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Robert A. Rastall
Editors

Prebiotics and Probiotics Science and Technology

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Dimitris Charalampopoulos, Robert A. Rastall (Eds.)

Prebiotics and Probiotics Science and Technology

With 67 figures and 97 tables

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To Elie Metchnikoff, Glenn Gibson and Marcel Roberfoid for originating the concept of probiotics and prebiotics

Preface

With growing consumer and industrial interest in self-care and integrative medicine, our understanding of the relationship between health and diet has grown stronger. As a result, the market for functional foods, dietary supplements and nutraceuticals is continuing to expand rapidly. Among these products, probiotics and prebiotics have carved their own special niche because of their scientifically-supported health promoting properties, and have been in the forefront of research over the past twenty years or so. This is driven by the realisation that the gut microbiota can play a critical role in human health. Important functions of the gut microbiota include the inhibition of the colonisation of the gut by potentially pathogenic microorganisms, the microbial fermentation of substrates yielding metabolic products which can serve as sources of energy for the gut cell wall, and the modulation of the immune system. A substantial amount of research has shown that the human gut microbiota can be modulated using probiotics and prebiotics leading to various beneficial effects.

The prebiotics and probiotics area is a fast evolving field that attracts significant interest by both the academic and industrial communities. As a result, a substantial amount of research is generated every year. The use of post-genomics, encompassing transcriptomics, proteomics, metabolomics and meta-genomics has helped greatly in making significant advances in the field, as they provide the means to carry out in-depth studies of the mechanisms involved and of the beneficial effects likely to be exerted to the host. The target of many probiotics and prebiotics is the prevention and treatment of disorders associated with the gastrointestinal tract (GIT), including infectious, traveller's, and antibiotic-associated diarrhoeas, *Clostridium difficile* infections, and constipation. They have also been suggested as therapeutic agents against irritable bowel syndrome and inflammatory bowel diseases. An increasing amount of evidence from *in vitro* and *in vivo* studies suggests that they are effective in the prevention of atopic allergies and may have potential anti-carcinogenic effects. In addition to the above, there has been considerable interest in

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extra-intestinal application of probiotics and prebiotics, such as in urogenital infections and oral health, and in their applications in animals.

This book will give a detailed and up to date account of the advances in the prebiotics and probiotics field, covering their taxonomy, the potential beneficial effects to humans and animals, the proposed mechanisms of actions, the molecular techniques used, and the challenges faced in manufacturing such products. The book consists of thirty one chapters. It starts with an introduction into the gastrointestinal tract ecosystem and its interaction with prebiotics and probiotics (Chapter 1), it then describes the molecular techniques used for analysing the complex gut ecosystem (Chapters 2 and 3), and discusses the design of human trials for evaluating their efficacy (Chapter 4). It then moves into the area of prebiotics and starts off by discussing the mechanisms of prebiotic impact on health (Chapter 5); it then provides a systematic overview of established as well as potential prebiotic oligosaccharides (Chapters 6 to 11). Each of these chapters covers the processes used to manufacture these compounds, and critically discusses their efficacy based on published data from *in vitro* and *in vivo* studies. The rest of the chapters in the prebiotics part cover the application of prebiotics in animals (Chapter 12), the analysis of prebiotic oligosaccharides (Chapter 13), and their manufacture from biomass sources (Chapter 14). The book then focuses on probiotics and begins with a taxonomic study of probiotic microorganisms (Chapter 15). It then discusses their interaction in the human gut (Chapter 16), provides an overview of functional genomic studies of probiotics (Chapter 17), discusses the challenges in their manufacture (Chapter 18) and in their addition to foods (Chapter 19), and presents the encapsulation methodologies used to improve probiotic delivery (Chapter 20). Subsequent chapters cover the potential beneficial effects of probiotics focusing on antibiotic-associated and *Clostridium difficile* diarrhea (Chapter 21), infectious and traveller's diarrhea (Chapter 22), immune modulation (Chapter 23), chronic gastrointestinal infections (Chapter 24), allergic diseases (Chapter 25), potential anti-carcinogenic effects (Chapter 26), urogenital applications (Chapter 27), oral health (Chapter 28), and on the development of mucosal vaccines based on lactic acid bacteria (Chapter 29). Finally, the remaining chapters cover the applications of probiotics in livestock animals (Chapter 30) and safety aspects of probiotics (Chapter 31).

The aim of this book is to provide a comprehensive overview of the research in the field of prebiotics and probiotics covering the achievements, challenges and future needs. As such, we hope that this book will be a valuable reference to both researchers and industrialists working in the field.

Dimitris Charalampopoulos
Bob Rastall

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1 Using Probiotics and Prebiotics to Manage the Gastrointestinal Tract Ecosystem

Randal Buddington

1.1 Introduction

Natural and man-made ecosystems are routinely managed to increase productivity and provide desired characteristics. The management approaches most commonly used include the addition of desired organisms, provision of fertilizers or feeds to encourage desired species, alteration of the physical or chemical features of the environment, and the selective removal of undesirable species. The selection of specific management strategies and their success are dependent on a thorough understanding of existing ecosystem characteristics and the short and long-term responses to the management strategy.

Ecosystems are generally recognized as consisting of structural elements that include living (biotic) and non-living (abiotic) components in a physically defined area and are maintained by functional elements that are involved in the cycling of materials and energy. The concept of the gastrointestinal tract (GIT) as an ecosystem is based on the interactions among the resident assemblages of microorganisms, the structural and functional characteristics of the GIT, and the responses to dietary inputs, and has proven useful for understanding the complexities of the interactions. Moreover, the application of ecological principles should assist in guiding "management" decisions to improve GIT characteristics and host health and nutrition.

The resident bacteria (the microbiome) are recognized to play a central role in GIT characteristics and host health (Falk et al., 1998; Martin et al., 2008; Tappenden and Deutsch, 2007). This has encouraged the development of approaches to manage the GIT bacteria to improve health and nutrition. Although the use of antibiotics for removal of undesired species has been a mainstay

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for managing the GIT bacteria, concerns about bacterial adaptation, development of antibiotic resistance, and destabilization of the commensal assemblages have stimulated the search for alternative strategies. At the forefront have been probiotic products, which include viable microorganisms that when administered in doses that reach the intestine in an active state are able to exert positive health effects. More recent is the introduction of prebiotic products, which are "selectively fermented food ingredients that elicit specific changes, both in the composition and/or metabolic activity of the gastrointestinal microbiota, and thereby confer health benefits to the host" (Roberfroid, 2007). The combination of pro- and prebiotics to obtain synergistic benefits has been termed synbiotics. The efficacy of probiotics, prebiotics, and synbiotics to improve host health and nutrition is dependent on the changes they elicit in the composition and metabolic activities of the resident assemblages of microorganisms.

This chapter describes the interactions among GIT structure and functions, the resident assemblages of bacteria, and dietary inputs, and how those interactions change from birth to senescence, are responsive to health status, and can be influenced by probiotics and prebiotics. Readers will first be introduced to the concept of the GIT as an ecosystem and provided with descriptions of the physical, chemical, functional, and biotic components. The objective is to encourage readers to consider the GIT from a different perspective. Examples and selected citations are then provided to demonstrate the application of probiotics and prebiotics as tools that can be used to manage the GIT ecosystem during development and in normal and disease states, and thereby improve the health and nutritional status of the host. The objective is to inform readers about the opportunities and challenges of using probiotics and prebiotics to manage the GIT ecosystem.

1.2 Gastrointestinal Tracts and River Ecosystems

The GIT ecosystem is in many ways similar to river systems that originate in a lake or reservoir (i.e., stomach) with an outflow into a fast flowing stream (i.e., small intestine) that receives inputs from other sources (pancreas and gall bladder) and eventually become slow moving, large rivers (colon) that empty into the ocean (*Figure 1.1*). Many of the tenets of the "river continuum concept" (Vannote et al., 1980) apply to the GIT. For example, both rivers and GIT's are characterized by physical and chemical gradients, which include regional differences in size, velocity of flow, and lumenal composition. The gradients shape the patterns of species distributions along the GIT, with the resident assemblages of biota

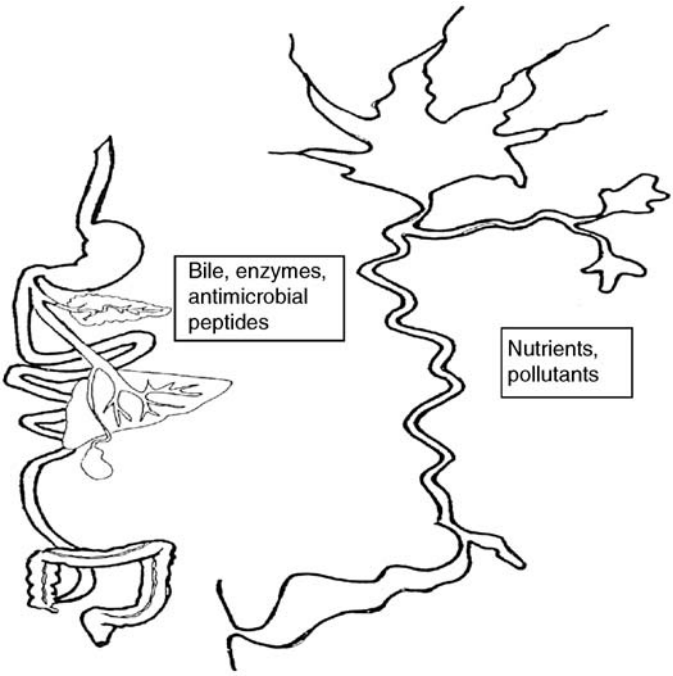

Figure 1.1
The gastrointestinal tract shares several similarities with many river ecosystems.

adapted to specific regions. Sharp gradients, such as the transition between the stomach and small intestine, result in distinct regional differences in biota, whereas the gradual gradient along the length of the small intestine results in a less distinct distribution of the resident biota. Furthermore, in both types of ecosystems the downstream communities of organisms are largely dependent on upstream inefficiencies that allow for the availability of needed nutrients.

Each region of the GIT represents a habitat with unique structural, chemical, and biotic components. Moreover, the regional processes of digestion and secretion alter the composition of the lumenal contents as dietary inputs are processed and flow distally, thereby influencing the species composition and metabolic activities of the resident bacteria. Exemplary are the profound differences between the stomach, the small intestine, and colon. Even within each GIT region there are sub-habitats. Specifically, the physical, chemical, and biotic characteristics of the lumenal contents differ from the layer of material that is immediately adjacent to the epithelial lining. Similarly, the physical, chemical, and biotic characteristics found in the middle of a stream or river differ markedly from

those at interface between the water and the land (riparian zone), resulting in different biotic communities.

Many of the ecological principles that apply to rivers are also relevant to the understanding of the GIT ecosystem. For example, the characteristics and functions of other ecosystems are closely related to the species composition, densities, and functions of the resident biota (Tilman et al., 1997). Of importance are the numbers of species and functional groups that constitute biodiversity (Hooper and Vitousek, 1997), with productivity and ecosystem stability increasing with greater species diversity. Similarly, the GIT and host health is related to the abundances, diversity, and metabolic activities of the commensal and pathogenic bacteria, and the responses of the bacteria to dietary inputs, including probiotics and prebiotics.

Differences do exist between rivers and GIT ecosystems. Unlike the abiotic substrates of rivers and streams, the living cells constituting the epithelium and underlying layers of the GIT are responsive to the chemical composition and bacterial assemblages of the lumenal contents. Exemplary are the changes in epithelial cell patterns of gene expression in response to different species of bacteria (Bry et al., 1996; Shirkey et al., 2006) and to changes in nutrient concentrations (Beaslas et al., 2008; Le Gall et al., 2007). Moreover, unlike river systems, signaling from distal to proximal regions of the GIT provides a mechanism whereby characteristics in the proximal regions of the GIT can be altered in response to events "downstream." This is exemplified by the regulatory peptides secreted by the ileum and colon (e.g., glucagon-like peptides 1 and 2, peptide YY) in response to the presence of nutrients and bacterial metabolites (e.g., short chain fatty acids) and the changes they elicit in the proximal small intestine (i.e., regulate digestion and stimulate growth and functions).

1.3 The Components of the GIT Ecosystem

The GIT ecosystem involves a dynamic balance between GIT structure and functions, the resident microbiota, and dietary inputs (*Figure 1.2*). The ability of bacterial populations to adapt to different GIT characteristics and dietary inputs (Dunne, 2001) results in GIT ecosystems that are unique for each individuals. Even monozygotic twins have different assemblages of GIT bacteria, though the differences are of lower magnitude than those of non-related individuals (Stewart et al., 2005). The following sections acquaint readers with the major components of the GIT ecosystem.

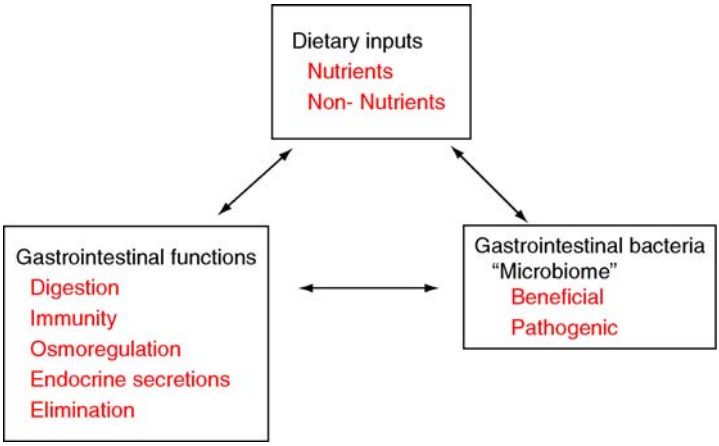

Figure 1.2
There are complex interactions among dietary inputs, gastrointestinal tract functions, and the resident assemblages of bacteria that influence host health and nutrition.

The physical features. Anatomically, the GIT is the most variable organ system of vertebrates. This is obvious by comparing GIT anatomy among carnivores, herbivores, and omnivores (Stevens and Hume, 1995). The different functional demands associated with processing the wide diversity of natural diets among species has led to adaptive changes in the structural features of the different GIT regions. Moreover, although the basic GIT plan for a species is established during evolution, adaptive changes in GIT structure can and do occur during development and in response to different diets.

The variation in flow rates that occurs in the different regions and during the processing of foods has an influence on the biotic components of the GIT ecosystem. Specifically, rapid movement of digesta in the proximal small intestine contributes to the reduced population densities by shortening residence time and hindering persistence. In contrast, the slower movement of digesta in the distal ileum and colon is associated with higher abundances and diversity of species.

The chemical features. The chemical composition in the different regions of the GIT is determined by the combination of dietary inputs and GIT functions. This includes secretions from the different regions of the GIT (stomach, small and large intestine) and the associated organs (i.e., pancreas and gall bladder). Correspondingly, the lumenal contents in the different regions of the GIT have unique chemical compositions. During the processing of meals, the contents of the stomach are nutrient rich, with nutrient levels declining along the intestine, into the colon. Regional differences in pH, bile acids, electrolytes, antimicrobial

peptides, and oxygen tension impose an additional selective pressure on the assemblages and metabolic activities of the resident bacteria. Notable is the presence of *Helicobacter* spp in the stomach, whereas populations of obligate anaerobes are highest in the colon.

The functional features. The functional features (physiology) of the GIT are key determinants of the chemical characteristics that influence the resident assemblages of bacteria. They also represent barriers to the introduction of species, such as probiotics as well as pathogens. The five basic functions of the GIT include (1) digestion of feedstuffs, (2) osmoregulation, (3) endocrine regulation of digestion and host metabolism, (4) immunity and defense against potential pathogens and harmful substances, and (5) the detoxification and elimination of toxic molecules originating from the environmental or the host.

Despite the diversity of anatomical structures, the functional characteristics of the vertebrate GIT are similar and are based on shared mechanisms. Hence, observed interspecies differences in GIT functions are related much more to adaptive responses of shared mechanisms to match dietary inputs and environmental influences rather than different mechanisms. For example, absorption of sugars and amino acids by the intestine is dependent on a diversity of apical membrane transporters that are shared among vertebrates. The higher rates of glucose absorption by omnivores compared with carnivores are caused by higher densities and activities of the apical membrane sodium glucose cotransporter SGLT-1, not because of a different type of transporter. Similarly, the exocrine pancreas of all vertebrates secretes a complex mixture of digestive enzymes and other proteins. The specific mixture of digestive enzymes secreted by the exocrine pancreas is modulated to match diet composition. Granted, over evolutionary time, individual proteins in pancreatic secretion have undergone changes in amino acid composition, thereby altering functional properties.

Following are brief reviews to acquaint readers with each of the GIT functions, with selected examples of the responses to the bacteria in the GIT. Extensive reviews of GIT physiology are available (e.g., Johnson, 2006).

Digestion. Feedstuffs are made available for metabolism by the host processes of secretion, motility, and absorption. Secretion of electrolytes, digestive enzymes, and bile by the GIT and associated organs is essential for the hydrolysis of the complex polymers in feedstuffs into the smaller molecules that are then absorbed and made available to the host. Although some food molecules are taken in by simple diffusion, the vast majority are absorbed from the GIT lumen by various specialized proteins (active and facilitative transporters) in the apical membrane. Additional transporters and ion channels are important for

recovering the electrolytes, water, and other molecules (e.g., bile acids) present in the digestive secretions. Contractions by the smooth muscle that lines the alimentary canal provides the motility that is necessary to mix foodstuffs with digestive secretions, reduce unstirred layer influences that reduce absorption, and the peristalsis that propels the food distally and reduces stagnation between the processing of meals. The patterns of intestinal motility are responsive to the resident bacteria (Husebye et al., 2001), including probiotics (Lesniewska et al., 2006).

Microbial fermentation, though not a host process per se, plays an important role in the ability of the host acquiring energy and nutrients from undigested feedstuffs. The products of bacterial fermentation, and particularly the short chain fatty acids (SCFA) provide up to 10% of the total metabolic energy requirement of humans and even higher proportions among animals with larger hindguts or rumens (Rechkemmer et al., 1988). Moreover, SCFA produced by the bacteria in the ileum and colon stimulates the secretion of regulatory peptides that enhance growth and functions of the proximal small intestine (Bartholome et al., 2004). Also to be considered is the interesting, but poorly understood competition between the host and the resident bacteria for nutrients. Obviously, it is in the best interest of the host to rapidly digest and absorb dietary nutrients before they can be metabolized by bacteria. By maintaining lower densities of bacteria in the proximal small intestine by peristalsis and antibacterial secretions from the pancreas and intestine, the host has first access to readily available, digestible nutrients. This leaves undigestible or poorly digested feedstuffs for the bacteria. Prebiotics represent dietary components that are not digestible by the host, but can be metabolized by some, but not all of the resident bacteria. The resulting products of fermentation from prebiotics and undigested foodstuffs are then available to the host, and provide energy, nutrients and additional health benefits.

Osmoregulation. The combination of dietary inputs and digestive secretions introduce about 10 L of fluid into the human GIT. Yet, only 100–150 ml are lost per day in the feces of normal individuals. A large fraction of the fluid is absorbed passively through the "leaky" epithelium lining the small intestine. The remainder is absorbed in the colon by the combination of ion channels, transporters, and aquaporins (Itoh et al., 2003), and the "tight" epithelium that lines the colon restricts the passive movement of water back into the colon. Small disturbances of the osmoregulatory functions can have profound consequences (Ewe, 1988), and particularly if they occur in the colon (Rolfe, 1999). Notably pathogens trigger diarrhea by disturbing osmoregulation.

The amounts of water and electrolytes absorbed by the colon are related to residence time of digesta. This is evident by the high water content of stools produced during diarrhea and by the characteristic desiccated stools of people with constipation and long residence times for materials in the colon. The importance of the colon in osmoregulation is also evident from the ability of the colon to compensate for the additional volumes of fluid that enter the colon of short bowel syndrome patients (Nightingale, 1999). Conversely if the colon is absent or bypassed (e.g., colectomized and jejunostomy patients) the increased loss of fluid and electrolytes requires interventions.

Endocrine secretion. Collectively, the various regions of the GIT and the associated organs represent the largest endocrine organ in the vertebrate body. The vast diversity of peptides regulate local (GIT) functions (e.g., gastrin, secretin) and metabolic processes throughout the host body (e.g., insulin, glucagon), with some GIT hormones regulating other host processes (e.g., cholecystokinin and satiety). The endocrine functions of the GIT act in concert with the enteric and central components of the nervous system to regulate digestion and host physiology and metabolism. It has been recently recognized that enteroendocrine cells can directly respond to resident bacteria by the secretion of hormones (Palazzo et al., 2007). However, the responses of enteroendocrine cells to probiotic bacteria are uncertain.

Immunity and host defense. The epithelium lining the GIT represents a critical interface between the external environment and the host and the combined surface area ($\sim$300 m^2) greatly exceeds the exposed surface area of the skin or the lungs. When one considers the combination of dietary inputs and the resident microorganisms, no other portion of the body is exposed to such a diversity and concentration of antigens. Corresponding with this, the cells constituting the enteric immune system exceed in number the immune cells associated with the rest of the body. Furthermore, the immune cells in the GIT have an even greater challenge of being able to distinguish between potentially harmful and beneficial molecules in the lumenal contents (Magalhaes et al., 2007; Tlaskalova-Hogenova et al., 2004). It is no surprise that the interactions between the enteric immune system and the resident bacteria are vital to host health (Schaible and Kaufmann, 2005; Tannock, 2007).

When considering the relationship between GIT immune functions and the resident bacteria, one is reminded of being asked as a small child to help remove weeds from the garden. Without instruction, one was unable to distinguish between weeds and flowers? They looked the same and one started to pull indiscriminately. Obviously, the outcome was not beneficial for the garden.

Once one was instructed, one was more selective in what plants were to be removed and those to be ignored. The result of one's actions thereafter will be beneficial. In some ways, the enteric immune system faces the same dilemma. It must learn to distinguish between potentially dangerous antigens and microorganisms and from those that are either neutral or potentially beneficial to the host. This "learning" process has occurred during the co-evolution of hosts with the bacteria in the GIT and during the life history of individuals and has resulted in the ability of the GIT to modulate immune responses to match the types of food and bacterial antigens (Comstock and Kasper, 2006).

The ability of the enteric immune system to differentiate between antigens that represent a threat and those that should be tolerated is dependent on a diversity of extracellular Toll-like Receptors (TLR's) and intracellular nucleotide-binding oligomerization domain (NOD) receptors. These provide intestinal cells with the ability to recognize a variety of pathogen associated molecular patterns (PAMP's) (Cario and Podolsky, 2005; Shaw et al., 2008). Importantly, during the co-evolution process, the TLR's and NLR's have acquired the ability to distinguish between pathogens and commensals, including probiotic bacteria. Furthermore, the patterns of expression for the TLR's and NLR's are modulated in response to the composition of the resident bacteria (Lundin et al., 2008).

The co-evolution between the GIT and the bacteria has selected for a set of innate and adaptive defense functions that respond to particular antigens. The innate responses of the GIT respond to antigens without prior exposure and include the secretion of antimicrobial peptides and other antimicrobial molecules, and the activation of macrophages and other defense functions (Eckmann, 2006). The adaptive components of the enteric immune system have similarly responded to co-evolutionary pressures and the populations of associated cells (i.e., T, B, and dendritic cells) have acquired characteristics that differ from those of cells in the systemic circuit (Coombes and Maloy, 2007) and prevent what could be massive and counterproductive immune response to the abundant and diverse antigens in the GIT. The adaptive components interact with the innate components of the GIT immune system to provide the GIT and the host with a comprehensive, multi-layered defense (Winkler et al., 2007) that is capable of "cultivating" the commensal assemblages of bacteria. When either component of the enteric immune system is compromised, the assemblages of bacteria are altered, and host health is compromised.

By changing the species composition of the GIT bacteria using probiotics and prebiotics it is possible to beneficially modulate enteric immune functions, thereby improving resistance to GIT pathogens and other health challenges

(Dogi et al., 2008). Importantly, the responses of the enteric immune system can be transferred to the systemic circuit enhancing disease resistance to systemic infections (Buddington et al., 2002).

Elimination. The GIT plays an important role in the detoxification and elimination of ingested toxins, including drugs, and metabolic wastes. The elimination functions include various GIT tissues that express numerous xenobiotic converting enzymes (e.g., cytochrome P-450's and other Phase I and II enzymes) (Kaminsky and Zhang, 2003; Kato, 2008; Lampe, 2007; Paine and Oberlies, 2007) and export transporters (e.g., P-glycoproteins, Multidrug Resistance transporters, and various ATP-binding cassette (ABC) transporters) (Oude Elferink and de Waart, 2007; Takano et al., 2006). The enzymes and transporters act in concert to detoxify and export toxic molecules.

A limited number of studies suggest management of the GIT bacteria can reduce accumulation and increase elimination of some environmental contaminants (Gratz et al., 2007; Kimura et al., 2004). Corresponding with this, changing the assemblages of the resident bacteria by dietary inputs or antibiotic regimens can influence the bioavailability and efficacy of some therapeutic compounds. For example, administration of some antibiotics can affect the efficacy of oral contraceptives (Dickinson et al., 2001). At the present time the potential influences of commensal, probiotic, and pathogenic bacteria on expression of xenobiotic converting enzymes and export transporters are poorly understood.

The biotic component. The GIT harbors a diverse collection of bacteria, generally estimated to include 400–500 species, with some estimates of >800 species and >7,000 strains (O'Keefe, 2008). Most of the GIT bacteria are unculturable, remain to be characterized, and there is little known about their functional characteristics and influences on host health (Flint et al., 2007). Collectively the GIT bacteria exceed the number of host cells, have a vast metabolic potential, and the interactions with the GIT have a profound impact on host health (Norin and Midtvedt, 2000). Comparisons of germ-free and conventional animals have revealed how the commensal bacteria are essential for normal GIT characteristics. Notable are the profound differences between germ-free and conventional rodents with respect to villus architecture, enterocyte proliferation, differentiation, and patterns of gene expression (Hooper et al., 2001). Significantly, there are differences in the responses to commensal and pathogenic bacteria (Lu and Walker, 2001). The resident bacteria also play an important role in host nutrition. Gnotobiotic rodents require 30% more dietary energy and vitamin supplements compared with conventional rodents harboring commensal bacteria that ferment undigested feedstuffs.

The interactions between the GIT bacteria and the host have been shaped by co-evolution (Ley et al., 2008) and have led to the emergence of commensal and symbiotic relationships (Hooper and Gordon, 2001). Additional interactions occur during the life history of individuals and these contribute to determining the characteristics of the assemblages (densities, diversity, evenness, regional distribution, and functional attributes). The interactions include the ability of some bacteria to alter the patterns of host gene expression, such as glycosylation patterns of extracellular proteins (Freitas and Cayuela, 2002), in ways that benefit both the bacteria and the host (Bry et al., 1996).

The regional distribution of the GIT bacteria. The densities, diversity, and evenness of bacterial species vary among the different regions of the GIT and over time (Franks et al., 1998). The persistence of a bacterial species in a specific region of the GIT is dependent on its being able to tolerate local environmental conditions, or exist in a refuge that provides shelter from adverse conditions (e.g., crypt regions), or adhere to the epithelium, or proliferate rapidly enough to avoid wash out from that region. The different attributes and environmental requirements of the various species and strains of GIT bacteria has resulted in an unequal distribution of species composition and densities along the length of the GIT.

Despite the high input of nutrients into the stomach of monogastric vertebrates, the density of viable bacteria in the stomach is typically low ($<10^4$ cfu/g or/ml). The diversity of species in the stomach is also low compared to the small intestine and colon. This can be attributed to the acidic environment. Although the assemblages of bacteria in the rumen of digastric animals (e.g., cows, sheep, goats) reach much higher densities and are more diverse (Larue et al., 2005; Nelson et al., 2003; Rodriguez et al., 2003) than in the monogastric stomach, bacterial densities and diversity are low in the acid secreting portion of the ruminant stomach (abomasum).

Bacterial densities and diversity in the intestine increase distally, with the highest densities (10^{11-12} cfu/gm) and greatest diversity in the colon. The relative distributions and proportions of aerotolerant and anaerobic species correspond with the declining gradient for oxygen from the proximal small intestine to the colon. Moreover, the rapid movement of the digesta in combination with the introduction of bile acids from the gall bladder and antibacterial peptides secreted by the pancreas and the intestine (e.g., defensins) result in comparatively low bacterial densities and diversity in the duodenum and proximal jejunum. Even within the colon, there is a proximal to distal distribution of species and metabolic activities. Notable is the higher proportion of saccharolytic bacteria and SCFA production in the proximal colon, whereas proteolytic bacteria and the

production of putrefactive metabolites are more prevalent in the distal colon. The higher incidence of colon cancer in the developed world has been attributed to a low fiber diet, causing lower abundances of saccharolytic bacteria and an increase in the proportion of proteolytic forms. The consequence is a higher production of ammonia, phenols, indoles, amines, and other toxic and carcinogenic metabolites.

Disturbances of the environment also play an important role in determining the densities, diversity, and relative proportions of resident species in ecosystems. Densities, diversity and evenness are reduced in environments that are harsh or highly disturbed as well as those that are highly stable. Typically, harsh or highly stable environments are dominated by relatively few species with a higher proportion of rare species. In contrast, the densities, diversity, and evenness of species are higher in environments characterized by intermediate frequencies and magnitudes of disturbances as this prevents one or a few species from establishing dominance. This "intermediate disturbance hypothesis" of species diversity (Connell, 1978) can be applied to the GIT ecosystem. Corresponding with the hypothesis, the harsh environments of the stomach and upper small intestine with the wide swings in chemical conditions and intermittently high flow rates, correspond with lower observed densities, diversities, and evenness of resident bacterial species. In contrast, the greatest densities, diversities, and evenness of bacterial species are found in the distal ileum and proximal colon where the environment is more benign, yet the conditions are subject to moderate fluctuations.

In addition to the longitudinal distribution of species in the GIT, there are vertical gradients in each region that extend from the epithelium into the lumen (Kleessen and Blaut, 2005). Although the populations of bacteria adherent or adjacent to the epithelium have a profound impact on the GIT and the host, they are less understood.

Adaptive responses of the GIT bacteria. The biotic components of ecosystems respond to environmental changes. The GIT ecosystem and the resident bacteria are somewhat unique in a two-way adaptation; bacteria adapt to changes in the characteristics of the GIT, and the GIT adapts to changes in the assemblages of bacteria. Of particular importance are how shifts in bacterial populations elicit responses by the GIT. The most obvious contrasts are the divergent responses to pathogenic versus the health promoting commensal and probiotic bacteria. The underlying concept of probiotics and prebiotics is that by increasing the beneficial and decreasing the pathogenic bacteria resident in the GIT, it is possible to improve GIT characteristics and have a beneficial impact on host health and nutrition.

1.4 Dietary Inputs and the GIT Ecosystem

The exclusion of terrestrial inputs into streams reduces the abundances, diversity, and production of the resident organisms. Similarly, during prolonged total parenteral nutrition (Alverdy et al., 2005), starvation (Nettelbladt et al., 1997), and hibernation (Banas et al., 1988) there are analogous changes in the assemblages of GIT bacteria. Elemental diets that are rapidly and efficiently absorbed by the proximal small intestine result in reduced amounts of nutrients entering the colon and thereby also disturb the resident bacteria (Alverdy et al., 2005). However, restriction (not exclusion) of complex feedstuffs is considered to have only a minor impact on the GIT bacteria (Henderson et al., 1998). Of particular concern during parenteral nutrition are the increases in pathogens and the elevated risk of secondary diseases (Deplancke et al., 2002; Harvey et al., 2006). These findings highlight the dependence of the GIT on dietary inputs for normal functions and health. Moreover, management approaches that change the species composition and metabolic activities can be expected to alter GIT characteristics (Tilman et al., 1997).

The dependence of the colonic bacteria on dietary inputs has led to the concept of "colonic foods." Simply increasing the amounts and types of feedstuffs that reach the colon increases bacterial diversity. Correspondingly, adding fermentable fibers, including prebiotics, to elemental diets partially restores the assemblages and metabolic activities of the colonic bacteria and improves GIT and host health (Schneider et al., 2006; Whelan et al., 2005). Importantly, the responses of the GIT bacteria and the production of SCFA and other metabolites vary among different types of dietary carbohydrate (Rabiu and Gibson, 2002; Silvi et al., 1999) and digestion-resistant proteins (Morita et al., 1998), and this impacts GIT functions and health (Topping and Clifton, 2001). Supplementing elemental diets with probiotics also reduces the magnitude of decline in GIT characteristics (Shen et al., 2006).

1.5 Applications of Probiotics and Prebiotics

There are complex interactions among the bacteria resident in the GIT that establish the commensal assemblages and exclude invading species by out competing for limiting resources (food, attachment sites, etc.) and by the production of metabolites (bacteriocins and SCFA) that inhibit the growth or kill outright invading bacteria. Additionally, the interactions between the commensal bacteria

and immune functions of the GIT can enhance the defenses against invading pathogens. Of particular interest is having an understanding of how probiotics and prebiotics can be used to manage the diverse and dynamic assemblages of bacteria such that pathogens are excluded or are unable to become established.

Even when probiotics are administered at relatively high doses (i.e., 10^{11} cfu/d), they represent <0.01% of the total number of bacteria in the GIT ($10^{14–15}$). In reality, the percentage is even less because a fraction of the probiotic bacteria do not remain viable. Moreover, competitive exclusion by the commensal assemblages can prevent probiotics from becoming established in the GIT and in most cases probiotics are transients. As a consequence, densities of probiotic bacteria in fecal samples are typically low. Yet, there are numerous examples that demonstrate the administration of probiotics provides health benefits. This emphasizes how "rare" species can affect the GIT ecosystem and provide health benefits. The success of a probiotic is dependent on the ability to survive in the GIT ecosystem and to interact with the other components of the ecosystem in a manner that fosters improved health. Corresponding with this, the responses to some probiotics are neutral.

Prebiotics encourage the proliferation of beneficial bacteria already resident in the GIT and can elicit larger changes than probiotics in the abundances of commensal bacteria that confer health benefits. Specifically, if the total mass of colon contents is 1 kg and a prebiotic increases the density of a bacterium from 10^7 to 10^9 cfu/g, the result is an almost 10^{12} increase in the total abundance (cfu) of that species of bacterium. Since several species can respond to prebiotics, the influence on the GIT ecosystem can be even more pronounced. Additionally, prebiotics may affects the assemblages of bacteria associated with the epithelial "biofilm" and the lumen (Kleessen and Blaut, 2005). However, the species that are responsive to prebiotics may not provide the same magnitude of benefits as some probiotics.

The following are examples of the application of probiotics and prebiotics to manage the GIT ecosystems of infants and the elderly, for prevention and treatment of acute and chronic GIT diseases, and for emerging health issues. Readers are referred to later chapters for more detailed descriptions.

GIT development. The two most dramatic periods of postnatal development of the GIT occur at birth with the onset of feeding and colonization with bacteria and again at weaning when dietary inputs changing from milk to the adult diet, with corresponding changes in GIT characteristics and assemblages of bacteria. At birth the sterile GIT of neonates is colonized by bacteria originating from the maternal GIT, vagina, skin, and the surrounding environment (Mackie et al., 1999).

Since the species composition and the relative abundances of the colonizers is not consistent, it is not surprising there is variation in the GIT bacterial assemblages that colonize the GITs among littermates and twins. After the initial period of colonization, the densities, diversity and distribution of species within the GIT ecosystems are determined by a combination of dietary inputs, GIT functions, and inter-bacterial interactions, including the processes of competition and facilitation. The process of facilitation, which can be described as cooperative relationships among bacteria, may not be considered enough in understanding the influences of probiotics and prebiotics, and particularly during development. An example is the concept of "metabolic cross-feeding" whereby metabolites produced by one group of bacteria are used or converted by other groups of bacteria (Flint et al., 2007). One of the better examples is the conversion of lactate produced by bifidobacteria into butyrate and other SCFA by anaerobic members of the GIT bacteria.

The interactions between dietary inputs, the resident bacteria, and health are particularly evident during infancy (Gil and Rueda, 2000) and can have profound health consequences. Necrotizing enterocolitis (NEC) is an inflammatory reaction that is the most common GIT disorder of neonates, and particularly those born premature (Schnabl et al., 2008). The NEC disease process is multifactorial and the protection provided by colostrum versus the increased risk associated with formula indicates dietary inputs play a central role. Moreover, the absence of NEC among germ-free animals, regardless of diet demonstrates the essential role of the resident bacteria. A review of trials that evaluated the efficacy of probiotics as a prophylactic for NEC suggest the disease risk is reduced (Barclay et al., 2007). However, the use of different strains and administration regimes confounds interpretations. Although the potential benefits of including prebiotics in formula fed to preterm infants are uncertain, animal models suggest including prebiotics may reduce the risk of NEC (Butel et al., 2002).

Dietary inputs can and do influence the successional changes that occur in the developing bacterial assemblages. By doing so, they influence postnatal GIT development as well as neonatal and infant health (Amarri et al., 2006; Moreau and Gaboriau-Routhiau, 2001; Walker, 2000). Of particular interest are the responses of the GIT bacteria to diet and the associated influences on the developing immune system of infants (Cebra, 1999; Gaskins et al., 2008; Haverson et al., 2007). This includes the relations with development of tolerance and allergy (Kukkonen et al., 2007, 2008). Coinciding with these findings, there is understandable interest in increasing the proportion of beneficial bacteria in the GIT of infants to improve health and disease resistance. There are three principle

approaches that have been used to date. The first is the administration of probiotics to infants. This has proven effective. However, the benefits are generally transient and do not persist after the probiotic is no longer administered. The second approach is to provide prebiotics to encourage the growth of beneficial bacteria. Human breast milk, which is high in oligosaccharides, encourages higher densities of health promoting lactobacilli and bifidobacteria. The addition of prebiotics to formulas has similarly increased the densities of lactobacilli and bifidobacteria (Veereman, 2007), thereby providing health benefits (Arslanoglu et al., 2007, 2008). The third approach has been to provide probiotic bacteria to pregnant women to facilitate colonization of infants born vaginally (Schultz et al., 2004). Since probiotics given to pregnant women can be detected in infants delivered by caesarian section (Schultz et al., 2004), colonization can be independent of exposure to the maternal GIT bacteria at the time of birth.

Elderly. The diversity, evenness, and relative proportions of bacterial species in the GIT typically decline with advanced age (Mitsuoka, 1992; Woodmansey, 2007). Of concern are the reciprocal declines in health promoting bacteria (e.g., *Lactobacillus* spp. and *Bifidobacterium* spp.) and increases in bacterial groups that are considered to be opportunistic pathogens or to be putrefactive. Although there is evidence that changes in diet during senescence contribute to the adverse shifts in the bacterial assemblages (Benno et al., 1989), the same patterns of changes during increasing age are known for animals that have consumed the same diet throughout life (Benno et al., 1992). Therefore, the changes in GIT structure and functions that occur during senescence must contribute to the changes of the bacterial assemblages.

The numbers and proportions of elderly subjects is increasing as is interest in alternative medicines to improve GIT health. Administering probiotics and prebiotics to elderly subjects has helped to restore the bacterial assemblages and apparently provide benefits (Hebuterne, 2003; Woodmansey, 2007). There is a need to expand these efforts to gain a better understanding of how probiotics and prebiotics can be used to manage the aging GIT ecosystem.

Diarrheal diseases. Diarrhea can be caused by food infections and toxins, by administration of antibiotics that disturb the commensal bacteria, and by dysfunctions of digestive functions (Huebner and Surawicz, 2006). To understand the application of probiotics and prebiotics for treatment of the various types of diarrhea it is important to first consider the impact of diarrhea on the GIT ecosystem. In many ways diarrhea is analogous to how floods affect river systems. Specifically, minor increases in the flow of rivers mainly disturb the center of the river, with little impact on the resident organisms and ecosystem functioning.

As flow rates increase, the disturbance radiates out toward the interface between the stream and substrate until at high flow rates (floods) the interface itself is disturbed, exchange between the substrate and stream is disrupted, and the residents are displaced. The disturbances associated with floods alter biodiversity, enhances the invasion of exotic species, and increases the abundances of "weed species."

Before the onset of diarrhea, anaerobes dominate the GIT bacteria, whereas the disturbance caused by diarrhea elicits an increase in aerotolerant forms, which includes the majority of pathogens, throughout the GIT (Balamurugan et al., 2008; Bhan et al., 1989; Neto et al., 1976). During diarrhea there are also concurrent declines in bacterial diversity and evenness of species (Mai et al., 2006), with adverse changes in bacterial metabolism and immune status (Khalif et al., 2005), thereby increasing the risk of secondary infections.

The length of time required for the GIT bacteria to recover from diarrhea is dependent on the severity and duration. It is of health importance to promote a rapid recovery of commensal assemblages of bacteria after diarrhea. Unfortunately, undesirable species (i.e., pathogens) usually recover faster and achieve higher densities. Corresponding with this, some of the strongest claims for the benefits of probiotics and prebiotics are for the treatment and prevention of diarrhea. Administration of probiotics during and after diarrhea are known to accelerate the recovery of the commensal bacteria, reduce the abundances of pathogens, and enhance GIT characteristics (Huebner and Surawicz, 2006; Sullivan and Nord, 2005). The adverse impacts of diarrhea on the GIT and the benefits of probiotic therapy are even more pronounced for infants with diarrhea (Johnston et al., 2007). However, the efficacy of probiotics is not universal and the benefits are widely variable, with some studies showing little if any benefit (de Vrese and Marteau, 2007). These findings highlight the need to identify strains of probiotics and establish doses that are most effective in accelerating recovery. There is evidence that probiotics can be used prophylactically to provide protection against diarrhea (Sullivan and Nord, 2005).

The benefits of using prebiotics, prophylactically and therapeutically, for diarrhea are not well established. Yet, supplementing an oral electrolyte solution with prebiotics accelerates recovery of the commensal bacteria and improves GIT structure and functions in an animal model of secretory diarrhea (Oli et al., 1998). However, caution must be exercised to not provide doses of prebiotics that elicit osmotic diarrhea.

Constipation. At the other end of the spectrum from diarrhea is constipation caused by one or more GIT dysfunctions that result in prolonged intervals

between defecations. The longer retention of digesta in the colon is associated with declines in commensal bacteria that promote health, increased abundances of potential pathogens, and alterations of digestive functions (Khalif et al., 2005; Zoppi et al., 1998). Although chemical laxatives are often effective and help to restore the assemblages of colonic bacteria, there is growing interest in identifying and using "natural" products.

The incidence of constipation is as high as 30% in developed countries with diets lower in fiber, and afflicts an even greater proportion of the elderly and individuals on hypocaloric diets. Increasing the consumption of fiber is often recommended to reduce constipation. Although including prebiotics as a fiber source for constipation can be predicted to provide benefits, studies directed at this possibility are limited. Those that are reported provide evidence of improved bowel habits and colonic assemblages of bacteria when the diets of constipated individuals are supplemented with prebiotics (Kleessen et al., 1997).

Studies of the efficacy of probiotics for treatment of constipation are actually more common than for prebiotics. There is evidence that probiotics can increase stool frequency (Drouault-Holowacz et al., 2008). However, these findings are not universal, with efficacy varying among strains of probiotic bacteria.

Chronic diseases of the GIT. The most common chronic diseases of the GIT include inflammatory bowel disease (IBD), irritable bowel syndrome with diarrhea (IBS-D) or constipation (IBS-C), celiac sprue, and colorectal cancer (CRC). These are reviewed elsewhere in this volume. Animal models have demonstrated the presence of bacteria is essential for triggering the damaging inflammatory reactions and for converting environmental contaminants into carcinogens (Bohn et al., 2006; Clavel and Haller, 2007; Geier et al., 2007). Hence, effective management of the resident bacteria can have a profound influence on the risk of chronic GIT diseases.

IBD involves a dysregulation of the immune responses to the GIT bacteria (Sartor et al., 2007) in conjunction with a genetic predisposition (Torres and Rios, 2008). The disease is characterized by chronic relapsing inflammation of the GIT, and includes Crohn's disease, ulcerative colitis, and the pouchitis associated with the formation of a "neo-colon" using segments of ileum after colectomy. Of concern is the increased risk of colorectal cancer among individuals with IBD (Geier et al., 2007). The assemblages of GIT bacteria are altered with IBD, including a decline in diversity (Dicksved et al., 2008; Xenoulis et al., 2008). The majority of evidence supporting the use of probiotics for treatment of IBD is largely based on various animals models that showed probiotics trigger signaling pathways that result in the secretion of anti-inflammatory cytokines and thereby

decrease the severity of IBD (Winkler et al., 2007). The findings from these studies have fostered the rationale and rapidly growing interest in the application of probiotics and prebiotics to restore the commensal assemblages for prevention and treatment of IBD (Quigley and Flourie, 2007). This is evident by more than 90% of the published reports having been published since 2000. Although clinical trials suggest probiotic preparations can be used for treatment of IBD, not all probiotic strains are effective (Heilpern and Szilagyi, 2008). The most effective probiotic preparations tend to be multi-species mixtures (Madsen et al., 2001). Still, the evidence for the efficacy of probiotics for treatment of IBD is not conclusive (Geier et al., 2007) and additional well-controlled studies are necessary to obtain definitive proof. Similarly, although there is evidence probiotics may reduce the incidence of IBD remission, the findings are inconclusive, largely because of the lack of adequately controlled and designed studies (Butterworth et al., 2008).

In light of the essential role of the resident bacteria in triggering IBD, the *a priori* expectation is that increasing the proportions of beneficial commensal bacteria by administration of prebiotics should stimulate anti-inflammatory responses and reduce the risk and severity of IBD. Yet, the application of prebiotics for prevention and treatment of IBD has received far less investigation. Still, the available evidence from animal models indicate that prebiotic-induced changes in the GIT bacteria can provide benefits (Leenen and Dieleman, 2007), and the limited, but promising results from clinical trials (Guarner, 2007). Although synbiotic combinations may provide the most effective approach, there are too few clinical trials to draw conclusions.

IBS is a growing health concern that afflicts up to 25% of the population in developed countries. Patients with IBS have chronic and recurring abdominal pain, altered bowel habits (diarrhea or constipation), and other GIT dysfunctions. There are multiple underlying causes of IBS, including the resident bacteria (Clavel and Haller, 2007), and curative therapies do not yet exist. A consistent finding is that the assemblages of GIT bacteria are disrupted with IBS, and there is evidence that suggests prior enteric infections increase the risk of developing IBS (Cuomo et al., 2007). These findings suggest therapeutic approaches that restore or improve the GIT bacteria may reduce the incidence or severity of IBS. A metanalysis of the limited number of studies that have been conducted suggests probiotics may be effective for IBS, but additional studies are needed (McFarland and Dublin, 2008). Again, there are fewer studies of the efficacy of prebiotics for treatment of IBS. One of the more promising applications of prebiotics is for treatment of IBS-C due to the laxative and bacterial normalization effects.

Celiac disease is an allergic response of the GIT to the gliadin component of wheat gluten that triggers an inflammatory reaction. Similar to IBD and IBS, there are shifts in the species composition of the resident bacteria of individuals with celiac disease (Sanz et al., 2007). This has fostered speculation that restoring the commensal assemblages of bacteria using probiotics and prebiotics should reduce the incidence and severity of celiac disease and other food allergies. Although cell culture studies have demonstrated probiotics abrogate the responses to the components of gliadin that elicit inflammation (Lindfors et al., 2008), clinical evaluations of efficacy are lacking.

There is also interest in exploring the efficacy of probiotics for preventing or reducing the severity of other food allergies (Mengheri, 2008). However, it can be expected that not all probiotic preparations will provide protection for enteric or systemic allergic reactions (Brouwer et al., 2006; Hol et al., 2008). Obviously, there is a need to identify the strains of probiotics that are effective.

There is increasing awareness of the relationship among diet composition, the GIT bacteria, and the risk of colorectal cancer (Juste, 2005). Of interest are the metabolic activities of the colonic bacteria that can activate procarcinogens or deactivate carcinogens. The majority of evidence demonstrating probiotics and prebiotics reduce the risk of colorectal cancer is based on animal models exposed to chemical carcinogens. A comprehensive description of the evidence is provided elsewhere in this volume. The best clinical evidence of a protective effect of probiotics and prebiotics is from the SYNCAN project (Pool-Zobel and Sauer, 2007).

Surgical interventions and dysfunctions that alter the GIT ecosystem. Fragmentation and reductions in the sizes of ecosystems, and changes in inputs are associated with shifts in the abundances and distributions of the resident organisms, and these can cause dramatic changes in the functions and productivity of the affected ecosystems. It is not surprising that surgical interventions and GIT dysfunctions similarly affect the species composition and metabolic activities of the resident bacteria. Exemplary is how resection of portions of the small bowel (e.g., short bowel syndrome) alters the species composition and metabolic activities of the GIT bacteria (Kaneko et al., 1997). Similarly, the bacteria resident in neo-colons (pouches) prepared using segments of ileum after colectomy differ from those in the normal colon and this has been associated with inflammation ("pouchitis"; Welters et al., 2002). In both cases, probiotics and prebiotics have been successfully administered to address the changes in the bacterial assemblages and to decrease the risk and severity of mucosal inflammation.

The popularity of gastric bypass surgery with the Roux-en-Y procedure for treatment of obesity has increased the number of individuals who have a GIT with vastly altered structural and functional attributes. This surgical procedure changes the resident bacteria throughout the GIT, including the stomach (Bjorneklett et al., 1984; Ishida et al., 2007). At the present time, there is very little known about whether providing probiotics or prebiotics to patients following bariatric procedures will provide health benefits.

Cholecystectomy and the loss of regulated bile acid secretion into the small intestine change the chemical composition of the GIT contents. There are corresponding changes in the metabolic activities of the colonic bacteria and the production of secondary bile acids and other metabolites. Although there is concern that these changes can increase the risk of colorectal cancer (Zuccato et al., 1993), the potential health benefits of probiotics and prebiotics after cholecystectomy have not been explored.

Exocrine pancreatic insufficiency and pancreatitis and the decline in secretion are associated with overgrowth of bacteria in the proximal small intestine and increase the risk of bacterial translocation and septicemia (Besselink et al., 2004; Simpson et al., 1990; Trespi and Ferrieri, 1999). Despite the decline in digestive efficiency, a large fraction of the malabsorbed feedstuffs are "salvaged" by bacterial metabolism and are available to the host as SCFA. Although probiotics are considered for prophylactic and therapeutic interventions with pancreatic dysfunctions, the benefits are not yet established and there is a need to identify strains that provide benefit without risk for such patients (Bengmark, 2008).

1.6 Considerations, Limitations, and Opportunities

The ability to manage ecosystems is dependent on being able to analyze, measure, and manage the biotic components. Important characteristics of the biotic component of any ecosystem include the abundances, diversity, and evenness (relative proportions) of the resident species. However, investigators and clinicians seeking to manage the GIT of human subjects face challenges. For obvious reasons, people know much more about fecal microbiology, yet many of the most important interactions among diet, the resident bacteria, and the host occur in the "upstream" habitats of the GIT. The difficulty of obtaining samples from throughout the GIT has impeded the understanding of this ecosystem and how it responds to dietary inputs, including probiotics and prebiotics, and other interventions. Similarly, if river ecologists were restricted to studying only water

samples collected at the beginning and termination of large rivers, their understanding of the entire river system would be limited, and the design of management strategies would be severely compromised. Moreover, even when other regions are accessible, the majority of information about the GIT bacteria is based on samples of the lumenal contents. Yet, the interactions between the host and the bacteria at the boundary layer have an impact on health, and this critical habitat should be considered as an important target of management strategies. Obviously, a better understanding of the entire GIT ecosystem is essential for developing and implementing management approaches based on probiotics, prebiotics, and other dietary interventions.

One of the foundations of community ecology is the relationship between the number of species and the area sampled. Determining the absolute and relative abundances of species, not just their presence or absence, is essential to understanding ecosystems. These principles highlight how appropriate sampling protocols (i.e., frequency, size, methods of identification and enumeration) are critical for investigating the biotic components of ecosystems. Also to be considered are the several orders of magnitude that separate the abundances of rare ($<10^3$ cfu/g) and common species ($>10^{11}$ cfu/g) of GIT bacteria. Rare species can be missed by both culture dependent and independent methods, particularly when the amount of material used for detection and identification is small. Yet, the ability to detect rare species is essential for characterizing and understanding ecosystems, including the GIT. Many studies of the GIT bacteria are dependent on a single sample, often less than 1 g. Because rare species may be detected only infrequently, they can be missed. Moreover, even with multiple samples the inconsistent detection may lead to the misinterpretation that such rare species are transient, even when they are continuously present.

The variation in bacterial populations (species composition and metabolic activities) among species, individuals, even among identical twins (Dicksved et al., 2008), and at different ages emphasize how GIT structure and functions and dietary inputs are important determinants of the characteristics and health of the GIT ecosystem, Although probiotics and prebiotics have proven to be effective tools to manage the GIT ecosystem, the responses and benefits are not universal. Coinciding with this, the patterns of gene expression by epithelial cells lining the small intestine and colon differ when exposed to a diversity of probiotic bacteria (Shima et al., 2008). These findings explain the divergent responses of host species and individuals to probiotics and prebiotics (Fujiwara et al., 2001; Simpson et al., 2000; Sullivan and Nord, 2005) and why a specific strain of probiotic or type of prebiotic does not elicit consistent responses among individuals.

As a consequence, there are difficulties associated with developing "one-for-all" management strategies. A better understanding of host-microbiome interactions and the responses to probiotics and prebiotics will greatly enhance efforts to improve management of the GIT ecosystem and thereby the health of individuals.

List of Abbreviations

ABC	ATP-binding cassette
CRC	Eolorectal cancer
GIT	Gastrointestinal Tract
IBD	Inflammatory bowel disease
IBS-C	irritable bowel syndrome with constipation
IBS-D	irritable bowel syndrome with diarrhea
NEC	Necrotizing enterocolitis
NLR	NOD-like receptor
NOD	Nucleotide-binding oligomerization domain
PAMP	Pathogen associated molecular pattern
SCFA	Short Chain Fatty Acids
SGLT-1	Sodium Glucose cotransporter-1
TLR	Toll-like Receptor

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2 Molecular Tools for Investigating the Gut Microbiota

Christophe Lay

2.1 Introduction

The "microbial world within us" (Zoetendal et al., 2006) is populated by a complex society of indigenous microorganisms that feature different "ethnic" populations. Those microbial cells thriving within us are estimated to outnumber human body cells by a factor of ten to one. Insights into the relation between the intestinal microbial community and its host have been gained through gnotobiology. Indeed, the influence of the gut microbiota upon human development, physiology, immunity, and nutrition has been inferred by comparing gnotoxenic and axenic murine models (Hooper et al., 1998, 2002, 2003; Hooper and Gordon, 2001).

First interventional human studies on the structure of the gut microbiota derived from a common initiative aiming to seek a link between bacterial inhabitants of the fecal microbiota and their implication in colon cancer (Finegold et al., 1983; Moore and Holdeman, 1974; Moore and Moore, 1995). Culture based methods have been applied to characterize the bacterial species diversity in fecal samples. According to Moore and Holdeman (1974), the number of species could be estimated at 400–500. Nevertheless, this extrapolated number may not reflect the reality. Indeed an inherent bias of classical culture techniques concerns the cultivability potential of bacteria. Several studies have indicated differential counts resulting from the comparison between enumeration of colony-forming units and bacterial concentrations determined by microscopy. This phenomenon has been called "the great plate count anomaly" (Staley and Konopka, 1985). According to studies, 15–54% of the fecal bacterial community is cultivable (Langendijk et al., 1995; Matsuki et al., 2002; Suau et al., 1999; Tannock et al., 2000). Apajalahti et al. (2003) estimated that one-third of

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the fecal bacterial community is not viable, and this mortality rate could partially explain "the great plate count anomaly."

Nucleic acid based methods have been developed to circumvent biases inherent to bacterial culture. Those approaches based on the analysis and comparison of ribosomal nucleic acid sequences, have permitted to unravel the hidden diversity of the gut microbiota. Microbial classification is mainly based on the 16S rRNA taxonomic gene marker. Currently, more than 700,000 16S rRNA gene sequences are available in the RDP or ribosomal database project (http://rdp.cme.msu.edu/). This valuable resource gathers sequences isolated from diverse microbial communities and comprises two types of sequences, those originated from cultivated bacterial strains and those generated from clone libraries. Using 16S rRNA gene sequences based approaches, an unprecedented microbial diversity has been described within the human colonic microbiota. The structure of the human gut bacterial community features mainly four phyla, Firmicutes, Bacteroidetes, Actinobacteria and Proteobacteria. Consistent data regarding bacterial members belonging to the "healthy gut" population have been gained. Members of the *Eubacterium rectale-Clostridium coccoides*, *Clostridium leptum* (both belonging to Firmicutes), *Bacteroides* (Bacteroidetes), *Bifidobacterium* and *Atopobium* (both belonging to Actinobacteria) groups form the main core representative of the bacterial collection resident in the human bowel (Eckburg et al., 2005; Harmsen et al., 2002; Hayashi et al., 2002; Lay et al., 2005a; Suau et al., 1999). Advances in genome sequencing technologies have opened up new perspectives in studying the gut microbiota. Beyond the structural parameters, an insight into the genetic potential of the human microbiome, a term referring to the total number of genes encoded by the collective genomes of the gut bacterial members, is being revealed through metagenomic approaches (Gill et al., 2006; Kurokawa et al., 2007; Manichanh et al., 2006).

Interest of the research community in the study of the intestinal microbial ecosystem is expanding. The Human Microbiome Project (HMP) has recently been launched by the National Institutes of Health (NIH) with the mission of generating resources enabling comprehensive characterization of the human microbiota and analysis of its role in human health and disease (Turnbaugh et al., 2007). In Europe, the MetaHIT (Metagenomics of the Human Intestinal Tract) project funded by the European Commission is investigating the role of the gut microbiota in obesity and inflammatory bowel diseases (Mullard, 2008). Topics surrounding the gut microbiota and its intrinsic relations with health and disease also interest the food and pharmaceutical industries. Nowadays, the concept of functional foods to maintain and enhance the bacterial balance of the gut microbiota through probiotic and prebiotic administration is anchored in

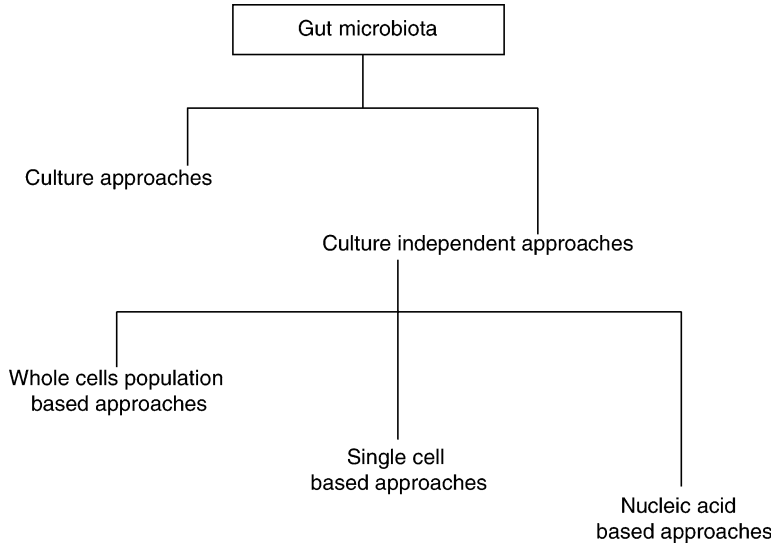

Figure 2.1
Molecular tools to investigate the gut microbiota.

our nutrition habits. This bacterial balance is dynamic and reflects the homeostasis of the intestinal microbial ecosystem, a concept to characterize this heterogeneous community of microorganisms, interacting with its host (Hooper et al., 1998, 2002, 2003; Hooper and Gordon, 2001), capable of self-regulation, even after allochtonous invasion (Tannock et al., 2000) or temporary antibacterial disturbance (De La Cochetiere et al., 2005).

In this chapter, a description of the molecular tools for investigating the gut microbiota and their applications in human intervention studies will be summarized. A review of what has been revealed on the structure and genetic potential of the human gut microbiota through the applications of these molecular tools will be conducted (*Figure 2.1*).

2.2 Culture-Dependent Molecular Approaches

2.2.1 PCR Screening and 16S rRNA Sequence Analysis

Identification of bacterial colonies using phenotypic approaches is laborious and time-consuming. Molecular methods based on 16S rDNA have been developed to circumvent these constraints.

Matsuki et al. (2002) designed and validated specific 16S rDNA PCR primers for the detection of *Bacteroides fragilis*, *Bifidobacterium*, *Clostridium coccoides*, and *Prevotella* groups, in order to characterize the cultivable fraction of the fecal bacterial community of six healthy adult volunteers. Fifty bacterial colonies were randomly selected on non-selective media from each individual. The 300 bacterial isolates were then tested with the group-specific primers, 117 isolates were affiliated to the *Bacteroides fragilis* group, 22 to the *Bifidobacterium* group, 65 to the *Clostridium coccoides* group, and 17 isolates to the *Prevotella* group. These results indicated that 74% of the bacterial colonies were identified with the four pairs of primers. The remaining 79 isolates were identified using 16S rRNA gene sequence analysis. Forty colonies were affiliated to *Colinsella aerofaciens*, 24 to the *Clostridium leptum* subgroup, and 15 isolates to disparate clusters. In this study the authors compared bacterial concentrations determined on non-selective media with microscopic counting. On average 46% of the total fecal bacterial population corresponding to the non cultivable fraction could not be characterized (*Figure 2.2*).

2.2.2 Ribotyping and Pulse-Field Gel Electrophoresis

Both techniques are based on RFLP (Restriction Fragment Length Polymorphism) and allow the discrimination of bacterial strains affiliated to the same species. Both molecular typing methods are culture-based dependent.

2.2.2.1 Ribotyping

Genomic DNA extracted from bacterial colonies randomly selected on selective medium is digested with appropriate restriction endonucleases. The resulting

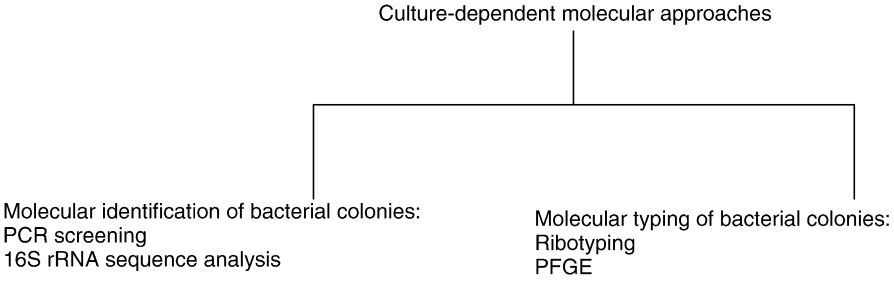

Figure 2.2
Culture-dependent molecular approaches.

restriction fragments are separated by agarose gel electrophoresis and transferred to nylon membranes. A radioactively labeled 16S or 23S rDNA group-specific probe is used to detect the restriction fragments in the digests that contains rRNA sequences. Hybridization is detected by autoradiography. Most bacteria carry multiple rRNA operons, therefore several restriction fragments of different sizes are detected after hybridization with the DNA probe. The hybridization pattern displays a genetic fingerprint or ribotype for the bacterial strain analyzed.

2.2.2.2 Pulsed-Field Gel Electrophoresis

Genomic DNA extracted from bacterial colonies randomly selected on selective medium is digested with an appropriate restriction endonuclease. The resulting restriction fragments are separated by PFGE (pulsed-field gel electrophoresis). Compared to conventional electrophoresis, PFGE separates large DNA fragments in the megabase range such as chromosomal DNA. After electrophoresis, gel is stained with ethidium bromide and examined by UV illumination. The gel obtained displays a genetic fingerprint or pulsotype for the bacterial strain analyzed.

2.2.2.3 Application in Human Intervention Studies

McCartney et al. (1996) applied ribotyping and PFGE to describe and monitor the *Bifidobacterium* and *Lactobacillus* populations in two adult volunteers over a period of one year. Five strains of *Bifidobacterium* were identified in one subject throughout the 12-month period. The *Bifidobacterium* population was relatively simple and stable over time, and among the isolates detected one strain was numerically predominant. In contrast, the other subject harbored a more complex and unstable *Bifidobacterium* composition, 36 sporadic or transient strains were identified during the 12-month period. A unique and distinctive collection of *Bifidobacterium* featuring its level of diversity and strain specificity was a characteristic of each subject. The authors also observed that both volunteers harbored a distinctive and predominant strain of *Lactobacillus*. McCartney and colleagues highlighted the complementarity of both molecular typing methods. Indeed the same ribotype was observed for both *Lactobacillus* isolates; however PFGE permitted to discriminate them in two distinctive strains. Kimura et al. (1997) corroborated the results of McCartney and colleagues in a similar study involving ten volunteers.

2.2.2.4 Application in Probiotic Intervention Studies

The composition of the *Lactobacillus* population of ten healthy subjects was monitored before (6 months control period), during (6 months test period), and after (3 months post test period) the consumption of a dairy product containing *Lactobacillus rhamnosus* DR20 (daily dose, 1.6×10^9 lactobacilli) (Tannock et al., 2000). PFGE was used to differentiate DR20 from the other strains present in the samples. Inter-individual comparisons of pulsotypes showed that each subject harbored a unique and distinctive collection of lactobacilli. Intra-individual comparisons revealed that DR20 was temporary detected in dominance among the subjects whose *Lactobacillus* populations fluctuated in terms of size or composition. In contrast DR20 did not predominate among subjects whose *Lactobacillus* composition was stable. Tannock and colleagues defined two types of bacterial strains characteristics of the *Bifidobacterium* and *Lactobacillus* populations. Those which permanently resided in the fecal bacterial community were defined as autochtonous or indigenous, and those which were detected occasionally were defined as allochtonous or transient.

2.2.3 Limits

The methods described can only be applied to the cultivable fraction of the fecal bacterial community.

Those 16S rRNA based approaches rely on the availability of group-specific PCR primers and probes.

2.3 Culture-Independent Molecular Approaches Based on 16S rRNA

2.3.1 Electrophoresis of PCR-Amplified 16S rDNA Amplicons

2.3.1.1 Principle

The technique allows profiling the genetic diversity and complexity of a bacterial community. The principle relies on: (1) the isolation of total genomic DNA or RNA (cDNA) from the sample; (2) amplification of 16S rRNA gene with specific

PCR primers; and (3) electrophoretic separation of 16S rDNA amplicons of equal length but differing in base-pair, and according to their sequence-dependent melting temperature. Separation occurs within a polyacrylamide gel where a denaturing gradient is generated either with a linearly increasing gradient of temperature over time (TTGE: temporal temperature gradient gel electrophoresis and TGGE: temporal gradient gel electrophoresis) or a linearly increasing gradient of chemical denaturants (urea, formamide) (DGGE: denaturing gradient gel electrophoresis). Once the amplicon reaches its melting temperature at a particular position in the denaturing gel, a conformational change from helical structure to partially melted molecule occurs, and the migration of the DNA fragment will stop. A GC-clamp attached to the 5′ end of one of the PCR primers prevents the amplicons from complete denaturation. The gel is then stained (ethidium bromide, silver staining or SYBR green), digitally captured and analyzed using specific software. The electrophoresis profile generated represents a 16S rDNA genetic fingerprint displaying a banding pattern. Differences in band intensities reflect a relative number of specific amplicons. The technique is considered as a semi-quantitative method rather than a quantitative one. Such approach has been applied to directly visualize and evaluate the heterogeneity or genetic diversity of the gut bacterial community. Zoetendal et al. (1998) estimated that PCR-TGGE profile generated with Eubacterial PCR primers displayed 90–99% of the amplicons. Therefore only dominant bacterial members are detected, nevertheless the use of group-specific PCR primers allows for analysis of specific bacterial sub-populations (*Figure 2.3*).

2.3.1.2 Application in Human Intervention Studies

Dynamic Characterization of the Complexity and Genetic Diversity of the Adult Fecal Microbiota

Zoetendal et al. (1998) applied RT-PCR-TGGE and PCR-TGGE to describe and characterize the species diversity of the dominant fecal bacterial community. The authors compared TGGE profiles derived from fecal RNA and DNA, generated from 16 healthy and unrelated adult volunteers. TGGE patterns derived from RNA and DNA extracted from the same fecal sample were similar. A closer comparison between RNA and DNA derived profiles showed the presence of predominant bacterial species but expressing a low metabolic activity and reciprocally. Inter-individual comparisons showed differences in the position of specific bands, the intensity and number of bands, demonstrating that

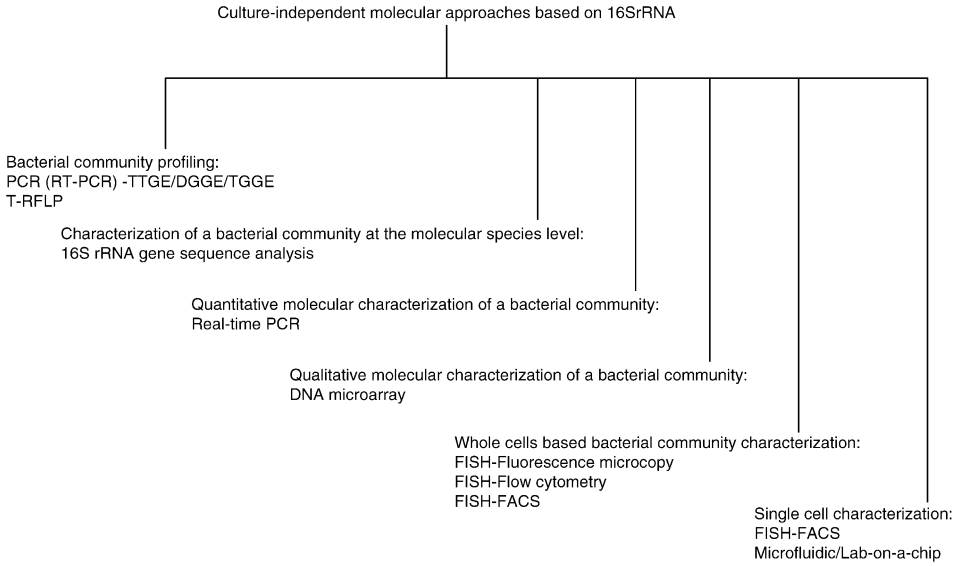

Figure 2.3
Culture-independent molecular approaches based on 16S rRNA.

each individual harbored its unique fecal bacterial community. The dominant fecal bacterial diversity of two subjects was monitored and showed a remarkable stability over time. These observations were corroborated by Seksik et al. (2003) and Vanhoutte et al. (2004). The uniqueness of the fecal bacterial community is not absolute since some distinct and dominant bands are found in all the subjects. Those bands were identified using a cloning and sequencing approach, and were affiliated to *Ruminococcus obeum*, *Faecalibacterium prausnitzii* and *Eubacteriuum hallii*.

According to Zoetendal et al. (2001) reasons of the uniqueness of the fecal bacterial community are likely to be found in host-related factors. The authors observed that PCR-DGGE profiles generated from monozygotic twins were significantly more similar than those derived from marital couples or unrelated individuals. Diversity, uniqueness and stability of the fecal microbiota were also demonstrated within specific bacterial groups. The *Bifidobacterium*, *Bacteroides* and *Clostridium leptum* groups were characterized by their genetic diversity, uniqueness and stability over time (Lay et al., 2007; Satokari et al., 2001a; Vanhoutte et al., 2004). In comparison, fluctuation of bacterial populations within the lactobacilli group was observed over time (Heilig et al., 2002; Walter et al., 2001).

Temporal stability of the fecal bacterial community is disrupted upon antibiotic administration. However, the fecal microbiota is able to cope with this challenge. De La Cochetière et al. (2005) investigated the ability of the dominant fecal bacterial community to return to its initial structure after temporary antibacterial treatment. The dominant species diversity of six healthy subjects was monitored before, during a 5-day course of amoxicillin, and after antibiotic therapy. Alterations of PCR-TTGE profiles were observed under antimicrobial chemotherapy. A return to the initial profile or resilience of the dominant fecal bacterial community was described in five out of the six volunteers within 2 months after the experimental period.

Zoetendal et al. (2002b) compared the distribution of the bacterial community adhering to the colonic mucosa to the luminal fecal bacteria. Fecal samples and biopsies from the ascending, transverse, and descending colons of ten individuals were analyzed by using PCR-DGGE. Stool samples were collected before the colonoscopy, and during the clinical examination three patients were diagnosed with an ulcer and one with a colon polyp. Intra-individual comparisons revealed that the predominant mucosa-associated bacterial community was uniformly distributed along the colon but significantly different from the fecal community. Inter-individual comparisons showed that the colonic mucosa-associated bacterial community is host specific. According to Zoetendal and colleagues these observations support the hypothesis that host-related factors are involved in the determination of the structure of the gut microbiota. No significant differences were observed between healthy individuals and those diagnosed with a disease. Group-specific PCR-DGGE on the lactobacilli showed low species diversity, and for 6 out of the 10 volunteers the *Lactobacillus* community adhering to the intestinal mucosa was similar to the luminal one. Profiles generated from biopsies taken from the descending colon were compared between them; one common band was found in nine individuals, and was affiliated to *Lactobacillus gasseri*. Results from this study were corroborated by Lepage et al. (2005) and Nielsen et al. (2003).

Dynamic Characterization of the Complexity and Genetic Diversity of the Infant Fecal Microbiota

Favier et al. (2002) combined PCR-DGGE and 16S rDNA sequence analysis to describe and characterize the establishment of the fecal bacterial community in two healthy infants during the first ten months of life. DGGE patterns revealed low species diversity during the first weeks following birth. Appearance and disappearance of bands occurred during the first two months. A certain number

of bands were identified by constructing and sequencing 16S rDNA clone libraries generated from both infant fecal samples collected at different ages. Electrophoretic mobility of the clones was also analyzed by PCR-DGGE and compared to the mobility of the amplicons separated in the former denaturing gel. Both infants showed similar bacterial colonization pattern featuring the detection of *Bifidobacterium*, *Streptococcus*, *Enterococcus*, *Ruminococcus*, *Clostridium*, and *Enterobacter*. The authors observed an increase in the complexity of PCR-DGGE profiles during the first ten months of life. These fluctuations of bacterial populations reflect a diversification in the species composition of the dominant infant bacterial community over time.

The species diversity of the fecal bacterial community of 13 infants was monitored at one and seven months following birth (Satokari et al., 2002). Seven babies were breast-fed and the others received an infant formula. Intra-individual comparisons of PCR-DGGE profiles at one and seven months showed fluctuations of the dominant fecal bacterial community. No significant differences were observed between the two experimental groups. Satokari and colleagues identified the species composition by constructing and sequencing 16S rDNA clone libraries. Common DGGE bands found in the majority of the infant profiles were affiliated to *Ruminococcus gnavus*, *Escherichia coli* and the genus *Bifidobacterium*. Group-specific PCR-DGGE on the genus *Bifidobacterium* revealed low species diversity and relative stability in half of the newborns. *Bifidobacterium infantis* was frequently found in both groups. Regarding the *Lactobacillus* group, a similar pattern was observed with however a variable stability. *Lactobacillus acidophilus* was the most detected.

Evidence of Vertical Transmission of Maternal Gut Bacteria to the Infant via Breast Feeding

The neonatal colonization of the infant gut may be promoted by the transmission of maternal gut bacteria through breast feeding. Perez et al. (2007) used eubacterial PCR primers and TTGE to detect and monitor the presence of bacteria in samples of maternal origin (feces, breast milk and blood) and in infant fecal samples. All samples were collected during lactation and over a 4-week period following birth. Control blood samples from non pregnant women were also analyzed. PCR-TTGE profiles generated from fecal samples were host specific, and species diversity was greater in maternal than in infant feces. Bacterial community detected in breast milk leukocytes was less complex than the maternal fecal microbiota. The authors built 16S rRNA clone libraries from breast milk leukocytes and revealed in addition of human milk bacterial

members, the presence of bacterial signatures from the colonic microbiota (*Bacteroides*, *Clostridium* and *Eubacterium*). Detection of bacteria in peripheral blood mononuclear cells was also observed and species diversity was greater in lactating women than in control women. Moreover PCR-TTGE profiles from lactating women were host specific whereas those generated from control women were similar. Common bacterial species were detected in infant feces and in samples of maternal origin. Those TTGE bands were identified by excision, cloning and sequencing, and were affiliated to *Bifidobacterium longum*, *Streptococcus thermophilus and Streptococcus epidermidis*. Perez and colleagues described the translocation of gut bacteria signatures from the mother to the infant via blood circulation and breast milk feeding. A clue of vertical transmission of maternal gut bacteria to the infant via breast feeding was also shown by Martin et al. (2007). The authors demonstrated with PCR-DGGE that the *Lactobacillus* populations detected in infant feces resembled those from the breast milk of the respective mothers.

2.3.1.3 Application in Probiotic and Prebiotic Intervention Studies

Tannock et al. (2000) applied PCR-DGGE to determine whether the daily consumption of a probiotic had an impact or not on the structure of the dominant fecal bacterial community. The bacterial species diversity of ten healthy subjects was monitored before (6 months control period), during (6 months test period), and after (3 months post test period) the administration of a dairy product containing *Lactobacillus rhamnosus* DR20 (daily dose, 1.6×10^9 lactobacilli). Long-term consumption of *Lactobacillus rhamnosus* DR20 did not affect the dominant members of the fecal microbiota regarding the PCR-DGGE profiles generated with universal PCR primers. Using *Lactobacillus* group-specific primers, Walter et al. (2001) investigated in 2 out of the 10 volunteers the impact of DR20 ingestion on the *Lactobacillus* populations. The authors visualized the transient passage of the probiotic strain within the *Lactobacillus* populations during the test period.

Satokari et al. (2001b) observed that the consumption of *Bifidobacterium lactis* Bb-12 and/or galacto-oligosaccharides did not affect the endogenous *Bifidobacterium* populations. Only a transient implantation of the probiotic strain was detected during the ingestion period.

Modulation of RT-PCR-DGGE profiles targeting the Eubacterial domain was observed following daily consumption of biscuits containing prebiotic (Tannock

et al., 2004). Appearance and intensification of bands in RNA-DGGE patterns was a response to galacto-oligosaccharides or fructo-oligosaccharides consumption. Conversely, DGGE profiles generated from bacterial DNA did not show any prebiotic effect. The authors identified the bacterial species incriminated in the alteration of RT-PCR-DGGE profiles. *Bifidobacterium adolescentis* and *Colinsella aerofaciens* were found to be metabolically stimulated when biscuits were consumed.

2.3.1.4 Limits

Heterogeneity between multiple 16S ribosomal RNA operons is characterized by the detection of multiple bands. 16S rRNA sequences are highly conserved among the genus *Bifidobacterium*, and there are multiple copies of the 16S rRNA gene per chromosome. In order to avoid those biases, Requena and colleagues developed a method that enabled the identification and detection of *Bifidobacterium* species by PCR targeting a conserved region of the transaldolase gene common to the *Bifidobacterium* genus (Requena et al., 2002).

Approximately, one band corresponds to one species, however amplicons with variable sequences can also migrate at the same position in the gel. Therefore, the bacterial diversity might be underestimated.

Methodological biases are encountered in all the experimental steps: nucleic acid extraction; PCR; cloning; sequencing and sequences analysis (von Wintzingerode et al., 1997).

2.3.2 T-RFLP

2.3.2.1 Principle

Terminal restriction fragment length polymorphism (T-RFLP) permits profiling the genetic diversity and complexity of a microbial community by analyzing the polymorphism of 16S rRNA gene. The approach is based on: (1) the isolation of total genomic DNA or RNA (cDNA) from the sample; (2) amplification of 16S rRNA gene using fluorescent labeled group-specific PCR primers; (3) restriction enzyme digestion of amplicons; and (4) separation of

the fluorescent terminal restriction fragments through either capillary or polyacrylamide electrophoresis in a DNA sequencer. Only the sizes of the different terminal fluorescently labeled fragments are determined by the fluorescence detector. T-RFLP profile displays a graph or electropherogram where the X axis represents the sizes of the fragment and the Y axis represents their fluorescence intensity. Putative phylogenetic identification of the terminal restriction fragments (T-RFs) generated can be predicted in silico using T-RFLP analysis program which assigns the T-RFs to existing sequences listed in database.

2.3.2.2 Application in Human Intervention Studies

Sakamoto et al. (2003) applied T-RFLP to describe and compare the fecal bacterial community of three healthy adult volunteers. T-RFLP patterns showed that each individual harbored a specific bacterial composition; this host specificity was seen at the dominant level and within the *Bifidobacterium* group.

Dicksved et al. (2007) investigated the impact of life styles on the composition of the fecal microbiota. A cohort of 90 children between 5 and 13 years-old from three European countries were involved in this study. Children were recruited and grouped in two main groups according to their life style. The anthroposophic lifestyle group with its reference group was compared to the farm lifestyle with its reference group. Inter-individual comparisons showed that each child had a unique fecal bacterial community. However, some terminal restriction fragments were observed in almost all the profiles, and those fragments were affiliated to the genus *Eubacterium* and *Clostridium*. Comparison between the experimental groups showed that anthroposophic children had significantly higher species diversity than farm children.

The species diversity of the dominant fecal microbiota was characterized in two groups of infants with and without atopic eczema (Wang et al., 2008). Selected from a cohort, fecal samples from 20 healthy newborns and 15 atopic infants diagnosed with atopic eczema at the age of 18 months were analyzed by T-RFLP and PCR-TTGE. Stool samples had been collected one week after birth, before tests for atopy were performed at the age of 18 months. Both molecular approaches showed a reduced diversity in infant fecal microbiota who later were diagnosed with atopic eczema.

2.3.2.3 Application in Probiotic and Prebiotic Intervention Studies

Jernberg et al. (2005) monitored the impact of antibiotic and probiotic administration on the fecal microbiota in healthy adults. Eight volunteers participated in this study, and were separated into two groups. The probiotic group ingested a dairy product containing *Lactobacillus acidophilus* NCFB 1748, *Lactobacillus paracasei* F19, and *Bifidobacterium lactis* Bb12 (10^8 CFU of each strain per ml) (250 ml) twice daily for 14 days. The placebo group received a yogurt without any of the probiotic strains. Both experimental groups were under antibiotic therapy (clindamycin) for 7 days, and the administration of clindamycin and dairy products were initiated on the same day. Fecal samples were collected before administration (day 0), on the last day of clindamycin administration (day 7), and one week after the end of the experimental diet on day 21. Terminal restriction fragment length polymorphism was used to describe and monitor the dominant fecal bacterial community during the experimental period. In both groups, the authors observed that the consumption of antibiotic induced alterations of the fecal bacterial community characterized by a marked reduction or complete eradication of the genus *Eubacterium*, and appearance of bacterial species belonging to the *Bacteroides* and *Prevotella* group, and the *Enterobacteriaceae*. By day 21, a reestablishment of the fecal microbiota to its initial composition was observed. Finally, a transit of both *Lactobacillus* probiotic strains was observed using *Lactobacillus* group-specific PCR primers.

The effect of prebiotic consumption on the mouse gut microbiota was monitored using T-RFLP approach (Nakanishi et al., 2006). Four experimental groups of mice were designed: a control group and three groups of mice (n = 7 per group) fed with short-chain fructo-oligosaccharide (scFOS) for 5 weeks. The composition of the prebiotic diet was different for each group of mice. Fecal and caecal samples were collected before and at 5 weeks after starting the experimental diet. Compared to the control group, T-RFLP profiles of the dominant microbiota generated from the prebiotic groups showed an increase in a particular group of terminal restriction fragments that were affiliated to the *Bacteroidetes* phylum.

2.3.2.4 Limits

Methodological biases are encountered in all the experimental steps: nucleic acid extraction; PCR; restriction enzyme digestion of amplicons.

The bacterial diversity may be underestimated because same T-RFs can be generated by different species of bacteria.

2.3.3 16S rRNA Gene Sequence Analysis

2.3.3.1 Principle

16S rRNA gene sequence analysis permits characterizing the members of a microbial community at the molecular species level. The method is based on the amplification and cloning of 16S rRNA gene from genomic DNA or RNA (cDNA) extracted from the total microbial community. Selected clones are then sequenced and analyzed in silico. Bioinformatic tools and phylogenetic approaches are utilized to align and affiliate the clones to their closest relatives. The aligned sequences are finally grouped in "an entity" named molecular species or OTUs (Operational Taxonomic Unit) or phylotypes and visualized in a phylogenetic tree. Stackebrandt and Goebel (1994) established that microorganisms having a 16S rRNA gene sequence similarity >97% belong to the same species. This threshold value delimitating two species is a reference in microbial ecology. Therefore a group of 16S rRNA sequences or clones having a sequence similarity >97% is affiliated to the same molecular species. One OTU or molecular species can be either affiliated to a cultivated bacterial species, or a not yet cultivated bacterial species. 16S rRNA gene sequences are a valuable resource because they constitute a database, that allows designing in silico oligonucleotide probes and primers.

2.3.3.2 Application in Human Intervention Studies

This molecular approach has been applied to explore in depth the bacterial diversity of the gut microbiota in healthy adults (Eckburg et al., 2005; Hayashi et al., 2002; Suau et al., 1999; Wilson and Blitchington, 1996). Large interindividual variations were observed reflecting the uniqueness of the gut microbiota in each subject. Phylogenetic distribution of the clones generated from those studies showed that three main bacterial groups, *Clostridium coccoides*, *Clostridium leptum* and the *Bacteroides* constituted the core of the colonic microbiota. A striking observation was that large numbers of the determined phylotypes were affiliated to novel and uncultivated species. Suau et al. (1999)

estimated the phylogenetic diversity of the human fecal microbiota by using the "Good's coverage" as a statistical model. The authors analyzed a 16S rRNA gene clone library generated from one healthy adult volunteer. The 284 clones isolated from the library were gathered into 82 OTUs for which 76% were affiliated to uncultivated species. The majority of the clones (95%) were distributed within the *Clostridium coccoides*, *Clostridium leptum* and *Bacteroides* groups. Proportions of OTUs corresponding to novel species were 85% for the *Clostridium leptum* group, 77.4% for the *Clostridium coccoides* group, and 62% for the *Bacteroides* group. Suau and colleagues statistically measured the coverage of their clone library in terms of species diversity. From the 284 clones analyzed, a global coverage of 85% was determined meaning that the isolation of 100 supplementary clones would have permitted to detect potentially 15 novel species. In other terms, wider species diversity would have been described.

Large-scale phylogenetic analyses of the human gut microbiota in terms of number of 16S rDNA clones generated, were undertaken in two studies. Bacterial and archaeal 16S rRNA gene libraries were generated from feces and biopsies taken at different colonic sites from three healthy adult subjects (Eckburg et al., 2005). From the pool of sequences generated (13,355) 395 bacterial phylotypes, and one single archaeal phylotype were determined. The bacterial phylotypes were mainly distributed within the Firmicutes and the Bacteroidetes phyla, and most of the sequences were affiliated to novel OTUs and derived from uncultivated species. Intra-individual comparisons showed differences in bacterial composition between the mucosal and luminal microbiota. Eckburg and colleagues observed large inter-individual variations, and those differences between individuals were greater than the differences observed between different sampling sites collected from one subject. Ley et al. (2006) monitored the fecal bacterial diversity of 12 obese volunteers randomly assigned to either a fat-restricted or to a carbohydrate-restricted low calorie-diet over a period of one year. Stool samples were collected before and at 12, 26, and 52 weeks after starting diet therapy. Two control lean humans also participated in this study. The authors built a 16S rRNA gene clone library to monitor the composition of the fecal microbiota. The large pool of 16S rDNA sequences generated (18,348) were classified into 4,074 OTUs, and were mainly distributed within the Bacteroidetes and the Firmicutes. Most of the phylotypes identified (70%) were unique to each individual. Intra-individual comparisons showed a stability of the fecal bacterial community over time. The authors observed that compared to the lean controls,

obese subjects had fewer Bacteroidetes and more Firmicutes before starting low calorie-diet. In the obese group regardless the type of diet, the relative abundance of Bacteroidetes increased and the proportion of Firmicutes decreased during the experimental period. Statistical analysis showed that the proportion of Bacteroidetes increased with weight loss on the two types of low-calorie diet.

2.3.3.3 Limits

The threshold limit for delimitating an OTU is variable according to studies (Martin, 2002). Therefore, determining the number of molecular species constitutive of the gut microbial community is rather subjective. The coverage is a biodiversity indices and its determination reveals that the total number of species constitutive of the gut microbiota is difficult to assess.

Methodological biases are encountered in all the experimental steps: nucleic acid extraction; PCR; cloning; sequencing and sequences analysis (von Wintzingerode et al., 1997).

2.3.4 Whole Cell Fluorescence *In Situ* Hybridization

2.3.4.1 Principle

Fluorescence *in situ* hybridization is based on the complementary binding of labeled nucleic acid probes to complementary sequences in cells or tissue sections. In microbial ecology, this technique is mainly applied to detect and quantify specific bacterial populations within a complex microbial community. Using specific 16S or 23S rRNA oligonucleotide probes (DNA probes) whole bacterial cells can be targeted *in situ* within an assemblage of mixed microbial populations. This strategy has the advantage of taking into account the cultivable and uncultivable fractions of a bacterial community. The DNA probes target the single strand rRNA molecules localized within the ribosomes. Probes are 5′ labeled with a fluorescent dye permitting detection and quantification of the specific bacterial population targeted. Two systems of fluorescence detection are combined with the technology of whole cell Fluorescence *in situ* Hybridization (FISH): Fluorescence Microscopy and Flow Cytometry.

2.3.4.2 Fluorescence *In Situ* Hybridization Combined with Microscopic Detection

Principle

According to Welling et al. (1997), the detection limit of whole cell fluorescence *in situ* hybridization combined with microscopic detection is estimated at 10^6 bacteria per gram of stool sample. Laborious and time consuming, visual and manual microscopic counting has been automated by coupling fluorescence microscopy to image analysis (Jansen et al., 1999). Using this automated counting system, the threshold is estimated at 10^7 bacteria per gram of stool sample. Therefore, only the dominant fecal bacteria can be detected. In addition to detection and quantification, *in situ* identification, morphological and topographical information are others advantages offered by fluorescence microscopy.

Application in Human Intervention Studies

DNA probes targeting diverse bacterial populations have been developed and applied to quantify and monitor the composition of the fecal microbiota in healthy volunteers (Franks et al., 1998; Harmsen et al., 1999, 2000, 2002; Langendijk et al., 1995; Suau et al., 2001). Large inter-individual variations in term of bacterial composition were observed; however three bacterial groups, *Clostridium coccoides*, *Clostridium leptum* and *Bacteroides* constituted the main core of the adult fecal bacterial community (Franks et al., 1998; Harmsen et al., 2002).

Application in Probiotic Intervention Studies

The composition of the fecal microbiota of ten healthy subjects was monitored before (6 months control period), during (6 months test period) and after (3 months post test period) the administration of a probiotic product containing *Lactobacillus rhamnosus* DR20 (daily dose, 1.6×10^9 lactobacilli) (Tannock et al., 2000). FISH combined with automated microscopy showed that long-term consumption of *Lactobacillus rhamnosus* DR20 did not affect the dominant members of the fecal microbiota, regarding the bacterial groups investigated: *Bacteroides*; *Clostridium coccoides-Eubacterium rectale*; *Atopobium*; *Bifidobacterium* and the gram-positive low-G + C content group 2 bacteria.

The establishment of the fecal bacterial community of breastfed infants at risk of allergy (n = 132) was monitored throughout the first 2 years of life (Rinne et al., 2006). Following birth, infants received either a probiotic *Lactobacillus rhamnosus* GG (daily dose, 1.0×10^{10} lactobacilli) or a placebo throughout the first 6 months of life. Fecal bacterial community were analyzed and monitored at 6, 12, 18, and

24 months. Results showed that administration of *Lactobacillus rhamnosus* GG in the first months of life did not significantly interfere with the establishment of the *Bifidobacterium*, *Lactobacillus-Enterococcus*, *Bacteroides-Prevotella* and *Clostridium histolyticum* groups.

In a preclinical study, Dinoto et al. (2006) investigated the effects of administration of raffinose and encapsulated *Bifidobacterium breve* JCM 1192^T on the rat cecal microbiota. Twenty-four rats divided in four groups, were fed for 3 weeks with four different diets: basal diet (group BD); basal diet supplemented with raffinose (group RAF); basal diet supplemented with encapsulated *Bifidobacterium breve* JCM 1192^T (group CB) and basal diet supplemented with both raffinose and *Bifidobacterium breve* JCM 1192^T (group RCB). The combination of raffinose and *Bifidobacterium breve* JCM 1192^T was referred as a synbiotic, an association of prebiotic and probiotic. A *Bifidobacterium breve* specific oligonucleotide probe, PBR2, combined with helper probes to increase its *in situ* accessibility, was developed in order to monitor the influence of the probiotic consumption on the rat cecal microbiota. Interestingly, the probiotic strain administrated was only detected in the RCB group (7.3% of the total bacterial population) compared to the CB group where no proliferation was observed. These results indicated that raffinose influences on the cecal proliferation of *Bifidobacterium breve* JCM 1192^T and supports the concept of combining a probiotic strain with its preferred substrate.

2.3.4.3 Fluorescence *In Situ* Hybridization Combined with Flow Cytometry Detection

Principle

Combined with flow cytometry, whole cell fluorescence *in situ* hybridization represents a high throughput quantitative and qualitative method of analysis. Rapid and easy to set up, flow cytometry associates quantitative and multiparametrics analysis system (size, internal granularity and fluorescence signal). According to Lay (2004), a threshold of 0.4% relative to the total number of bacteria determined with the probe EUB338 was shown. A limit of detection around 10^4 CFU/ml was demonstrated using FISH and serial dilutions of *Escherichia coli* cells suspension (unpublished data).

Application in Human Intervention Studies

Flow cytometry has been used as a powerful tool to determine the specificity and labeling efficiency of DNA probes (Fallani et al., 2006; Fuchs et al., 1998; Lay et al.,

2005b; Rigottier-Gois et al., 2003b; Saunier et al., 2005). 16S rRNA group and species-specific probes validated with fluorescence microscopy, and newly developed probes, have been applied to quantify and monitor the composition of the fecal microbiota with flow cytometry (Fallani et al., 2006; Lay et al., 2005b; Rigottier-Gois et al., 2003a; Zoetendal et al., 2002a). Rigottier-Gois et al. (2003a) applied a panel of 6 DNA probes to investigate the fecal bacterial community in 23 healthy adult volunteers. Lay et al. (2005a) expanded the panel of probes to 18, and analyzed the bacterial composition of fecal samples (n = 91) collected from five northern European countries (Denmark, France, Germany, the Netherlands, and the United Kingdom). On average 75% of fecal bacteria could be identified with the extended panel of probes, whereas a proportion of 51% of fecal bacteria were recognized in the study of Rigottier-Gois and colleagues. Large inter-individual variations were observed. Each individual harbors a specific fecal bacterial community or signature in term of bacterial composition. The *Clostridium coccoides* and *Clostridum leptum* groups were co-dominant and constituted more than 50% of the total bacterial population, followed by the *Bacteroides*, *Bifidobacterium* and *Atopobium*. No significant correlations between the composition of the colonic microbiota and the parameters; age, gender, and geographical origin were found. Conversely, in a study involving a cohort of 230 healthy volunteers (85 adults and 145 elderlies (>60 years)) recruited from four European countries (France, Germany, Italy, and Sweden) some associations were identified (Mueller et al., 2006). The Italian cohort was characterized by a higher proportion of *Bifodobacterium*, two- to threefold higher than in the three others groups of European volunteers. Independently of the geographical location higher proportions of enterobacteria were found in elderly subjects. Male volunteers harbored a higher proportion of *Bacteroides* than female.

Mah et al. (2007) monitored the establishment of the fecal bacterial community of 37 infants at risk of developing allergic disease, from birth to one year. FISH combined with flow cytometry (FISH-FC) showed that the colonic bacterial colonization followed a pattern featuring an expansion of the bifidobacterial population during the first three months of life. Whereas enterobacterial and *Bacteroides-Prevotella* populations decreased over time, *Eubacterium rectale-Clostridium coccoides* and *Atopobium* groups gradually increased. Members of the *Clostridium leptum* subgroup formed only a small fraction of the total fecal microbiota at one year of age.

Application in Probiotic Intervention Studies

Garrido et al. (2005) monitored the composition of the fecal bacterial community in ten volunteers, who ingested daily different amounts of the probiotic

preparation *Lactobacillus johnsonii* La1 (La1): 100 ml of 10^8 CFU/ml of La1 during the first week; 200 ml of 10^8 CFU/ml of La1 during the second week and 500 ml of 10^8 CFU/ml of La1 during the third week. A baseline period (before administration, 5 days) and a post-ingestion period (7 weeks) were also monitored during the trial. FISH-FC showed that La1 intake increased the populations of *Clostridium histolyticum*, *Lactobacillus-Enterococcus* and *Bifidobacterium*, and decreased those of *Faecalibacterium prausnitzii*. These bacterial groups returned to their basal levels during the post-ingestion period. No control group was defined in this study.

The influence of consumption of the fermented milk containing *Lactobacillus casei* DN-114001 (daily dose of 3.0×10^{10} CFU) on the fecal bacterial community was monitored in 12 healthy subjects, before (control period of 1 week), during (10 days supplementation) and after (10 days post-ingestion) the probiotic administration (Rochet et al., 2006). Compared to the study of Garrido et al. (2005), the probiotic supplementation did not affect the dominant members of the fecal microbiota regarding the seven bacterial groups investigated.

Mah et al. (2007) monitored the fecal bacterial community of 37 infants at risk of developing atopy, with (n = 20) and without probiotic administration (n = 17) during the first year of life. The probiotic group received daily an infant formula containing *Bifidobacterium longum* BB536 (1.0×10^7 CFU/g) and *Lactobacillus rhamnosus* GG (2.0×10^7 CFU/g) during the first six months. The authors observed that the intestinal colonization followed a pattern regardless of probiotic administration.

2.3.4.4 Phylogenic Gap

The determination of the composition of the fecal bacterial community using FISH has shown that the panel of existing phylogenetic probes does not cover the totality of the colonic bacterial population. This cellular fraction, phylogenetically unaffiliated has been defined as the phylogenetic gap (Lay et al., 2005a). Twelve DNA probes were applied in the study of Harmsen et al. (2002) permitting to take into account more than 56% of the total cell population. Rigottier-Gois et al. (2003a) observed that 49% of the total fecal bacterial population was not recognized by any of the six phylogenetic probes selected. The characterization of the fecal microbiota composition of 91 individuals from five northern European countries showed that on average, a proportion of 25% of the fecal bacteria still remained unidentified (Lay et al., 2005a). Such proportion of untargeted

bacteria also exists within specific bacterial groups. Rigottier-Gois et al. (2003b) determined 34% of unidentified species within the *Bacteroides* group. Lay et al. (2007) observed a phylogenetic gap of 9% within the *Clostridium leptum* subgroup.

2.3.4.5 Fluorescence Activated Cell Sorting

Flow cytometry equipped with a cell sorting function allows sorting specific bacterial cell populations from a heterogeneous community of bacteria. This approach was applied to identify the phylogenetic gap observed within the *Clostridium leptum* subgroup (Lay et al., 2007). Using FISH-Fluorescence activated cell sorting (FISH-FACS) combined with 16S rRNA gene cloning strategy the authors identified new molecular species within this Gram-positive bacterial group.

Amor et al. (2002) used FACS and fluorescent viability probes to sort viable, injured, and dead bacteria from fecal microbiota. Three bacterial sub populations were sorted and characterized by PCR-DGGE, and cloning of PCR-DGGE dominant bands. Members of *Clostridium coccoides*, *Clostridium leptum*, and *Bacteroides* were found in the three physiological groups of sorted bacterial populations.

2.3.4.6 Limits

Parameters Affecting the Fluorescence Detection

Cell Wall Permeability In particular, the cell wall of Gram-positive bacteria represents a protective structure to crossover. Therefore, a standardized hybridization procedure including a permeabilization step is needed (Lay et al., 2005b). Once within the cell, probes face with another physical hurdle, the secondary structure of rRNA molecules and their molecular interactions within the ribosome, which may hinder the access of the probes to their target sites.

***In Situ* Accessibility** The *in situ* accessibility of a probe to its target site determines the probe-conferred fluorescence or probe-mediated fluorescence (Fuchs et al., 1998). The concept relies on the principle that high *in situ* accessibility would facilitate the binding of the probe to its target site, and hence allowing the probe to emit a bright fluorescence signal. The determination of the brightness of fluorescence, or probe relative fluorescence conferred by a probe is a means to

evaluate its *in situ* accessibility (Fuchs et al., 1998; Lay et al., 2005b). Modeling secondary structure of the 16S rRNA has permitted an in silico diagnostic of the *in situ* accessibility of the entire molecule (Fuchs et al., 1998; Kumar et al., 2005; Saunier et al., 2005). Therefore, the target site of a probe can be assessed in terms of *in situ* accessibility. If the target region is determined as a lower or non-accessible site, a strategy using helpers or unlabeled oligonucleotide probes can be developed in order to promote the binding of the probe to its target and hence, amplifying the fluorescence signal emission (Fuchs et al., 2000). Helpers are designed in silico to open inaccessible regions and are complementary to regions adjacent to the probe's target site (Dinoto et al., 2006; Fuchs et al., 2000; Saunier et al., 2005).

Ribosomal Content or Metabolic Activity of the Cell The ribosomal content is linked to the physiological state or metabolic activity of the cell. Therefore, bacterial cells in a quiescent state i.e., harboring a low number of ribosomes have weak fluorescence emission and may escape detection.

Hybridization Conditions The stringency of hybridization depends on three parameters: temperature; salt concentration and formamide concentration. Manipulation of these factors influences the specificity of hybridization and hence the fluorescence signal detection.

Others Parameters Affecting the Detection and Quantification

Beside the parameters influencing the fluorescence signal detection, specificity and coverage of the probes may also impact on the outcome. In gastrointestinal microbial ecology, 16S rRNA probes mainly have been developed to quantify and monitor the composition of the fecal microbiota. Cross-hybridization with non-target cells and partial coverage of a bacterial group are the main criteria of exclusion in probes selection. So far, more than 700,000 16S rRNA sequences are available from the Ribosomal Database Project website (http://rdp.cme.msu.edu), permitting the in silico development and validation of a large panel of phylogenetic probes. Several probes targeting members of the gut microbiota were designed using the previous version of the Ribosomal Database Project website (Release 8.1). With the increasing number of 16S rRNA sequences available, reassessment of the specificity and coverage of those oligonucleotide probes is essential in order to update and reconfirm their reliability. The use of competitor strategy has enhanced the specificity of several DNA probes, previously and newly designed (Lay et al., 2005b). The approach consists to

combine the "specific" probe with unlabeled oligonucleotides targeting the non-specific targets.

2.3.5 Quantitative Real-Time PCR

2.3.5.1 Principle

Quantitative real-time PCR (qPCR) enables to amplify, detect and quantify a target DNA or RNA. Frequently, real-time PCR is combined with reverse transcription to quantify RNA. A system of detection based on fluorescence signal emission is applied to monitor and quantify the accumulation of amplicons during the reaction. The fluorescence signal emission is proportional to the accumulation of amplicons, therefore the initial amount of DNA or RNA present in a sample can be indirectly quantified by determining the cycle threshold (Ct). The cycle threshold corresponds to the point at which the reaction reaches the fluorescence intensity above the background. The Ct is conversely proportional to the initial amount of DNA or RNA present in the sample. The comparison of the Ct value to a standard curve determined from samples of known concentrations enables to extrapolate the initial quantity of target DNA or RNA. Two fluorescence based technology of amplicons detection are used in quantitative real-time PCR, the fluorescent dye SYBR Green that intercalates with double-stranded DNA and fluorescent reporter probes that fluoresce when hybridized with a complementary DNA.

2.3.5.2 Application in Human Intervention Studies

SYBR Green-based real-time quantitative PCR has been used to detect and quantify different fecal bacterial populations. Matsuki et al. (2004) described the distribution of *Bifidobacterium* in 46 healthy adult volunteers. The *Bifidobacterium catenulatum* group, *Bifidobacterium adolescentis* group, and *Bifidobacterium longum* species were the most predominant populations detected. The authors validated their results with those derived from culture, and FISH combined with fluorescence microscopy. Real-time quantitative PCR was 10–100 times more sensitive than plate-count and FISH methods. The composition of the elderly-like fecal bacterial community was described in healthy volunteers (n = 35), hospitalized patients (n = 38), and hospitalized patients receiving antibiotic treatment for non-intestinal affections (n = 31) (Bartosch et al., 2004).

Compared to the healthy control group, both cohorts of hospitalized patients were characterized by a marked reduction in the *Bacteroides-Prevotella* group. Antibiotic treatment was correlated with an increase of *Enterococcus faecalis*.

Another alternative to the fluorescent dye SYBR Green is the TaqMan hydrolysis probe. TaqMan probes are oligonucleotides designed to hybridize specifically between the forward and reverse primer to an internal region of the target sequence, and are labeled with a reporter fluorophore at the 5′ end, and a quencher at the 3′ end. A phosphate group is also added at the 3′ end to prevent extension of the reporter probe by the Taq polymerase. During the amplification the 5′ exonuclease activity of the Taq polymerase degrades the hybridized probe, releasing the reporter from the quencher, and as a result the fluorescence intensity of the reporter dye increases. The sensitivity and specificity of TaqMan probes have been increased by combining at the 3′ end conjugated minor groove binders (MGBs), which form extremely stable duplexes with the target DNA. Compared with unmodified DNA probes, MGB probes have higher melting temperature and increased specificity (Ott et al., 2004). PCR primers in combination with TaqMan probes targeting diverse bacterial groups and species have been developed and applied to quantify and monitor the composition of the gut microbiota (Gueimonde et al., 2004; Huijsdens et al., 2002; Ott et al., 2004; Requena et al., 2002).

In a large scale epidemiologic study the composition of the fecal samples of 1,032 infants collected at 1 month of age were analyzed by quantitative real time PCR (Penders et al., 2006). Penders and colleagues examined external factors influencing the composition of the fecal bacterial community in infants, and particularly regarding the *Bifidobacterium* group, *Escherichia coli*, *Clostridium difficile*, *Bacteroides fragilis*, and lactobacilli. Factors influencing the gut microbiota development include mode of delivery, hospitalization, prematurity, antibiotic intake, breast feeding versus infant formula and presence of siblings.

2.3.5.3 Application in Prebiotic Intervention Studies

Haarman and Knol (2005) designed and validated a panel of primers and probes targeting several *Bifidobacterium* species in order to monitor their distribution in infant fecal samples. Three groups of infants participated in this study, formula-fed (n = 10), formula-fed supplemented with galacto-oligosaccharides and fructo-oligosaccharides (n = 10), and a breast-fed control group (n = 10). Fecal samples were collected at the beginning of the study and after six weeks of intervention. An increase in the total number of *Bifidobacterium* was observed in

the prebiotic group, and the species diversity was similar to that of the breast fed group. *Bifidobacterium infantis*, *Bifidobacterium breve*, and *Bifidobacterium longum* were the predominant species detected in both groups. At the end of the study, the standard formula group was characterized by a decrease in *Bifidobacterium breve* and an increase in *Bifidobacterium catenulatum* and *Bifidobacterium adolescentis*.

2.3.5.4 Limits

Methodological biases are encountered in all the experimental steps: nucleic acid extraction; PCR (von Wintzingerode et al., 1997).

Biases are also introduced into the analysis with the different methods to express qPCR results using the standard curve.

2.3.6 DNA Microarray

2.3.6.1 Principle

DNA microarray analysis is based on nucleic acid hybridization and relies on the complementary binding of labeled amplicons to complementary nucleic acid probes pre-immobilized onto a surface. Multiple 16S rDNA probes targeting a panel of dominant fecal bacterial species are pre-immobilized onto a surface, and those probes are potentially revealed after hybridization with labeled polynucleotides amplified by PCR from fecal DNA or RNA (cDNA). Hybridization is detected either with a colorimetric- enzymatic reaction or a physical reaction based on the excitation of fluorochrome. The approach is rather qualitative than quantitative, indeed according to the signal intensity generated from the hybridization reaction, only positive or negative detection is revealed.

2.3.6.2 Application in Human Intervention Studies

Wang et al. (2002) developed and validated a nitrocellulose-based membrane-array featuring a panel of 20 triplets of 16S rDNA probes targeting 20 predominant bacterial species of the fecal microbiota. The full length 16S rRNA gene was amplified from fecal samples collected from three volunteers. Amplicons labeled

with digoxigenin were hybridized to the membrane-array and colorimetric-enzymatic reaction was used to reveal the hybridization. Results showed that some species were common to all subjects; however each volunteer had its unique bacterial species composition. Following the development of this membrane-array, Wang et al. (2004) designed an oligonucleotide-microarray for the detection of 40 bacterial species. Fecal samples collected from 11 volunteers were screened. 16S rDNA amplicons labeled with a fluorochrome were hybridized to the array of probes pre-immobilized on an epoxy-based matrix. A microarray laser scanner was used to read the fluorescence emission corresponding to a positive signal of hybridization. Inter-individual comparisons revealed that the bacterial species composition is host-specific. The authors observed that 25–37 of the 40 bacterial species could be detected in each fecal sample, and that 33 of the species were found in a majority of the samples.

Palmer et al. (2007) developed and validated a 16S rDNA microarray to describe and monitor qualitatively the bacterial colonization of the infant gut. The authors also investigated the provenance of the colonizers by looking at the bacterial composition of samples (stool, vaginal and breast milk) collected from parents and siblings. The microarray featured 9,121 group and species-level taxonomic probes. Palmer and colleagues tested the performance of their microarray with a set of biological samples. For each individual sample, they compared the 16S rDNA profile generated from the microarray with sequence analysis generated from a clone library. Results obtained from both strategies of 16S rDNA analysis were similar and showed that Bacteroidetes, Firmicutes, Proteobacteria, and Actinobacteria were the main phyla represented in the samples. Fecal samples from 14 healthy babies were collected from birth to the age of one year. 16S rDNA microarray approach was used to profile and monitor the fecal bacterial community of each infant over a 1 year period. On average 26 fecal samples per infant were analyzed. The majority of the bacterial species detected was distributed within the Bacteroidetes, Firmicutes, Proteobacteria, and Actinobacteria. Inter-individual comparisons showed that each infant had its unique collection of fecal bacteria. Interestingly, similar bacterial profiles were observed for a pair of twins included in the study. Intra-individual comparisons revealed a dynamic fecal bacterial community characterized by periods of relative stability punctuated with the occurrence of marked fluctuations in bacterial populations. Similarities in bacterial composition were found between some infant samples and samples of their respective mothers. At the age of one year, the composition of the infant fecal microbiota is predominated by the Bacteroidetes and Firmicutes, a characteristic of the adult-like fecal microbiota.

2.3.6.3 Limits

The approach is qualitative rather than quantitative.

Methodological biases are encountered in all the experimental steps: nucleic acid extraction; PCR; hybridization.

Using universal PCR primers, only dominant bacterial species can be detected.

The coverage is a biodiversity index and its determination reveals that the total number of species constitutive of the gut microbiota is difficult to assess. Therefore, the development of a microarray that allows covering the species diversity of the colonic microbiota is difficult to consider.

2.4 Culture-Independent Genomic Approaches

2.4.1 Metagenomic Approach

2.4.1.1 Principle

The study of the genome of one single organism is called genomics. Metagenomics (also called environmental genomics or community genomics) is a culture-independent approach allowing the analysis of multiple bacterial genomes extracted from environmental samples. Accessing the collection of bacterial genomes belonging to a particular environmental niche (also termed the metagenome) through metagenomic methods allows investigation and characterisation of the genetic potential of the microbial community, and hence inferring its potential activity within the ecosystem. In gastro-intestinal microbial ecology, the term microbiome refers to the total number of genes encoded by the collective genomes of the gut bacterial members. Metagenomics analysis of the gut microbiota has been investigated in several studies. The approach relies on: (1) the isolation of the metagenomic DNA from the bowel sample; (2) cloning of large fragments of bacterial genomic DNA into BAC (bacterial artificial chromosome) or fosmids; and (3) analysis of the metagenomic library. Several approaches can be applied to analyze a metagenomic library. A genetic and functional screening is used when the gene of interest is known. A random approach based on shotgun sequencing is applied when the genetic information encoded by the metagenome is deciphered. Recently, metagenomics has been combined to an emerging high-throughput DNA sequencing strategy termed as pyrosequencing (Margulies

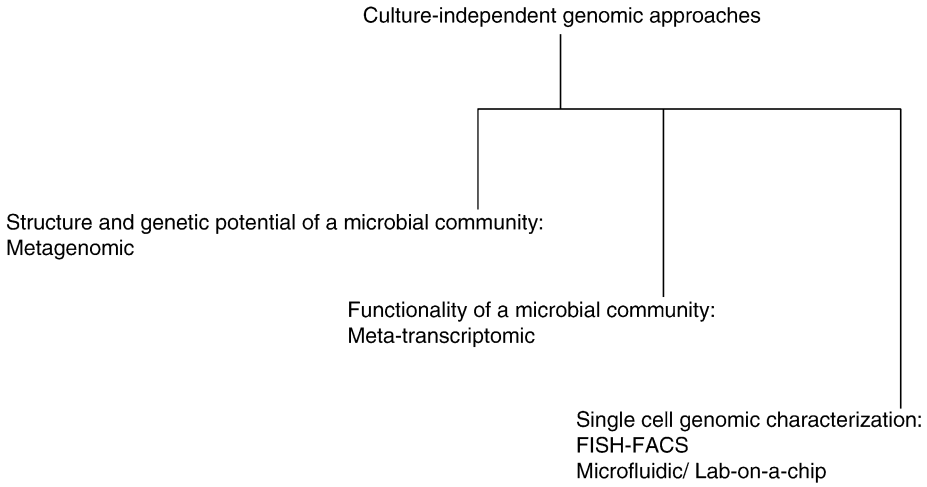

Figure 2.4
Culture-independent genomic approaches.

et al., 2005). Pyrosequencing is a clone-free sequencing-by-synthesis technology, which allows sequencing directly circumventing the need for clone library construction (*Figure 2.4*).

2.4.1.2 Application in Human Intervention Studies

Manichanh et al. (2006) developed a metagenomic approach to investigate the fecal bacterial diversity in Crohn's disease patients in remission (n = 6) and in healthy donors (n = 6). Two metagenomic libraries were generated from these two groups of volunteers. The clone libraries were screened for 16S rRNA gene using a DNA hybridization approach. Positives clones carrying 16S rDNA insert were then sequenced and analyzed. Four bacterial phyla, Bacteroidetes, Firmicutes, Actinobacteria and Proteobacteria were described in the fecal microbiome of both groups of volunteers. The authors observed a reduced bacterial diversity within the Firmicutes phylum and particularly within the *Clostridium leptum* subgroup in patients with Crohn's disease. Following this phylogenetic characterization, those metagenomic clones carrying a taxonomic signature were screened *in vitro* for the modulation of epithelial cell growth (Gloux et al., 2007). Gloux and colleagues developed a high throughput method to detect the presence of bacterial molecular signals involved in the stimulatory or inhibitory effects

of eukaryote cell proliferation. The authors tested *in vitro* the cell growth using bacterial lysates extracted from the metagenomic clones. Putative clones displaying an effect were then screened by transposon mutagenesis and subcloning in order to map and identify the candidate loci. Ubiquitous genes were a common feature of those modulatory clones.

Gill et al. (2006) described the bacterial and functional genetic diversity of two healthy human fecal microbiomes. Phylogenetic analysis of the metagenomic libraries showed that both human gut microbiomes were dominated by the Firmicutes and the Actinobacteria. Functional annotation of the metagenomic DNA sequences showed that the human gut microbiome is a reservoir of genes encoding different metabolic pathways: metabolism of glycans, amino acids, and xenobiotics, methanogenesis, biosynthesis of vitamins.

Kurokawa et al. (2007) performed a comparative metagenomic analysis of fecal samples collected from 13 healthy individuals of various ages including infants. The phylogenetic distribution of the adult-type microbiome showed three distinct phyla, Bacteroidetes, Firmicutes and Actinobacteria. In the infant-type microbiome, members of the Actinobacteria and Proteobacteria phyla were predominant. Compared to the adult-type one, large inter-individual variations were described in the infant-type microbiome. The predicted functional genes identified in both metagenomic libraries showed that the colonic microbiota uses different adaptive strategies to thrive in the intestinal ecosystem, and establish symbiosis with its host. Interestingly, the authors described a rich reservoir of mobile genetic elements in the microbiome suggesting that the gut is a "hot spot" for horizontal gene transfer between intestinal bacterial members. This metagenomic analysis also revealed that 25% of the total genes identified were orphan.

2.4.1.3 Application in Animal Studies

Walter et al. (2005) prepared a metagenomic library from the large bowel microbiota of mice. The clone library was screened for the expression of β-glucanases. Three positive clones were detected and sequenced. Besides the genes encoding glucanolytic enzymes, other putative genes were annotated on the metagenomic DNA inserts. Predictive functions in nutrient acquisition, host-bacterial interactions and bacterial coaggregation were described. Among the three metagenomic clones, two originated from uncultivated bacteria and one featured sequence similarity with *Bacteroides* species.

In a preliminary study based on 16S rRNA gene sequence analysis, Ley et al. (2005) revealed that genetically obese mice compared to their lean counterparts, had a 50% reduction in the abundance of Bacteroidetes and a proportional increase in Firmicutes. Using the same analytical approach, the authors observed the same microbiota structure in obese versus human volunteers (Ley et al., 2006). In this study, Ley and colleagues monitored the fecal bacterial diversity of obese human volunteers randomly assigned to either a fat-restricted or to a carbohydrate-restricted low calorie-diet over a period of one year. In the obese group regardless the type of diet, the relative abundance of Bacteroidetes increased and the proportion of Firmicutes decreased during the experimental period. Also the increase proportion of Bacteroidetes was correlated to weight loss on the two types of low-calorie diet. In order to determine if the collection of bacterial genomes present in obese subjects had an association with obesity, Turnbaugh et al. (2006) used metagenomic to characterize the genetic potential of the bacterial community present in genetically obese mice. Shotgun sequencing of metagenomic DNA prepared from the caecal contents of obese mice and their lean counterparts, revealed that compared to the lean microbiome, the obese microbiome harbored a higher proportion of Firmicutes and had an increase genetic capacity for fermenting polysaccharides. This genetic potential of the gut microbiome to harvest more energy from the diet was demonstrated in another elegant study. Turnbaugh et al. (2008) created a mouse model of obesity by raising conventional mice on a western diet high in saturated fats, unsaturated fats, and carbohydrates. Using 16S rRNA gene sequencing and metagenomic approaches, the authors observed that conventionalized mice initially on a low-fat diet, when switched to a western diet harbored a distinctive cecal microbiota characterized by a higher relative abundance of Firmicutes and a lower proportion of Bacteroidetes. The shift towards the Firmicutes was due to the expansion of an uncultivated Mollicute lineage. Moreover the authors revealed that the western diet-associated gut microbiome was enriched in the import and fermentation of carbohydrates.

2.4.1.4 Limits

Methodological biases are encountered in all the experimental steps: nucleic acid extraction; cloning; genetic (sequencing) or/and functional screening of the metagenomic library.

Linking a metagenomic sequence to its microbial origin is a difficult task.

2.4.2 Meta-Transcriptomic Approach

2.4.2.1 Principle and Recent Applications in Studying Complex Microbial Communities

An inventory of the microbial genes content is performed when metagenomic is applied to investigate the genetic potential of a microbial community. However, this approach does not reflect what genes are actually being expressed within the community. Meta-transcriptomic is the logical continuation of metagenomics. The approach to study the expression of genes in environmental samples relies on: (1) the isolation of the environmental transcripts; (2) reverse transcription of the transcripts to cDNA; (3) synthesis of double-stranded cDNA; (4) cloning the cDNA; and (5) sequencing and analysis of the cDNA clone library. Such method has been applied to investigate gene expression in marine and fresh water bacterioplankton communities (Poretsky et al., 2005). A recent application of meta-transcriptomic approach to investigate the activity of the gut microbiota was performed by Turnbaugh et al. (2008). The authors built a cDNA clone library from enriched mRNA isolated from the cecum of a mouse model of obesity fed with a western diet high in saturated fats, unsaturated fats, and carbohydrates. Phosphotransferase systems involved in the transport of sugars were expressed in the diet-induced obesity cecal microbiome's transcriptome.

Recently, meta-transcriptomic analysis has been combined to pyrosequencing, a clone-free sequencing technology (Margulies et al., 2005). So far, there have been few studies on the application of meta-transcriptomic combined with pyrosequencing to analyze gene expression in complex microbial communities. Frias-Lopez et al. (2008) developed a method to analyze bacterial gene expression in seawater. The authors used an approach based on polyadenylation-dependent RNA amplification to isolate and convert bacterial messenger RNA into cDNA. The bacterial community cDNA was then sequenced by pyrosequencing and analyzed. Genes involved in photosynthesis, carbon fixation, and nitrogen acquisition, were highly expressed in the ocean bacterial community transcriptome. Frias-Lopez and colleagues observed that half of the bacterial messengers identified were novel.

Urich et al. (2008) applied a meta-transcriptomic approach to simultaneously gain insight on both structure and function of a soil microbial community. Total RNA extracted from a soil sample was used as a template for random-hexamer primed reverse transcription. The generated cDNA was directly subjected to

pyrosequencing resulting in 258,411 RNA-tags with an average size of 98 bp. The authors set up a two-step analysis process to identify and differentiate rRNA-tags from mRNA-tags. All RNA-tags were first compared against a compiled rRNA database gathering a small subunit and a large subunit rRNA reference database. In the second step, all unassigned RNA-tags were compared against the Genbank non-redundant protein database to identify mRNA-tags. Seventy five percent of the RNA-tags derived from rRNA, 8% from mRNA, and 17% could not be assigned. The rRNA-tags or ribo-tags were distributed within the three domains of life, Eukaryota, Bacteria and Archaea. Bacterial ribo-tags were the most abundant (85.5%) and were characterized by their wide range of diversity. Among the phyla detected, Actinobacteria, Proteobacteria, Firmicutes, Planctomycetes and Acidobacteria were the most encountered prokaryotic groups. The Crenarchaeota phylum was the predominant archaeal ribo-tag (<2% of the total ribo-tags) identified in the soil sample. Eukaryotal rRNA-tags represented 11% of the total ribo-tags and were affiliated to the Fungi, Viridiplantae, Metazoa and Protist kingdoms. Global functional analysis of the mRNA-tags was compared to metagenomic data derived from the same soil habitat and a farm soil microbial community. Differences in RNA, protein and carbohydrate metabolism were observed between the meta-transcriptome and the metagenomes.

2.4.2.2 Limits

Methodological biases are encountered in all the experimental steps: nucleic acid extraction; reverse transcription; cloning and sequencing or pyrosequencing of the metatranscriptomic library.

2.4.3 From Population Based Analysis to Single Cell Analysis

Metagenomic studies look at the collection of bacterial genomes present in the microbial community. It involves deciphering the genome of an entire community rather than that of an individual bacterial species. A major issue encountered in metagenomic approach concerns the genome assembly which raises the difficulty to assign a functional gene to a bacterial species. Nevertheless, the assignment of a metabolic gene of interest is deconvoluted when a phylogenetic marker

is located on the same large environmental DNA insert. Sorting and reassembling the environmental bacterial chromosomes which were in first instance truncated and then displayed in a metagenomic library comes to resemble searching for a needle in a haystack. However, the ability to obtain a complete genome sequence from an individual bacterial cell directly isolated from the environment has been demonstrated using single-cell techniques. Fluorescence activated cell sorting and innovative technology based on microfluidics have recently been used to sort or isolate single bacteria cells from complex microbial communities. Once isolated, genome analysis at single-cell level can be carried out by combining whole-genome multiple displacement amplification (MDA) to PCR screening or genome sequencing.

2.4.3.1 Fluorescence Activated Cell Sorting

Stepanauskas and Sieracki (2007) combined high-speed droplet based FACS, whole-genome multiple displacement amplification (MDA), and PCR screening to investigate the marine bacterioplankton. Individual bacterioplankton cells fluorescently labeled and displaying high nucleic acid content were sorted from coastal water sample. Single bacterial cells sorted into 96-well plates were then lysed before performing single-cell whole-genome MDA. Large quantity of genomic DNA was generated from single-MDA reaction, which allowed the authors to use PCR approach to screen the presence or absence of specific phylogenetic and functional markers. Using this approach Stepanauskas and Sieracki were able to detect, identify and analyze multiple metabolic genes in not yet cultivated marine bacteria.

Podar et al. (2007) combined FISH, FACS, MDA and cloning-sequencing to characterize an uncultivated bacterial group of the soil microbial community. Present in low abundance among the soil bacterial community, bacteria belonging to the TM7 phylum were detected by hybridization with a fluorescently labeled DNA specific probe, then analyzed and sorted with a fluorescence activated cell sorter. The authors determined empirically that with five sorted cells, an efficient MDA genomic DNA amplification could be achieved with a low level of non-target bacterial cells contamination. 16S rRNA clone library and TM7 genomic DNA library were constructed from the MDA-amplified genomic DNA. With this approach Podar and colleagues inferred some phylogenetic and metabolism features of bacterial members belonging to the TM7 phylum that have not yet been cultivated.

2.4.3.2 Microfluidic Based Devices

Microfluidics is a multidisciplinary field of research requiring competencies from engineering, physics, chemistry, microtechnology and biology. Microfluidic based technology deals with the behavior, precise control and manipulation of fluids that are geometrically constrained to a small volume, typically sub-millimeter scale. The physical dimensions of bacteria matches with such micron-scale size and therefore rendering possible the experimental manipulation of individual bacterial cells. Recently, microfluidic based devices have been developed to analyze directly complex microbial community at single-cell level without any cultivation step.

Ottesen et al. (2006) used an innovative approach based on microfluidic digital PCR technology to separate and interrogate individual bacterial cells harvested from the hindguts of wood-feeding termites. Diluted gut content samples from termites were mixed with PCR reagents and then loaded onto the microfluidic PCR chips. The microfluidic device displayed an array of thousands of interconnected nanoliter reaction chambers. Micromechanical valves were used to randomly partition single PCR mixture into thousands of independent nanoliter reaction chambers. A flat-block thermocycler was used to carry out the PCR, and the amplification reaction was monitored with TaqMan probes. Fluorescent signal generated during the reaction was detected with a customized microarray laser scanner. Amplicons were then retrieved from the reaction chambers and reamplified off-chip, cloned, sequenced and analyzed. Using this microfluidic PCR chip the authors demonstrated the co-amplification of eubacterial 16S rRNA gene and a metabolic gene of interest from single bacterial cells partitioned in nanoliter reaction chambers. Looking for the identity of bacteria encoding homoacetogenesis, Ottesen and colleagues identified not yet cultivated *Treponema* species as carrier of this fermentation activity.

Marcy et al. (2007) designed a microfluidic device allowing to select, isolate and amplify the whole genome of single bacteria from the human oral microbiota. Biofilm samples were collected from periodontal pockets by scraping subgingival tooth surfaces of a healthy volunteer. Samples were loaded onto the chip which was placed on an optical microscope. The single-cell genome amplification device includes series of interconnected nanoliter reaction chambers. The authors targeted an unexplored bacterial phylum of the oral mouth bacterial community, TM7, and particularly a subset of this phylum represented by long filamentous bacteria with a rod-like morphotype. Using this criterion of selection, the single bacterial cells of interest were optically selected and isolated before

undergoing the whole genome amplification process. Sequentially after the on chip optical trapping, the single cell is conveyed to the sorting, lysis, neutralization, and reaction chambers allowing the whole genome amplification reaction to be performed. The chip was placed on a hot plate to carry out the isothermal MDA reaction. The amplified genomic DNA was then recovered from the microfluidic chip and re-amplified off-chip in order to increase the amount of genomic material. 16S rRNA amplification and direct sequencing were performed from the amplified genomic DNA in order to identify the filamentous isolated rod-like cells. Several bacterial cells were affiliated to the TM7 phylum. In order to gain insight into the genome of TM7 bacterial members, high-throughput pyrosequencing and genome assembly were performed on the amplified genomic DNA. Using this approach the authors identified several metabolic pathways within this particular group of bacteria from the human mouth, which has no cultivated members.

2.4.3.3 Limits

Methodological biases are encountered in all the experimental steps: single bacterial cell isolation; whole genome amplification; cloning and sequencing or pyrosequencing.

2.5 Conclusion

This chapter describes the molecular tools that have been applied to investigate the structure and genetic potential of the human gut microbiota, and their applications in human intervention studies. Knowledge on the gut bacterial residents has been gained through a variety of techniques based on 16S rRNA. Beyond the structure of the human gut microbiota, insight into its genetic potential is being deciphered through metagenomic. Once a comprehensive human microbiome metagenomic database is available, the global analysis of transcribed genes expressed by the colonic microbiota will be achievable through meta-transcriptomic. Finally, the possibility to identify which proteins are expressed through meta-proteomic approach, will pave the way to characterize the functionality of the human microbiome (Klaassens et al., 2007). The determination of which genes the gut microbiota is using in its daily activities, or when and in which circumstances those genes are expressed, will allow

profiling the functional signatures of the human microbiome in relation to nutrition, health and disease. Understanding how the microbiota and the microbiome respond to disease or specific diet, and how both entities are regulated will bring clues on how we could improve health by manipulating our intestinal microbiota. The bacterial balance of the gut microbiota is not altered under probiotic supplementation, only a transient intestinal passage of the probiotic is detected. Application of "omics" approaches may certainly elucidate the functional effect of probiotic and prebiotic supplementation on the gut bacterial balance.

What could we expect in gastro-intestinal microbial ecology in the future?

The human fecal viral community is largely unexplored. The implication of bacteriophages in the regulation of the structure of the gut bacterial community, and their suspected role in inflammatory bowel diseases interests the research community (Breitbart et al., 2003, 2008; Lepage et al., 2008). Recently, Finkbeiner and colleagues applied metagenomic to characterize the diversity of the viral gut structure in pediatric patients suffering from diarrhea (Finkbeiner et al., 2008).

From bacterial colonies isolation to total population based approaches, that has been the way microbiologists are used to study complex microbial community. We are now moving to a new dimension, towards single cell technology. Deciphering or interrogating the genome of single bacteria directly isolated from the environmental sample without the need for culture will be the new tendency. The possibility to separate and interrogate hundreds of individual bacteria in parallel to investigate for instance the heterogeneity of clonal bacterial population, or perform a metabolic mapping of uncultivated microorganisms will revolutionize microbiology. With the growing interest in lab-on-a-chip technologies development, on chip cultivation of not yet cultivated bacteria might not be unfeasible, as well as the study on chip of simplified model of bacterial community (Balagadde et al., 2005, 2008).

2.6 Summary

- The bacterial diversity of the human intestinal microbiota has been investigated using a battery of techniques, which has highlighted its complexity, uniqueness and stability.
- Consistent data regarding bacterial members belonging to the "healthy gut" population have been gained. Members of the *Eubacterium rectale-Clostridium coccoides*, *Clostridium leptum* are predominant and represent more than half of the total

bacterial population, followed by the *Bacteroides*, *Bifidobacterium* and *Atopobium* groups. These bacterial groups form the main core representative of the microbial collection resident in the human bowel.

- Each individual harbors a specific gut microbiota signature in terms of bacterial composition reflecting a bacterial balance unique to each individual. Not yet identified host-related factors are involved in the determination of the structure of the gut microbiota.
- The bacterial community adhering to the intestinal mucosa is uniformly distributed along the colon but significantly different from the luminal fecal community.
- The dynamic and idiosyncratic colonization of the infant gut is characterized by substantial demographic change occurring during the first year of life. Clues of vertical transmission of maternal gut bacteria to the infant via breast feeding have been demonstrated.
- Temporal stability of the fecal bacterial community is disrupted upon antibiotic administration, nevertheless the fecal microbiota is able to recover its bacterial balance.
- A probiotic supplementation does not alter the dominant bacterial members of the fecal microbiota. However, modulation at the subdominant level is detected through the transient passage of the probiotic strain during the ingestion period. The bacterial balance of the fecal microbiota withstands the long-term establishment of exogenous invaders. This ecological barrier fulfils its entire function from birth.
- Prebiotic effect is characterized by either the proliferation of a particular bacterial population or the stimulation of the metabolic activity of a particular bacterial population.
- The human gut microbiome is a reservoir of genes encoding different metabolic pathways. A "hot spot" for horizontal gene transfer between intestinal bacterial members has also been described within the microbiome.
- Studies with animal models of obesity have shown that the gut microbiome plays important roles in nutrient and energy harvest, moreover a bacterial signature of obese microbiota has been observed.
- The study of complex microbial communities can be performed at single cell level.

List of Abbreviations

BAC	bacterial artificial chromosome
BD	basal diet
Ct	cycle threshold

DGGE	denaturing gradient gel electrophoresis
FISH	Fluorescence *in situ* hybridization
FISH-FC	FISH combined with flow cytometry
FISH-FACS	FISH-Fluorescence activated cell sorting
HMP	human microbiome project
MDA	multiple displacement amplification
MGBs	minor groove binders
NIH	National Institutes of Health
OTUs	operational taxonomic units
PFGE	pulsed-field gel electrophoresis
qPCR	quantitative real-time PCR
RDP	ribosomal database project
RFLP	restriction fragment length polymorphism
scFOS	short-chain fructo-oligosaccharide
TGGE	temporal gradient gel electrophoresis
T-RFLP	terminal restriction fragment length polymorphism
T-RFs	terminal restriction fragments
TTGE	temporal temperature gradient gel electrophoresis

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3 Post-Genomics Approaches towards Monitoring Changes within the Microbial Ecology of the Gut

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3.1 Introduction

The human gut microbiota, comprising many hundreds of different microbial species, has closely co-evolved with its human host over the millennia. Diet has been a major driver of this co-evolution, in particular dietary non-digestible carbohydrates. This dietary fraction reaches the colon and becomes available for microbial fermentation, and it is in the colon that the great diversity of gut microorganisms resides. For the vast majority of our evolutionary history humans followed hunter-gatherer life-styles and consumed diets with many times more non-digestible carbohydrates, fiber and whole plant polyphenol rich foods than typical Western style diets today. Adaption of the Western-style diet over the past 50 years may thus have resulted in a significant down-shift or adverse modulation in the fermentative and metabolic output of the colonic microbiota. Short chain fatty acids (SCFA), the major end-products of bacterial carbohydrate fermentation, impact on many critical physiological processes including energy metabolism, lipid and cholesterol levels, carcinogenesis and gene expression. Additionally, the close inter-relationship between bacteria within our gut and the gastrointestinal lymphatic tissue is critical to the optimal functioning of our immune system. Modulation of these microbiota related processes as a result of our modern dietary environment may have played a role in the growing incidence of allergic diseases and chronic diseases like cardiovascular disease, obesity and cancer, often called the diseases of affluence. Recent advances in

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functional foods, particularly prebiotics, offer a means of modulating the gut microbiota and potentially redressing some of the deleterious consequences of modern diet on our gut microbiota and health, both locally within the gut and systemically. There is a growing body of evidence, mainly from animal studies, but also from notable human interventions, that prebiotic dietary supplementation can improve host health. Similarly, prebiotics have been repeatedly shown in human and animal interventions to modulate the relative abundance of bacteria within the gut microbiota, particularly bringing about an increased abundance of bifidobacteria, seen as beneficial microbiota constituents. However, in many cases the underlying mechanisms of effect remain to be determined. Until recently, studies examining the impact of the gut microbiota on human health or the efficacy of microbiota targeted functional foods were greatly limited by the fact that up to 70% of the gut microbiota are resistant to culture under laboratory conditions. Additionally, many gut bacteria are new to science, lacking even closely related representatives within microbial culture collections. This made assigning function to particular intestinal bacteria difficult if not impossible in most cases. Recent advances in post-genomics technologies such as genomics, metagenomics and metabonomics, are allowing researchers to derive the metabolic and microbiological consequences of microbiota modulation in a culture independent manner and link these to physiological biomarkers. These culture independent approaches allow the determination of not just the metabolic potential of the gut microbiota, in terms of the genes comprising the microbiota metagenome, but the metabolic kinetics of the gut microbiota in terms of metabolic fluxes or changes in metabolite profiles upon microbiota modulation. In this chapter, we will discuss how metagenomics and metabonomics are being applied to study the consequences of diet:microbe interactions in the gut and particularly, the microbiological and metabolic consequences of prebiotic ingestion. These technologies hold great promise in elucidating the underlying molecular basis of prebiotic induced health effects.

3.2 From Genomics to Metagenomics

The Human Genome Project (HGP) (Abdellah et al., 2004; Venter et al., 2001) provided a human genetic blueprint which has proved an extremely useful tool in identifying determinants of heritable diseases and providing the possibility of linking particular genotypes to disease risk (Desiere, 2004). During its completion the HGP also gave rise to many of the bioinformatic tools necessary for the emergence of other high resolution, data rich molecular technologies such as transcriptomics, proteomics and metabolomics. Additionally, it created the

necessary scientific and commercial interest which has lead to technological leaps in terms of DNA sequencing capabilities. However, it has become apparent that this genetic blueprint does not tell the whole story of our individuality and other factors, environmental factors, play an important role in human health and disease susceptibility. These extra-genomic environmental factors which include diet, xenobiotic compounds (e.g., cooked food mutagens, drugs, environmental chemicals) and our intestinal microbiota closely interact with an individuals genome to regulate gene expression, metabolic pathways and epigenetics, and consequently, impact on an individuals health and disease susceptibility (Johnson et al., 2008; Martin et al., 2007). The advent of high through-put sequencing has enabled the rapid genetic characterization of microbial genomes and whole microbial community genomes or "metagenomes" derived directly from microbial consortia in a culture independent manner (Goldberg et al., 2006). Metagenomics is defined as "the application of modern genomics techniques to the study of communities of microbial organisms directly in their natural environments, bypassing the need for isolation and lab cultivation of individual species" and has provided unparalleled insight into the composition and metabolic potential of microorganisms comprising important communities in the terrestrial and marine environments (Chen and Pachter, 2005; McHardy and Rigoutsos, 2007; Rusch et al., 2007). This approach has now been adopted by a large scale international sequencing project, the Human Microbiome Project, run by the NIH in the United States (see Turnbaugh et al., 2007). This multi-disciplinary project aims to sequence and characterize the gut microbiota giving a global view of the metabolic potential encoded by the gut microbiota. The collaborative sequencing effort will result in the completion of an estimated 1,000 microbial genome sequences as well as metagenomic analysis of microbial communities resident in different regions of the body including the gastrointestinal and urogenital tract, oral and nasal cavities and the skin. This genomic encyclopaedia will revolutionize both our understanding of the human associated microbiota and the way in which human microbiology is carried out in the future, given the enormous reference database of sequence information on both culturable and un-culturable microbiota members that will be generated.

3.3 The Human Gut Microbiota as an Extension of the Human Genome

The human gut microbiota is estimated to comprise up to 1,000 different microbial species which colonize to various degrees and in different ecological

niches along the length of the alimentary canal, from mouth to anus. The composition and activity of the gut microbiota differs between the different geographical regions of the gut. Host physiology plays an important role in nurturing these gastrointestinal microbial communities and can also play a regulatory role in bacterial colonization. Gastric acid, rapid flow of digesta, enzymatic and bile secretions all limit the growth of bacteria in the upper gut and it is not until the colon that sizable bacterial populations develop (Conway, 1995; Hayashi et al., 2005; Wang et al., 2005). In the healthy adult, a climax microbial consortium resides within the colon and this microbiota displays a remarkable compositional stability and a high degree of homeostasis and self-regulation which greatly impedes colonization by allochthonous microorganisms ingested by the host (Marchesi and Shanahan, 2007). Additionally, many bacteria, even distantly related species share phenotypic traits and there is a degree of functional redundancy shared between some microbiota components, ensuring for the host a steady supply of metabolites of microbial origin including vitamins and SCFAs for example. This homeostasis also ensures essential functions within the gut microbiota are maintained irrespective of fluctuations in the relative abundance of individual bacterial strains e.g., upon bacteriophage attack (Kurokawa et al., 2007). These activities include fermentative and enzymatic transformation of plant storage and structural carbohydrates, many of which are recalcitrant to degradation and digestion in the upper gut, allowing the host to derive energy from otherwise inaccessible substrates. The gut microbiota also enables host access to plant derived polyphenolic compounds most of which escape digestion in the upper gut of which some possess strong biological activities including antioxidant, phytoeostrogenic, vaso-dilation activities. Important co-metabolic processes occur between host and microbiota, explemified by the entero-hepatic circulation of bile acids (Ley et al., 2008; Nicholson et al., 2005). The gut microbiota is largely fermentative, converting un-digested carbohydrate and protein into short chain fatty acids and there is also a complex array of mutualistic, symbiotic and cross-feeding interactions between bacteria within the gut microbiota in SCFA transformation and metabolism (Duncan et al., 2004; Morrison et al., 2006).

3.4 Limitations of the Microbiological Culture Based Approaches

The extent to which the gut microbiota contributes towards human health is only becoming clear now and until relatively recently the colon was viewed solely as a

retention tank for faeces, where water absorption was considered its most important contribution to systemic biological function. A major contributor towards this lack of understanding and a major hurdle to studying the gut microbiota is the fact that the vast majority of intestinal bacteria are recalcitrant to growth under laboratory conditions and up to 30% of bacterial species within an individual's gut microbiota may be novel phylotypes and new to science (Eckburg et al., 2005; Suau et al., 1999). Therefore there are major limitations to the traditional culture based microbiological approach for studying the gut microbiota. These include;

- Traditional culture based approaches are limited to monitoring only the culturable minority of the gut microbiota. About 70–80% of intestinal bacteria are considered recalcitrant to cultivation in pure culture, many requiring thus far unidentified growth factors or co-culture with other intestinal microorganisms to fulfil their mutualistic or symbiotic life strategies.
- Many phylogenetically diverse bacteria share phenotypic traits and can grow under the same growth conditions and on the same nutrient media, even in the presence of selective supplements (Tuohy and McCartney, 2006). This necessitates sub-culturing followed by biochemical and genetic characterization before the microorganisms may be phylogenetically positioned; a time consuming and costly process.
- Bacteria show a high degree of genetic plasticity, acquiring genetic determinants through horizontal DNA transfer from even distantly related species and genome duplication followed by divergent evolution within bacterial species is common. This genomic plasticity is driven by environmental selective pressure and often involves transfer of environmentally important genetic traits. Repeated passage of bacterial cultures under the selective pressure of pure culture growth conditions in the laboratory will thus result in loss of traits relevant to the natural environment from which these organisms were isolated (Lee et al., 2008). Environmentally driven genomic instability in bacteria should thus be taken into consideration when conducting functional studies on bacteria, such as probiotics, isolated from gastrointestinal ecosystems.

Thus, the reasons why we can not culture the majority of gut bacteria include the following factors; the unknown growth requirements of the bacteria, the selectivity of the media that are used, the stress imposed by the cultivation procedures, the necessity of strictly anoxic conditions, and difficulties with simulating the interactions of bacteria with other microbes and host cells. The traditional microbiological culture approach is therefore is not well suited to the enumeration of intestinal bacteria or species diversity measures especially for complex

microbial consortia, such as the human gut microbiota. Advances in microbial genetics, and the adoption of the 16S rRNA gene as a universal microbial molecular chronometer, have allowed the development of tools which greatly facilitate culture independent microbial ecology studies (Frank and Pace, 2008; Handelsman, 2004; Tuohy and McCartney, 2006). Ribosomal RNAs are excellent molecules for measuring evolutionary relationships among organisms (Olsen et al., 1986). In contrast to traditional taxonomy, which is based on phenotypic traits, this kind of taxonomy reflects natural evolutionary relationships among organisms (Woese et al., 1990). Prokaryotic ribosomes contain two subunits, the sizes being 50S and 30S. The 50S subunit contains about thirty-four proteins as well as 5S rRNA (120 bases), and 23S rRNA (about 2,900 bases), and the 30S subunit contains about twenty-one proteins and 16S rRNA (about 1,500 bases). The 16S rRNA has been the most widely employed molecule to develop the phylogeny of prokaryotes. Within the various regions of 16S rRNA, the degree of conservation differs considerably. Analyses of rRNA sequences have revealed signature sequences, short stretches of rRNA, that are unique to a certain group or groups of organisms enabling the phylogenetic placement and identification of bacteria (Blaut et al., 2002).

Recent large scale sequencing projects have significantly increased the number of 16S rRNA genes archived in public databases such as GenBank, enhancing our understanding of how bacteria are related to one another at the phylogenetic level (Frank and Pace, 2008). However, many of these 16S rRNA gene sequences were derived from direct cloning experiments where environmental DNA is extracted, purified and sequenced either directly or upon generation of clone libraries and as such do not correspond to previously cultured bacteria. Indeed, many phylotypes identified within the gut microbiota are only distantly related to previously cultured bacteria. Where close relatives have been cultivated, comparisons have been drawn between these known and cultured bacteria and close unculturable novel relatives in terms of putative physiology and ecological function. However, such functional inferences are limited since many core metabolic functions appear to be shared between distantly related bacteria whilst traits enabling occupation of a particular ecological niche may be strain specific and may not be shared with close relatives. The gut microbiota in particular appears to be a hot bed of bacterial promiscuity, with many intestinal bacteria showing evidence of high-frequency heterologous DNA transfer. Many of the genes carried by highly transmissible genetic elements within the gut microbiota encode ecologically important functional genes, involved in substrate metabolism, drug resistance or antimicrobial production (Salyers et al., 2004; Tuohy et al., 2002, 2004).

Recent comparative genomic studies on the intestinal microorganism *Bifidobacterium longum*, clearly illustrated the significance of DNA transfer within the gut microbiota. This important member of what may be considered the beneficial gut microbiota, and close relative of common probiotic microorganisms, proved to be highly susceptible to deletion and loss of genetic traits upon repeated passage through pure culture. Upon comparative genomic analysis, several DNA regions, encoding intestinally important traits including oligosaccharide and polyol utilization, arsenic resistance and lantibiotic production, unique to the intestinally isolated *B. longum* strain DJO10A appeared to have been deleted from the culture collection strain, *B. longum* NCC2705. The authors were able to demonstrate this loss of intestinally relevant traits upon culturing *B. lonugm* for more than 1,000 generations under laboratory conditions (Lee et al., 2008). Similarly, comparative genomics studies with the important gastrointestinal and nosocomial pathogen, *Clostridium difficile* showed that a core of only 19.7% of genes were shared between 75 clinically relevant strains compared to a *C. difficile* 630 whole genome DNA microarray (Stabler et al., 2006). Thus despite the growing number of novel 16S rRNA species identified, we know little about how intestinal bacteria behave in their own specific ecological niche within the gut.

3.5 Gut Microbiota Community Level Phylogenetic Analysis

Studies on the composition and species richness of the human gut microbiota have been greatly facilitated by the development of molecular techniques and tools based around the phylogenetic information encoded by the bacterial 16S rRNA gene (Tuohy and McCartney, 2006; Zoetendal et al., 2004). The gut microbiota species composition may be determined by directly isolating bacterial DNA from intestinal samples followed by separation and DNA sequencing of the 16S rRNA genes present. Upon phylogenetic positioning, these 16S rRNA gene sequences can be used to generate a picture of the relative abundance of different bacterial species or phylotypes present in the original environmental sample. Using this and similar approaches a detailed picture of the composition of the human gut microbiota has now emerged. Suau et al. (1999) showed that 95% of 16S rRNA gene sequences recovered from a single fecal sample of a healthy adult volunteer fell within one of three phylogenetic groupings: the *Bacteroides* group, the *C. coccoides-E. rectale* group, and the *C. leptum* group. The *Clostridium*

groups, *C. leptum* and *C. coccoides-E. rectale* include many bacteria previously described as important members of the gut microbiota including species of *Eubacterium*, *Ruminococcus*, *Butyrivibrio* and *Faecalibacterium prausnitzii*. More recently the divisional structure of the gut microbiota at the phylum level has been described using a similar approach with the two most abundant bacterial phyla being the Gram negative *Bacteroidetes* and the Gram positive, low GC% *Firmicutes* (Eckburg et al., 2005; Louis et al., 2007; Wang et al., 2005). Seventy two per cent of the 395 phylotypes detected by Eckburg et al. (2005) belonged to the clostridial group of Firmicute bacteria, most in clusters XIVa (also referred to as the *Clostridium coccoides* group) or IV (*Clostridium leptum*) and the majority of these were either novel, or unrelated to species held in culture collections. Other important though less abundant phyla include the *Proteobacteria*, *Actinobacteria*, *Fusobacteria* and the *Verrucomicrobia* phyla (Eckburg et al., 2005; Wang et al., 2005). These lesser phyla none-the-less contain many bacteria important for human health and disease including the enterobacteria and bifidobacteria. Moreover, the bacterial community in the stomach and jejunum has been shown to differ from that in the distal ileum, ascending colon and rectum, with the major phylogenetic groups being similar in the distal ileum and rectum (Bik et al., 2006; Suau et al., 1999; Wang et al., 2005) also showed that a major proportion, up to 70% of the 16S rRNA species present in this fecal microbiota belonged to novel phylogenetic lineages all be it within the three dominant groupings. Only 24% of clones corresponded to previously identified bacterial phylotypes. More recently, Gill et al. (2006) using a direct high throughput sequencing of metagenomic DNA present in fecal samples collected from two American individuals found that 22.2% of phylotypes were novel and 83.3% of phylotypes corresponded to previously uncultured bacterial species. It is likely that as more metagenomic-level studies add 16S rRNA gene sequences onto the databases, the proportion of un-recognized phylotypes within an individual's gut microbiota will fall considerably. However, as discussed above, full community phylogeny does not necessarily translate into an understanding of bacterial function or community function and other approaches are needed to link bacterial identity and ecological functioning.

3.6 Community Finger-Printing Techniques (e.g., DGGE)

Fingerprinting techniques have been used to study bacterial communities and appear to be ideal for monitoring community shifts and comparing communities between GI sites and among animals.

Denaturing gradient gel electrophoresis (DGGE) and similar techniques (e.g., temperature gradient gel electrophoresis, TGGE and temporal temperature gradient gel electrophoresis, TTGE) profile or finger-print of the gut microbiota by generating snap-shots of bacterial richness encoded by 16S rRNA gene fragments within environmental samples (Muyzer et al., 1993). Two additional microbial community fingerprinting techniques are single strand conformation polymorphism (SSCP) and terminal-restriction fragment length polymorphism (TRFLP) analyses. DGGE, TGGE, and TTGE are based on sequence-specific melting behavior of amplicons, SSCP on the secondary structure of single stranded DNA, and T-RFLP on specific target sites for restriction enzymes. Double stranded DNA (ds-DNA) melts into single-stranded DNA at different rates across a gradient of denaturant (e.g., urea, or temperature) depending on its DNA sequence. DGGE takes advantage of this differential ds-DNA melting to separate 16S rRNA gene fragments of equal length amplified from microbial communities across a denaturing urea and formamide gradient on a polyacrylamide gel. The resultant pattern of DNA bands on the DGGE gel constitutes a fingerprint of the different 16S rRNA gene fragments amplified from the original sample and thus the species make-up of the sampled microbial community. This approach generates a snap-shot that can then be used to assess changes in species richness over time or in response to different environmental stimuli, such as the presence of growth substrate, antibiotics or xenobiotics. The phylogenetic identity of differential bands can be determined following band excision and sequencing. It has been reported that DGGE or TGGE are sensitive enough to detect bacteria that constitute up to 1% of the total bacterial community. This means that only the most dominant bacteria will be represented. Theoretically, each DGGE band corresponds to a single operational taxonomic unit (OTU), where the total banding pattern is reflective of a community's species richness and diversity (Muyzer et al., 1993). Earlier workers have excised and directly PCR-amplified and sequenced (Ampe et al., 1999; Ovreas et al., 1997) or PCR cloned and sequenced DGGE bands to successfully identify the taxonomic units of interest (Iwamoto et al., 2000; Zwart et al., 1998). Conversely, recent investigators (Abecia et al., 2007; Ercolini, 2004; Ercolini et al., 2003; Kowalchuk et al., 1997; van Beek and Priest, 2002) have reported that band excision and sequencing of DGGE bands might not provide unequivocal identifications as a result of the co-migration of DNA fragments from different taxa to the same positions within DGGE gels.

This approach has been employed to show that individuals harbor unique collections of intestinal bacteria, and that the species composition of the adult gut microbiota is remarkably stable over time (Zoetendal et al., 1998). DGGE has

recently been employed to follow the compositional changes that occur within the human gut microbiota upon ingestion of prebiotic functional foods (Tannock et al., 2004); to characterize the mucosa associated microbiota in Crohn's disease patients (Martinez-Medina et al., 2006); to monitor microbiota changes within the infant gut upon dietary intervention (Nielsen et al., 2007); to monitor the impact of antibiotics and probiotic intervention on the gut microbiota (Saarela et al., 2007; Yap et al., 2008) and has shown that the gut microbiota profile presented by the patient before pharmaceutical intervention can predict individual susceptibility to diarrhoea (De La Cochetière et al., 2008).

3.7 Limitations of PCR Based Techniques

Many of molecular microbiology tools rely on efficient extraction of community DNA, PCR and cloning to characterize community 16S rRNA. This approach has a number of limitations, and bias towards recovery of particular 16S rRNA species may be introduced at different stages. Firstly, the method used to isolate bacterial DNA from faeces or mucosal specimens may select for a certain bacteria based on cell wall conformations; Gram positive bacteria by and large are more difficult to disrupt than Gram negative species. PCR is a competitive reaction governed by melting and renaturation efficiencies of the target DNA sequences and therefore PCR based approaches may select for particular pools of 16S rRNA based on PCR amplification efficiency rather than the relative abundance of bacteria and their 16S rRNA genes in the test sample. High GC, Gram positive bacteria, such as the bifidobacteria, consequently are often under-represented in 16S rRNA gene libraries derived from competitive PCR reactions (Farris and Olson, 2007). Primer sequences to amplify bacterial DNA are based on highly conserved regions within the 16S rRNA gene. Even slight differences in the DNA sequence in these primer binding regions might result in bacteria not being detected and information on the highly conserved regions within the 16S rRNA gene derives only from already sequenced species. Finally, cloning of 16S rRNA gene fragments may introduce bias since different cloning vectors and host strains favor certain DNA sequences (Bonnet et al., 2002; Sipos et al., 2007). The limitations of these approaches must be recognized particularly when considering the relative abundance of bacteria within an ecosystem and methods which enable direct visualization and enumeration of phylogenetically related groups of bacteria within environmental samples may be more suited to this task. Hongoh et al. (2003) detected a significant increase in the expected number

of phylotypes by lowering the annealing temperature, and a significant decrease in the proportion of clones belonging to the predominant group by raising the number of PCR cycles.

3.8 Molecular Characterization of the Gut Microbiota *in situ*

Fluorescence *in situ* hybridization (FISH) using 16S rRNA targeted oligonucleotide probes provides a direct means of enumerating specific bacterial populations in environmental samples without the need for microbiological culture and in a phylogenetically relevant manner (Amann et al., 1992). Here, bacterial cells are visualized either by epifluorescence microscopy or flow cytometry. The advantage of this approach is that phylogenetically related bacteria ranging from kingdom or phylum levels to species level, depending on probe design, may be enumerated directly in situ. This approach is now commonly applied to enumerate changes in relative abundance of bacteria within the human gut microbiota. As with other molecular approaches it is important to realize the limitations of FISH, which include: variability in permeabilization of target cells and the need for prior 16S rRNA gene information for probe design. FISH does however, allow the estimation of relative bacterial population levels within mixed microbial consortia without the bias introduced by DNA recovery and subsequent PCR and cloning (Amann and Fuchs, 2008). FISH gives an accurate picture of relative abundance of bacteria in mixed consortia because the bacteria are enumerated directly without selective amplification or culture (Amann and Fuchs, 2008). *Table 3.1* shows the relative abundances of bacteria within the human gut microbiota as determined by FISH.

Use of flow cytometry allows fluorescence activated cell sorting of bacterial cells. Where 16S rRNA targeted FISH is employed to label bacteria, FACS can be used for the physical separation or sorting of microorganisms according to their phylogenetic groupings from mixed microbial consortia (Ben-Amor et al., 2005; Kalyuzhnaya et al., 2006; Lay et al., 2007). The cell sorted bacterial groups can then be subjected to other molecular procedures to identify bacteria enumerated by the particular FISH probe employed or to investigate the genetic potential of cell sorted bacteria following cloning and DNA sequencing in a culture independent manner. This approach has been used to identify the species make up of viable, injured and dead fractions in human faeces (Ben-Amor et al., 2005; Lay et al., 2007) also used this approach to monitor which bacterial species were

Table 3.1

Adapted from Flint (2006) and Stewart et al. (2006)

Bacterial group	Abundance (typical % of total bacteria)	Fermentation end-products
Phylum Firmicutes		
Clostridial clusters XIV a + b		
Eubacterium rectale – Clostridium coccoides	14.5–33.0	Butyrate, formate, lactate
Eubacterium hallii	0.6–3.8	Butyrate, formate, acetate
Ruminococcus obeum	2.5	Acetate
Lachnospira spp.	3.6	Formate, acetate, lactate, succinate
Clostridial cluster IV		
Clostridium leptum	21.7–26.8	
Faecalibacterium prausnitzii	4.9–20.4	Butyrate, formate, lactate
Ruminococcus bromii, Ruminococcus flavefaciens	1.8–10.2 0.4–1.3	Acetate, formate, lactate, succinate
Clostridium viride	0.5–2.6	Acetate, propionate, butyrate, valerate, ammonia
Eubacterium desmolans	0.1–0.4	Acetate, butyrate
Clostridial cluster IX		
Veillonella spp.	0.9–2.5	Propionate, various minor acids
Clostridial cluster XVI		
Eubacterium cylindroides	0.3–1.7	Butyrate, acetate, lactate, succinate, formate
Phylum Firmicutes		
Lactobacillus/Enterococcus	0.2–2.7	Lactate, acetate
Phylum Actinobacteria		
Bifidobacterium spp.	1.1–5.8	Lactate, acetate, formate
Atopobium spp.	0.8–6.3	Acetate, formate, Lactate
Phylum Bacteroidetes		
Bacteroides-Prevotella gp.	3.9–13.6	Acetate, propionate, succinate
Bacteroides putredinis	0.1–0.8	Acetate, succinate
Bacteroides fragilis	0.4–4.2	Acetate, propionate
Phylum Enterobacteriaceae		
Escherichia coli *Salmonella* *Klebsiella*	0.1–0.2	Lactate, acetate, succinate, formate
Desufovibrio	<5.5	SCFA cross-feeding, H2S production
Kingdom Archaea		
Methanobrevibacter smithii	<0.2	SCFA cross-feeding, CH4 production

Relative abundance of dominant human gut bacterial groups and corresponding main acidic fermentation end-products. The abundance is expressed as mean values of the percentage of total bacteria using FISH, based on data from Lay et al. (2005) and Mueller et al. (2006). The reported fermentation end-products are indicative of cultured representatives

enumerated by *C. leptum* group specific FISH probes by carrying out PCR-TTGE (a DGGE type method) on cell sorts of fecal bacteria labeled with the *C. leptum* probes (Clep866-CY5/cp or Fprau645-CY5). The *C. leptum* group, which include important butyrate producing bacteria, constitute one of the dominant groupings within the gut microbiota and covers many different phylogenetic clades. Since the majority of bacteria within the gut are unculturable such culture independent techniques which have the potential for separating out bacterial species without the need for microbiological culture may prove invaluable in assigning ecological function to these dominant and prevalent unculturable moieties.

The prebiotic concept, defined as "non-digestible (by the host) food ingredients that have a beneficial effect through their selective metabolism in the intestine" requires that certain dietary fibers are selectively metabolized by the gut microbiota leading to a concomitant improvement in host health (Gibson and Roberfroid, 1995). The FISH technique has recently been employed to demonstrate that the ability of various foodstuffs and ingredients to bring about a change within the relative abundance of gut bacterial populations seen as beneficial towards human health. The fructans, inulin and oligofructose, galactooligosaccharides, lactulose, resistant starch, whole grain wheat have all been shown to elicit a significant relative increase in numbers of bifidobacteria within the human gastrointestinal tract upon dietary intervention using FISH (Costabile et al., 2008; Depeint et al., 2008). Indeed, true prebiotics can reproducibly be shown to bring about a selective modulation of the gut microbiota, whereby relative abundance of what are termed the beneficial microbiota (currently including bifidobacteria and/or lactobacilli) using either FISH or more traditional culture based approaches. In parallel, as outlined elsewhere in this volume, there is a growing body of evidence from both animal and human studies that prebiotics can bring about improvements in mineral absorption and bone health, improved immune function and relief of allergic and inflammatory disease; protection from gastrointestinal infections including infectious diarrhea and travellers diarrhea; reduced risk of colon cancer and through modulation of blood lipid profiles and energy metabolism, reduce the risk of the metabolic syndrome, type 2 diabetes and coronary vascular disease. However, there is a lack of mechanistic data linking changes within the gut microbiota with biological mechanisms underpinning the observed health effects. The challenge for the prebiotics field of research in the coming years is to bridge this gap and link prebiotic induced modulations within the gut microbiota with specific physiological processes responsible for improved health or protection from disease.

3.9 Culture-Independent Functional Characterization of the Gut Microbiota's Metabolic Potential

The 16S rRNA based molecular tools mentioned above have contributed to a picture of the gut microbiota as being made up of mainly unculturable bacteria, with many novel species. This approach has also contributed greatly to the extensive 16S rRNA gene database used for bacterial phylogeny and taxonomy and has proved invaluable in characterizing species make up and composition of the gut microbiota. However, knowing the phylogeny of a microorganism may not necessarily shed light on its ecological function. These issues highlight an important problem, how to assign ecological function to novel or unculturable bacteria? Recent advances in high throughput sequencing allow the rapid genome sequencing and more over rapid and accurate compilation of community metagenomes (Committee on Metagenomics, 2007). Sequence based metagenomics applies shot-gun sequencing of total DNA extracted from an environmental sample and was originally pioneered in marine and terrestrial environments (Gill et al., 2006; Kurokawa et al., 2007; Turnbaugh et al., 2007). This approach has also recently been applied to study the human gut microbiota, e.g., the Human Microbiome Project described above. The functional or metabolic potential of the human gut microbiota may thus be accessed through these large scale sequencing projects targeting not just community 16S rRNA genes, but potentially all bacterial genes present.

Gill et al. (2006) examined the fecal metagenome of two unrelated healthy American adults. They found that these metagenomes were enriched for genes involved in the metabolism of carbohydrates, amino acids and xenobiotics, methanogenesis and the biosynthesis of vitamins and isoprenoids, compared to the human genome itself and other bacterial communities. They also pointed out that the fecal metagenome encoded many functions not represented in the human genome, in particular, genes involved in the metabolism of major plant structural and storage polysaccharides such as xylans, pectins, arabino-containing carbohydrates and fructans. The abundance of such genes within the gut microbiome and their rarity within the human genome is of key importance to the overall metabolic capacity encoded in the combined human-microbiota metagenome. Some 81 different glycosyl hydrolase families as well as key genes involved in the production of short chain fatty acids (SCFA, acetate, butyrate, propionate and succinate) could be assigned to the gut microbiota highlighting the fact that carbohydrate fermentation appears to be a major energy

source in the colon, driving the energy economy within the microbiota, building cross-feeding interrelationships and providing about 50% of the daily energy requirements of the gut wall. In a second metagenomics study, Kurokawa et al. (2007) compared the metagenomes of 13 healthy Japanese individuals of different age, with two American fecal metagenomes (Gill et al., 2006). They found that genes involved in carbohydrate metabolism and transport were enriched in all 13 metagenomes, compared to a database constructed from COG (Cluster of Orthologous Groups) assigned genome sequences of 243 different bacteria not commonly found within human gut microbiota. Genes involved in lipid metabolism however, were under-represented in the gut metagenomes compared to this reference database. These observations support the notion that the human gut microbiota has evolved along-side its human host to complement human encoded functions and allows the host access to dietary nutrients not digested or absorbed in the upper gut like complex plant storage polysaccharides. SCFA derived from microbial fermentation in the colon contribute about 10% of our daily energy intake, and play important physiological roles including regulating cellular differentiation and proliferation in the gut wall, cholesterol synthesis and *de novo* lipogenesis in the liver, and may act as an energy source systemically (Macfarlane and Gibson, 1997). Similarly, 90% of plant derived polyphenolic compounds reach the colon where they are transformed into biologically active and available intermediates by the resident microbiota (Spencer et al., 2001). This interaction between our intestinal microbiota and plant foods, both carbohydrate and polyphenolic moieties has important implications for human health and disease and in devising optimal human diet as well as functional food design. For much of our evolutionary history, humans followed a hunter-gatherer life strategy and consumed diets rich in whole plant foods particularly high in dietary fiber and polyphenols. Whole plant foods (fruits, vegetables, whole grain cereals) and dietary fiber are recognized as beneficial for human health and in epidemiological studies these foods are often inversely related to risk of chronic human diseases including coronary vascular disease and cancer. Both for present day humans and for our prehistoric ancestors the maintenance of a stable, fermentative gut microbiota would have been essential to maximize energy and nutrient recovery as well as non-nutrient functional benefit from our diet (Cassidy, 2006; Pompei et al., 2007). However, our modern, Western style diets, low in fiber and whole plant foods, and high in refined sugars, protein and saturated fat, appear to be out of step with our co-evolved hunter-gatherer intestinal microbiota. It is not surprising that there appears to be a strong link between adoption of this Western style diet and increased incidence of certain cancers, coronary

vascular disease, obesity and the metabolic syndrome, and immunological diseases, like inflammatory bowel disease and allergy, in which the gut microbiota have been proposed to play an aetiological role and for which certain whole plant foods are proving protective. Modulating our modern gut microbiota through dietary supplementation with Prebiotics in particular can mediate positive health effects on a number of important physiological functions including *de novo* lipogenesis Beylot (2005), mineral absorption (Abrams et al., 2005; Holloway et al., 2007), regulation of satiety and body fat deposition (Delzenne et al., 2007; So et al., 2007) and importantly, providing butyrate as an energy supply to the colonic mucosa (Pool-Zobel and Sauer, 2007).

One of the more surprising insights provided by recent metagonomic studies has been the observation that the gut microbiota may be involved in the aetiology of obesity and that the composition of the gut microbiota is fundamentally different in the obese compared to the lean individuals at the phylum level. There is currently an epidemic of obesity which appears to develop at the population level upon adoption of a Western-style diet. In the UK there has been a sharp increase in the incidence of obesity over the past 15 years and it has been estimated that by 2,050 60% men in the UK will be obese Foresight (2007). There is a genetic component to obesity with over 600 genes reported to play a role in energy metabolism and body weight, and there is a sub-population genetically predisposed to obesity (Wardle et al., 2008). However, the sudden increase in the incidence of obesity since the 1980s has occurred at a rate which far outstrips that of human genomic evolution, showing that the obesogenic environment has a major role to play. This obesogenic environment impacts on the quantity of food we eat, the types of foods we eat, satiety, energy recovery from the diet, epigenetic programming, mental state and exercise, all of which play important roles in determining the risk of obesity. There are strong epidemiological data linking diets high in fat and refined carbohydrates with obesity (Johnson et al., 2008). Conversely, diets rich in fiber and whole plant foods are inversely associated with obesity (Astrup et al., 2008). Gordon and co-workers have recently shown that the gut microbiota differs between obese and lean individuals at the phylum level in both animal models of obesity and in human subjects (Ley et al., 2005, 2006; Turnbaugh et al., 2006). These authors report that obesity is associated with a reduced abundance of intestinal Bacteroidetes and increased abundance of Firmicutes in the genetic *ob*/*ob* mouse model of obesity and in humans. This altered microbiota is associated with increased fermentation end products in caecal and fecal samples, and differences in the metabolic potential of the gut microbiota with an increased energy harvest from

the diet (Ley et al., 2005, 2006). Interestingly, obesity appears to be transferred concomitantly with this obese type microbiota and when germ-free animals are associated with caecal contents of obese mice they too become obese despite despite eating less food (Turnbaugh et al., 2006, 2008). However, it also appears that diet has a strong influence on the composition and activity of the obese-associated microbiota. It appears that the gut microbiota of obese humans who lose weight after one year on either a low carbohydrate or a low fat diet returns to a lean type profile (Ley et al., 2006). Similarly, there is some evidence from animal studies that certain fibers, particularly prebiotics may reduce the risk of obesity itself and its associated pathologies. Cani et al. (2007a) showed that when conventional mice are placed on a high fat diet obesity and insulin resistance may be induced by increased plasma levels of the highly inflammatory Gram negative bacterial wall fragment, lipopolysaccharide. They observed a die-off in the intestinal microbiota in high fat fed animals. This high-fat diet with low fermentable carbohydrate may have contributed towards increased availability or though depleted mucosal barrier function have contributed to increased uptake of intestinal LPS thus providing the immunological trigger for the chronic low grade inflammation characteristic of obesity and predisposing to insulin resistance. The same authors later found that dietary supplementation with the prebiotic oligofructose, in these same high-fat fed animals, resulted in reduced plasma LPS, reduced inflammation and improved insulin sensitivity. These physiological changes were strongly associated with an increased abundance of intestinal bifidobacteria in these animals (Cani et al., 2007b). Further studies are required to investigate in more depth any apparent aetiological role of the obese-type microbiota in body weight gain and conversely, in the ability of different diets to induce an obese-type microbiota or reduce the risk of becoming obese by modulating gut microbiota composition and activity through dietary interventions for example with prebiotics.

The sequence based metagonomics is a powerful tool for measuring the metabolic potential of the gut microbiota and recent high level investment in metagenomic studies of the gut microbiota will generate a wealth of sequence data for data-mining and the design of future gut microbial ecology studies. Identifying the genetically encoded functions which are enriched or under-represented within a microbial community can shed light on the ecological role of that community as a whole (Turnbaugh et al., 2007). Additionally, identifying ecologically sensitive genes or sets of genes which enable a particular strain to successfully colonize an ecological niche within the gut will greatly enrich our understanding of how bacteria interact with each other, our diet and their human

host to mediate either beneficial health effects or induce disease. A good place to start may be to characterize the composition and commonality of ecologically important genes constituting the gut microbiota mobile metagenome, encoded by transmissible plasmids, transposons and bacteriophages, which is becoming recognized as an important determinant of strain identity and ecological function within the gut (Lee et al., 2008). However, as observed by Kurokawa et al. (2007) not all open reading frames (ORFs) identified in metagenomic studies can be annotated to known function by comparisons with reference databases. In the existing gut microbiota metagenomes between 45% and 80% of protein-coding genes observed in the 13 Japanese (Kurokawa et al., 2007) and two American (Gill et al., 2006) fecal metagenomes respectively could not be assigned a metabolic function at the 90% threshold identity upon BLASTP analysis against their reference database of 243 non-gut bacterial genomes. Another limitation of the metagonomic approach is that it generates data on the metabolic potential of a microbial ecosystem, the potential encoded by the genotype of the organisms present which is only translated into metabolic kinetics or phenotype in response to particular environmental stimuli. Another post-genomics approach has recently emerged with the potential to measure the metabolic kinetics or community phenotype of the gut microbiota and possibly relate these changes in microbial derived metabolites to particular intestinal bacteria irrespective of whether they can be cultured under laboratory conditions or whether they are new to science.

3.10 Measuring the Metabolic Kinetics of the Human Gut Microbiota Through Metabonomics

Metabonomics is defined as "a systems approach to examining the changes in hundreds or thousands of low-molecular-weight metabolites in an intact tissue or biofluid" (Nicholson et al., 2005). The human metabonome thus comprises; the metabolites derived from human genome encoded determinants, metabolites of derived from the human microbiota, and the flux in these combined metabolite profiles under different environmental perturbations e.g., interactions with diet, pharmaceuticals, carriage of parasites, and chronic disorders like cardiovascular disease and cancer. This technology employs ^{1}H-nuclear magnetic resonance (NMR) spectrometry and mass spectrometry-based techniques to profile metabolites in biofluids like urine, plasma and fecal water, generating a picture of the metabolic kinetics of an organism at a particular point in time. By applying image

analysis followed by multivariate statistics, metabonomics is currently being used to track changes in these metabolite profiles in response to dietary modulation, including prebiotic, or pharmaceutical interventions, and to generate distinctive metabolite profiles in disease states such as inflammatory bowel disease, bowel cancer and cardiovascular disease. Such studies offer the possibility of developing new diagnostic tools or novel therapeutic targets and importantly deliver tools with the breath and resolution to derive functional data on the behavior of the gut microbiota in situ and in response to dietary change (Marchesi et al., 2007; Martin et al., 2008; Holmes et al., 2008; Solanky et al., 2005). The metagenomic studies described above illustrate clearly the degree of co-operation between the human genome and the gut microbiota and that the gut microbiota has co-evolved alongside mammals over time (Ley et al., 2008; Nicholson et al., 2005) also put forward the concept of the gut microbiota co-evolving and metabolically complementing the human genome resulting in a close symbiotic relationship involved in co-metabolism of a range of dietary and xenobiotic compounds. These authors suggested that gut microbiota structure and composition reflects this symbiotic relationship with only the bacterial populations beneficial to the host predominating in the human microbiota in health and contributing to co-metabolic activities. Of course this symbiotic relationship is impacted by other life-style influences such as diet, exercise, stress and drug or xenobiotic intake. Recognizing the contribution of microbiota derived compounds observable in metabolite profiles of human biofluids to metabolic processes at the whole organism level, Nicholson et al. (2005) combined high resolution analytical techniques with image analysis and multivariate statistics to establish the metabonomics concept.

Recent metabonomics studies have highlighted the potential contribution of microbiotal derived metabolites or co-metabolites in the aetiology of chronic human diseases. Dumas et al. (2006) found that in mice genetically predisposed to impaired glucose homeostasis and non-alcoholic fatty liver disease (NAFLD) maintained on a high fat diet, microbial activities within the gut lead to reduced choline bioavailability. This mimics choline-deficient diets already known to induce NAFLD and insulin resistance (IR), key initial steps in the development of the metabolic syndrome and obesity. ^{1}H-NMR metabolite profiling using principle component analysis was able to identify low circulating plasma phosphatidylcholine and high urinary excretion levels of methylamines (dimethylamine, trimethylamine and trimethylamine-N-oxide), gut microbe:host co-metabolites, as characteristic of high-fat fed animals with NAFLD and impaired glucose metabolism. Holmes et al. (2008) later illustrated the power of the metabonomics

approach in grouping individuals according to their urine metabolite profiles at the population level, and relating these profiles to geography, diet and disease risk. These authors showed that urinary formate (and, to a lesser extent, hippurate) was inversely related to blood pressure (BP) and coronary vascular disease (CVD) risk. Again ^{1}H-NMR was used to generate urinary metabolite profiles of 4,630 human volunteers in the UK, the USA, China and Japan. Individuals could be separated in a blind manner according to geography with East Asians separating from UK/USA populations; Japanese living in Japan separating from Japanese living in the USA, and populations of northern (Guangxi) and Southern (Beijing and Shanxi) China showing distinct urinary metabolite profiles. The authors reported that urinary profiles of people in the UK and USA were similar. The main differentiating metabolites between the populations were of dietary origin, including amino acids, creatine and trimethylamine-*N*-oxide; acetylcarnitine, tricarboxylic acid cycle intermediates involved in energy metabolism and dicarboxylic acids like suberate. Holmes et al. (2008) also identified a group of microbial compounds or which derive from the gut microbiota:host co-metabolic processing including hippurate, phenylacetylglutamine, methylamines and formate. Formate can either be formed from endogenous one-carbon metabolism or upon fermentation of non-digestible carbohydrates in the colon by diverse bacteria including certain clostridia (mainly belonging to clusters XVI, IV, XIV), the *Actinobacteria* including *Bifidobacterium* species, and to a lesser extent, the *Proteobacteria*, see *Table 3.1*. Hippurate, an end product of aromatic (benzoic acid) co-metabolism and a likely end product of polyphenolic metabolism by the host and gut microbiota, was also inversely related to BP and positively correlated with fiber intake, while high BP was associated with diets high in animal protein and the urinary metabolite, aniline (*Table 3.2*).

The same group have also recently shown that the close relationship between mammals and their intestinal microbiota extends through-out the life-span. Distinct ^{1}H-HMR urinary metabolite profiles were found in dogs in response to dietary change (a calorie restricted diet compared to normal chow) and at different ages in this longitudinal study. Urinary metabolite profiles shifted rapidly before age 1 year (early life) after which the metabolic signature stabilized between 1 and 2 years of age. This change in metabolite profiles in to first 12 months of life corresponds to the emergence and successive development of the gut microbiota and the dietary change upon weaning. A second metabolic shift was observed in middle-age (years 5–9) before profiles again underwent a metabolic transformation in old age after about 10 years. Many of the differentiative metabolites had their origins in the microbiota:host co-metabolism highlighting the role of

Table 3.2

Estimated daily fiber intake in palaeolithic diet and modern diet

Dietary pattern	Fiber content (g)	Reference
Palaeolithic diet modified in 1997 (50% meat, 50% vegetables)	104	Eaton et al. (1997)
Rural Chinese diet	77	Campbell and Chen (1994)
Rural African diet	60–120	Dunitz (1983)
Current US diet	12–18	Institute of Medicine (2002)
Recommended fiber content in US	20–35	Institute of Medicine (2002)
Current UK diet	12	British Nutrition Foundation (2004)
Recommended fiber content in UK	18 (minimum)	British Nutrition Foundation (2004)

the gut microbiota in the ageing process, from successive development of the gut microbiota in puppies to modulation of the gut microbiota in middle and old age, times often associated with the onset of chronic disease (Wang et al., 2007).

3.11 Metabonomics and Disease States (IBD and Colon Cancer)

Metabonomics has been applied to investigate changes in metabolic profiles in order to identify mechanisms involved in certain diseases. Metabonomic analysis of either fecal extracts (Marchesi et al., 2007) or colonic mucosa (Balasubramanian et al., 2008) in patients with active inflammatory bowel disease (IBD) both showed a reduction in SCFA in these patients compared to control individuals, in particular in acetate but also in butyrate. Balasubramanian et al. found however, that in patients in remission the values for these metabolites were similar to control. They also reported that the concentration of formate was significantly lower in patients with active ulcerative colitis (UC) compared to patients with active Crohn's disease (CD) and that this difference may serve as a biomarker for the distinction between active UC and CD. It is important to accurately diagnose IBD at an early stage as a correct differentiation between CD and UC defines treatment and prognosis.

The above metabolites differentiating UC and CD include microbial metabolites originating from the fermentation of carbohydrates by the gut microbiota highlighting the importance of the microbiota in the aetiology of IBD. Similar findings have been observed in studies looking at blood metabolites in different mouse models of colitis (Chen et al., 2008). The metabonomics analysis revealed increased levels of stearoyl lysophosphatidylcholine and lower levels of oleoyl lysophosphatidylcholine in blood which the authors traced to an inhibition of stearoyl-CoA desaturase 1 (SCD1) expression in the liver. As this inhibition did not only occur in a dextran sulphate sodium (DSS)-induced colitis model but also in *Citrobacter rodentium*-induced colitis Chen et al. concluded that the observed inhibition of SCD1 is highly likely to be due to the disruption of the intestinal microbiota and the resulting inflammation. Furthermore Marchesi et al. reported higher quantities of amino acids lysine, leucine, isoleucine, valine and alanine – products of bacterial protein metabolism – in faeces of CD patients compared to controls.

Although these studies identified metabolites of microbial origin as playing important physiological roles at the whole body or system level, they did not attempt to link these metabolite profiles with specific bacteria within the gut microbiota.

A study in colon cancer and polypectomized patients attempted to attribute changes in metabolic profiles to changes in bacterial diversity by combining DGGE and metabonomics analysis of fecal water (Scanlan et al., 2008). The authors reported a significantly increased diversity in the *Clostridium leptum* and the *Clostridium coccoides* subgroups as well as relatively higher levels of amino acids such as valine, leucine, isoleucine, glutamate and tyrosine and lower levels of methylamine in fecal water of colon cancer and polypectomized patients compared to control individuals. The altered amino acid profile together with the increased diversity may suggest a higher incidence of potentially detrimental species of clostridia.

The modulating effect of microorganisms on systemic metabolite profiles (blood, jejunal wall and longitudinal mysenteric muscle tissue) was also confirmed by infection with *Trichinella spiralis* in NIH Swiss mice which subsequently caused post-infective irritable bowel syndrome (IBS) – an intestinal disorder characterized by abdominal pain, vomiting and either diarrhoea or constipation (Martin et al., 2006). The metabonomic signature of the *T. spiralis*-infected mice revealed an increased energy metabolism, fat mobilisation and a disruption of amino acid metabolism as well as muscular hypertrophy. The treatment of the infected mice with probiotic *Lactobacillus paracasei* resulted in

metabolic profiles closer to those of uninfected mice indicating a partial normalisation of the muscular activity and the disordered energy metabolism.

3.12 Measuring the Impact of Microbiota Modulation Using Metabonomics

As described above, the gut microbiota interacts intimately with host metabolism to mediate health and disease. These interactions are complex, often involving multiple and interconnected metabolic pathways, which makes a classical approach whereby one or a few metabolites are monitored impractical for investigating microbe:host metabolic interactions. Recently, metabonomics has been employed to measure the consequences of microbiota modulation. Yap et al. (2008) investigated the impact of the broad spectrum glycopeptide antibiotic, vancomycin, on the gut microbiota and the metabonome of female mice. Vancomycin was chosen because it is active against Gram positive bacteria and is poorly absorbed across the gut wall which means it will reach the large bowel. Vancomycin induced changes in the composition of the gut microbiota as determined by 16S rRNA targeted PCR-DGGE was reflected in changes in metabolite profiles in faeces and urine. Vancomycin intervention had a dramatic impact on phenolic regions of the NMR spectrum, with reduced levels of urinary hippurate and phenylacetylglycine which are produced through microbiota:host co-metabolic pathways. Although these changes in urine metabolites appeared to be transitory, particular metabolites took longer to return to pre-treatment levels, with hippurate in particular only returning to pre-vancomycin levels 19 days after the vancomycin intervention. Microbiotal choline metabolism also appeared to be disrupted by vancomycin treatment. Reduced concentrations of trimethylamine (TMA) and trimethylamine-N-oxide (TMAO), gut microbial and hepatic detoxification end products of choline metabolism respectively, were observed in urine post-vancomycin treatment. Vancomycin intervention had a dramatic effect on carbohydrate fermentation, a key functional activity of the gut microbiota, with reduced levels of acetate, propionate and n-butyrate and elevated fecal oligosaccharide concentrations. Considering the important and diverse biological roles of these SCFA in the host, antibiotic disruption of this key microbiotal function may have a significant impact on host health. Such an impact on carbohydrate fermentation and SCFA production may have been expected considering the key roles played by Gram positive bacteria like the bifidobacteria and species belonging to the *C. leptum* and *C. coccoides* groups in polysaccharide

and oligosaccharide fermentation and in cross-feeding on SCFA. Interestingly, although alterations in fermentation end products lasted 13 days post-vancomycin treatment, populations of *C. leptum* and *C. coccoides* appeared to recover from day 2 onwards, indicating that disruption of other bacterial groups present may have had a more dramatic impact on SCFA production than bacteria belonging to these clostridial groupings. Vancomycin intervention also disrupted protein handling by the gut microbiota with elevated levels of amino acids in faeces and reduced levels of creatine and α-ketoisocaproate in urine.

Using a defined microbiota animal model of the infant gut microbiota, Martin et al. (2008) recently described the impact of probiotics (*Lactobacillus paracasei* and *L. rhamnosus*) and prebiotics (two different GOS preparations) on the gut microbiota and host metabonome. This simplified animal model of the human infant gut microbota comprised ex-germ-free animals colonized with strains of *E. coli*, *B. breve*, *B. longum*, *Staphylococcus epidermis*, *S. aureus*, *C. perfringens* and *Bacteroides distasonis* isolated from a healthy twenty day old breast fed human infant. The authors showed distinct metabolite profiles in urine, plasma, fecal extracts and intact liver tissue upon prebiotic induced microbiota modulation using NMR based metabonomics. Supplementation with either prebiotic resulted in elevated population levels of the bifidobacterial strains present, *B. breve* and *B. longum* and reduced levels of *C. perfringens*. When given in combination with *L. paracasei*, reduced numbers of *Bacteroides distasonis* were observed, while reduced numbers of fecal *E. coli* were observed in animals dosed with prebiotic and *L. rhamnosus*. Prebiotic treatment appeared to reduce bacterial proteolysis, with lower concentrations of lysine observed in faeces, isobutyrate in the caecum and *N*-acetyl-glycoproteins in urine. Changes were also observed in choline metabolism upon prebiotic intervention. Co-metabolic processes in the metabolism of dietary choline have been previously shown to impact on insulin resistance, non-alcoholic fatty liver disease and type 2 diabetes in animal models. Both TMA and TMAO concentrations were altered in the urine and liver respectively, indicating prebiotic induced changes in choline metabolism by the gut microbiota. Prebiotic intervention impacted significantly on levels of lipids stored in the liver, with reduced triglycerides and increased concentrations of polyunsaturated fatty acids. Similarly, Prebiotic intervention resulted in increased hepatic glutamate, gutamine, branched-chain amine acids and alanine, and when mice were dosed with prebiotic plus *L. paracasei*, increased hepatic glycogen was observed indicating a stimulation of gluconeogenesis and glycogenesis. Prebiotic intervention also appeared to stimulate animal energy expenditure as indicated by increased levels of taurine and creatine in urine post-prebiotic

supplementation which derive from increased muscular activity. Changes in SCFA within the gastrointestinal tract were measured by gas chromatography and correlated with prebiotic induced alterations in microbiota population levels. In general there was a negative association between SCFA concentrations and population levels of *C. perfringens* and *E. coli* and a positive association between SCFA and bifidobacteria, the lactobacilli and *Bacteroides diastasonis*, corresponding with perceived prebiotic modes of action within the gut microbiota. However, it is difficult to extrapolate these data to the human situation since there are a number of major differences between these model microbiota systems and humans. Principally, in the lack of microbiota complexity, differences in gut physiology (rodents are copiophagious, and their upper gut are colonized by large populations of bacteria unlike healthy humans) and bacterial species may have different biological roles in different animals.

Recently, Li et al. (2008) in an attempt facilitate direct human microbial ecology studies at the "omics" level, combined both metagenomics (16S rRNA targeted PCR-DGGE) and metabonomics (metabolite profiling by NMR) to generate a matrix of differential urinary metabolites and unique bacterial genotypes present in fecal samples collected from four generations of a single Chinese family. These authors were thus able to correlate individual bacterial species identified within the fecal microbiota of the human volunteers by 16S rRNA gene targeted PCR-DGGE with particular profiles of metabolites present in urine. This powerful approach offers for the first time a real insight into the *in vivo* functioning of even unculturable and previously uncharacterized members of the gut microbiota directly without the need for bacterial cultivation and in biological samples which can be collected in a non-invasive manner.

3.13 Conclusion

Recent insights into the composition and make up of the human gut microbiota and the evolution of powerful and high resolution data rich analytical techniques are revolutionizing the way we view the human intestinal microbiota. It is clear that our resident microbiota, which has co-evolved with us over the millennia, impacts on a range of human metabolic processes and appears to be particularly effected by recent population level changes in human diet, particularly the reduction in fiber and whole plant food ingestion and adoption of the Western-style diet which as occurred with growing affluence over the past 50 years. Nowhere is this more clearly illustrated than in the fact that the gut microbiota

of the obese people appears to be different to lean people and that this obese-type microbiota returns to a lean type profile upon weight loss. The application of metagenomics approaches, particularly the recent Human Microbiome Project which aims to genome sequence up to 1,000 gastrointestinal bacteria as well as directly sequence functional communities in different body sites will provide a valuable encyclopaedia of genetic information mapping out the metabolic potential of bacteria residing on or in the human body. Metabonomics, on the other hand offers the possibility of tracking changes in metabolite profiles at the systems level allowing direct measurement of metabolic kinetic or metabolite flux over experimental time courses such as before and after dietary intervention or in the presence or absence of disease. A key recent development has been the combining of these two approaches (Li et al., 2008) offering a powerful tool for direct study of the human gut microbiota *in vivo* and upon dietary modulating. These omics based approaches are thus providing tools of sufficient resolution to allow researchers to realistically address one of the most fundamental and tantalizing questions in the area of functional foods research, "how do probiotics and prebiotics really work."

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4 Designing Trials for Testing the Efficacy of Pre- Pro- and Synbiotics

Stephen Lewis · Charlotte Atkinson

4.1 Introduction

Providing an evidence base for the rational delivery of medicines and treatments is the cornerstone of modern health care delivery. Much of this evidence base is gained through conducting clinical trials.

Superficially, designing a clinical trial seems straightforward. However, in practice many unforeseen difficulties arise with long setting up times, poor recruitment rates and patients or interventions not behaving in the way expected. Unfortunately, clinical trials examining the efficacy of pre-, pro- and synbiotics have developed a reputation for being published in low impact journals and reaching unconvincing conclusions. As a generalization, the reason for this poor reputation is that the trials have tended to be too small and have not used meaningful clinical endpoints. The level of evidence required to alter clinical practice is expected to be high and robust. Trials of drugs such as those used to treat hypertension are often very large with hundreds (if not thousands) of patients and have hard clinical end points, such as stroke, myocardial infarction or death. Many clinical trials involving pre-, pro- or synbiotics have less than 200 patients and often use surrogate markers of health benefit as main outcome measures. This chapter sets out to give an overview of how to design and run a clinical trial highlighting examples and problems related to studies using pre-, pro- and synbiotics.

4.2 Before You Begin

A thorough literature search is required to ensure that any prospective trialist has an in-depth knowledge of the subject area to be researched. Traditionally a trialist would start by looking in electronic databases (e.g., PUBMED, COCHRANE),

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then searching through the references of relevant published papers. Writing to the manufacturers of a given pre-, pro- or synbiotic can be helpful as can writing to other trialists with experience in the field. In particular, it is worth checking to see if any other researchers are studying the chosen or a similar subject such that any new study can complement, rather than duplicate, existing trials. A number of web sites provide up-to-date information on currently registered clinical trials (e.g., www.controlled-trials.com, http://clinicaltrials.gov and http://eudract.emea.europa.eu/index.html).

Much attention is now paid to health claims for functional foods, especially those containing pre or probiotics. The Process for the Assessment of Scientific Support for Claims on Foods (PASSCLAIM) project provides a scheme by which health claims for functional foods could be justified in a scientific manner. The project was initiated by the International Life Science Institute (ILSI) Europe (http://europe.ilsi.org/), which is a European Union backed multiprofessional organization, principally funded by industry. The published criteria (Aggett et al., 2005) provide an excellent source of information for researchers designing both non-clinical and clinical trials. In particular the authors promote the importance of high scientific standards and the requirement to examine physiologically relevant end-points.

Collaboration with other researchers may be desirable. Indeed grant awarding committees are often impressed by a multidisciplinary team approach, which will draw on a breadth of expertise, e.g., pharmacologist, health economist, statistician as well as an experienced trialist.

The International Conference for Harmonization of Good Clinical Practice in Research (ICH-GCP in research) has produced a combined framework for research conducted in Europe, Japan and the United States of America. Researchers are obliged to be compliant with this framework and thus should ensure they are familiar with its requirements. It is also important to ensure that the appropriate resources and motivation are available, as most studies will take a minimum of 3–4 years from conception to publication.

4.3 Hypothesis

The hypothesis is the main focus of the study. It cannot be emphasized how important it is to define clearly the question you would like to answer. The hypothesis is usually based on previous observations or assumptions, and the goal of the study is to either prove or disprove the hypothesis. Keeping the question

simple and focused will greatly increase the study's success in proving or disproving the stated hypothesis. It is often desirable also to examine any underlying mechanisms that support the hypothesis, to explain why the trial either produced the desired result or to give an insight into the reasons for any unexpected results. Generally, researchers test the validity of the "null" hypothesis. The "null" hypothesis is where the assumption is that no clinically important difference of interest in the outcome exists between groups. For example, a trialist may set out to show that an interventional agent such as a probiotic yogurt when taken by patients does not alter the risk of developing endocarditis when compared with patients not taking the probiotic yogurt. An "alternative" hypothesis is that patients taking the probiotic yogurt will have a lower or higher risk of developing endocarditis when compared with patients not taking the probiotic yogurt.

4.4 Choosing an Interventional Agent, Placebo and Packaging

When choosing a pre-, pro- or synbiotic as an interventional agent, justification is required for the choice of agent and the proposed dose to be given. A trialist may be guided by factors such as faecal recovery rates as an indicator of intestinal viability, the demonstration of, such as: immune stimulation in previous studies, or other studies suggesting a benefit in terms of health outcomes for the target population of patients.

If a trialist chooses to use live microorganisms as the interventional agent, quality control and storage are important considerations as inappropriate bacteria may be present (e.g., through contamination) or viable counts of organisms may be less than desired if incorrectly stored (Gilliland and Speck, 1977; Hamilton-Miller et al., 1996). If a clinical trial requires multiple batches of microorganisms to be prepared or a long period of storage then repeated quality control is required.

In most countries pre-, pro- and synbiotic preparations are considered as foods or food additives. It is possible that some preparations – especially, if genetically modified to have specific characteristics (e.g., produce cytokines) would be classified as drugs. In such cases further approvals would be required (in the UK by the Medicines and Healthcare products Regulatory Agency (MHRA)) and considerably more stringent monitoring throughout the trial, especially for complications.

The power of suggestion should not be underestimated. It has been clearly shown that patients do better if they feel that they are receiving an active

intervention rather than placebo. Likewise, if the treatment arm is known to the trialist then there may be a temptation for them to interpret the data according to their personal views of the expected outcome for those taking the active intervention. As such it is desirable that the patient and the treating physician are both unaware of which intervention the patient is receiving (e.g., a "double-blind" trial). This can be achieved by using a placebo. The placebo, where possible, should be indistinguishable in sight (including packaging), smell and taste from any interventional agent. Trials that do not use a placebo are usually much diminished in terms of credibility.

When choosing or developing a placebo, it is important to avoid the use of any materials that could affect the outcome measures or potentially affect recruitment rates. For example, "carrier" substances such as lactose in capsules or milk products in yogurts may cause diarrhea in some people, and therefore must be provided in similar amounts (if at all) to participants taking either the active or placebo intervention. Using products like gelatin or additives (for coloring, taste etc) may exclude some potential volunteers to a study because of vegetarianism or allergy to certain additives. Such products should be avoided where possible.

Where an intervention is compared with a standard treatment, e.g., an anti-motility agent against a probiotic to treat diarrhea, the principles of blinding still apply and both products should be indistinguishable from one another. Alternatively placebos can be given for both products. However, care should be taken when considering such a trial, as it is not always wise to assume that a standard treatment is effective particularly for disorders such as irritable bowel syndrome. Any trial that involves the use of a licensed drug will have to comply with further sets of regulations (see above). Indeed there will be a requirement for the drug, placebo and packaging to be prepared by a licensed facility, which will increase the cost of the trial and also increase the administrative burden on the trial due to the requirement for increased monitoring.

4.5 Choosing the Primary Study End-Point

Choosing the principal outcome measure of any given study, e.g., pneumonia, diarrhea etc is critical. Above all the principal end-point being studied has to be clinically relevant and represent an improvement, which if achieved is likely to alter clinical practice. Generally clinical trials are designed to examine mortality, morbidity, quality of life and economic benefits. Ideally, a clinical trial should be able to isolate the effects of a treatment on a study outcome and provide results

that are free from bias. Surrogate markers of benefit, such as inflammatory or immune function markers are good for exploring mechanisms of action and for planning large, more definitive trials. However, surrogate markers of benefit, even if improved by the intervention, may not imply clinical benefit, especially if seen in healthy people and thus unlikely to alter clinical practice. A chosen primary end-point should be practical to measure and occur frequently enough in the target population to be statistically viable. If more than one principal end-point is chosen, then this usually will require the study to be larger, and thus increases its cost and duration and may reduce its chances of success. End-points can be binary, i.e., Yes/No, or continuous, e.g., blood pressure. A chosen endpoint must be clearly defined using a recognized and relevant definition. This can be difficult; for example what constitutes a wound infection? Trials examining the benefit of postoperative enteral nutrition revealed wound infection rates between 0–33% (Lewis et al., 2001), the large variation being due primarily to the different definitions used to describe a wound infection. A clinician may feel that a relevant definition of wound infection would include the need (or not) for treatment, increased cost, or patient discomfort, but the degree of erythema may be irrelevant, whereas another clinician may place more emphasis on the degree of erythema than other factors. It is also critical that any definition of an endpoint is reproducible; this subject has its own literature, which will not be covered in detail here but should be researched. For example what is a clinically meaningful and reproducible definition of diarrhea? Definitions range from one to three loose bowel motions over 1–3 days. Even trying to define what a loose bowel motion is can be fraught with difficulty, as different observers will interpret definitions such as semi-solid, loose and watery differently. Graphical representations of stool form have been developed such as the Bristol stool form scale, but even this scale has an intra and inter-observer error (Lewis and Heaton, 1997). Creating your own definitions of end-points will be open to criticism unless they have been substantiated. Again, any end point has to be clinically meaningful. If a clinical trial was to show that an intervention led to the resolution of diarrhea say one day earlier than the control group, would this change clinical practice? Probably not, but the answer to this would depend on a number of factors including the cost and ease of taking the intervention. It can be meaningful for trialists to adapt existing definitions or scales to complement their trial. Using the example of diarrhea, as well as collecting data on stool form it would also be relevant to know what a patients degree of urgency to defecate was or whether the presence of faecal incontinence was altered by an interventional agent.

In some clinical trials, assessing the economic benefits may be relevant, though this is difficult to do well. In order to do meaningful healthcare economic analysis it is usual to involve a heath economist early on within the trial design process. The most frequently used health economic measure is quality-adjusted life-years (QALYs) gained; this measurement takes into account the patient's duration of life, and heath related quality of life. QALYs can be used to calculate a cost-effectiveness ratio (the cost of gaining an extra year of good quality life). However, collecting data on costs can be problematic as identifying every health care cost may not be technically possible in many healthcare systems.

Many disease-specific as well as generic scoring systems are available to assess improvement in quality of life, or degree of dependence on carers, severity of illness and predicting outcome from illness. These can usually be easily incorporated into most trial designs. Indeed, it is becoming fashionable to use "composite" measures as the main end-point of clinical trials. Composite end-points may include several relevant outcome measures (e.g., for a study looking at outcomes of patients after a myocardial infarction the following measurements may be relevant: quality of life, heart rate, blood pressure and exercise tolerance tests) grouped together to give an overall score.

Secondary end-points may ask other relevant questions. When considering the study size, it may be appropriate to power the study to look at secondary end points if they are of sufficient interest to justify a larger study.

If possible, data on endpoints should be recorded prospectively rather than retrospectively. If the trial involves collecting data from a patient's notes going through their notes after the end of a study is inadequate. Data may have been either poorly recorded or not recorded at all, and it may not be possible to obtain the data at a later stage. Collecting data as the trial progresses will also allow interim analysis to be conducted if appropriate (see below). Furthermore, it could enable the identification of problems in data recording (e.g., patients may not understand a particular questionnaire and thus fill it in inadequately) or obtaining test results, which can be rectified quickly and with minimal loss of useable data.

4.6 Independent Variables

Independent variables are factors that may influence the main study outcome measure. A thorough review of previous literature to identify independent variables is important, and similar to primary end-points they need to be robustly defined. With regards to studies looking at *Clostridium difficile* related diarrhea, patient age, degree of disability, immune suppression, taking of proton pump inhibitors and

recent antibiotic use need to be recorded, as they can all influence the development of the disease. It is likely that any study will have to be larger to be suitably powered to examine the influence of independent variables on the main outcome end-point, e.g., the use of immune suppressing drugs. The most commonly used approach to remove the potential effects of an independent variable on the outcome of interest is to use a randomized trial design where patients are randomly allocated to an intervention. The independent variables are recorded and if recruited numbers are large enough, then these independent variables will be equally distributed between study groups and their influence on the final outcome (e.g., *C difficile* related diarrhea) should also be equally distributed. If there is concern over the influence of a variable on the main trial outcome measure, e.g., immunosuppressive drugs on the development of diarrhea due to *C difficile*, then the patient's random allocation to different groups can be stratified by the variable. Where stratification is used, this may result in the requirement for greater numbers of patients in the trial to ensure reasonable numbers in each subgroup.

4.7 Clinical Trials

Any new medical drug has to go through various levels of clinical trials, often described as first, second, third and fourth phase trials. Pre-, pro- and synbiotics have not gone through these hurdles because in most countries they are considered to be nutritional supplements and are not subject to the same rules and regulations as drugs. Trialists are, however, interested in substantiating health claims, including benefit and lack of harm, for a given pre-, pro- or synbiotic. Whilst most pre-, pro- or synbiotics are considered safe, it must not be assumed that they are free from potential side effects. Indeed, there is a considerable literature on septicemias caused by many probiotic bacteria and yeasts. Furthermore, prebiotics may cause diarrhea. Thus, it would be wise to pay considerable attention to possible detrimental effects of an intervention when given to certain groups of patients such as those who are immunosuppressed or who are prone to diarrhea (e.g., those with ulcerative colitis).

4.7.1 Phase I Trials (Clinical Pharmacology and Toxicity, Typically 20–80 People)

These are the first experiments in humans (usually healthy volunteers or patients in whom usual treatments have failed), and are primarily concerned with drug

safety and drug pharmacokinetics. Trials often involve small numbers of people and dose escalation studies using predetermined criteria can be conducted.

4.7.2 Phase II Trials (Initial Clinical Investigation of Treatment Effect, Typically 40–100 People)

These are small-scale investigations (although usually larger than phase I trials) into the effectiveness and safety of a drug, and require close monitoring of patients. Usually, but not always, there is no randomization process.

4.7.3 Phase III Trials (Evaluation of Intervention, Typically Greater than 200 People)

After a drug has been shown to be potentially effective in a phase II trial, it is essential to compare this drug against the current standard treatment or a placebo within a real life environment in order to provide definitive data on the effectiveness of an intervention. Phase III trials may require relatively large sample sizes and lengthy follow-up of study participants.

4.7.4 Phase IV Trials (Post Marketing Surveillance)

Large, long-term follow-up studies looking at morbidity and mortality are termed phase IV trials, and may detect uncommon problems, which were not picked up in phase III trials.

4.8 Trial Design

This chapter is principally concerned with phase III trials that are designed to show clinical benefit. The simplest and most widely used design involves an intervention and placebo randomly allocated to subjects or group of subjects, these are known as randomized placebo controlled trials. Where both the trialist and trial subject are blinded to the intervention these trials are called, "double blinded, placebo controlled" (DBPC) and are considered the "gold standard" of clinical trial design. Other ways of designing a clinical trial include studying cohorts of subjects or using a crossover design so that all subjects receive both

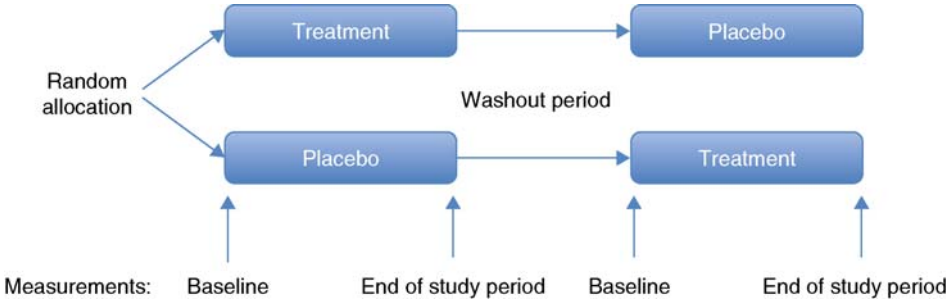

Figure 4.1
Basic cross-over design for a clinical trial Patients are randomly allocated to one of two groups (treatment and placebo groups), they are assessed at the beginning and end of a study period. After a washout period the patients undergo another study period with beginning and end assessment. If there has been no "carry over of effect" then the two baseline assessments for each individual should be similar.

the interventional agent and placebo. The best trial design will depend on the study question, the study population and the nature of the end-points being studied.

If the end-points of a trial can be measured repeatedly over time (e.g., serum cholesterol) then using more complex trial designs such as crossover trial (*Figure 4.1*) can be more economic in terms of numbers of subjects required for adequate statistical power. Any carry-over of effect between different phases of a trial can be assessed by collecting further "baseline" data after the washout period (but prior to the start of the next period) for comparison with the initial baseline data collected at the start of the trial. Using a crossover design any number of study periods can occur, though increasing the length of the time subjects are in the trial may increase the likelihood that they may withdraw from the trial due to the additional burden. If a subject withdraws from a crossover trial the loss of statistical power may be greater than if a simpler trial design had been used, because all of that subjects data from each of the study periods may then be unavailable for analysis.

There are many other design options and the exact format of any trial will depend exactly on what is being studied.

4.9 Protocol and Other Study Documents

Often, trial protocols go through many versions prior to being finalized, which enables ideas to be developed and refined (*Table 4.1*). It is important to discuss

Table 4.1

Ideal contents of a protocol

A clear statement of the clinical question being asked, then how the trial will answer the question
Scientific background, and why the trial results will be meaningful
Trial design and methodology compliant with Good Clinical Practice (GCP) guidelines in research
Study population and how they will be recruited
How the trial will be analyzed then presented

trial design with people who may have done similar trials, in order to identify potential problems - many of which may not be initially obvious.

Protocols are commonly structured along the lines of: Title, Hypothesis, Background/Rationale, Objective/Value of research, Design, Methods, Timetable, Statistics, References, Costings. Some medical journals, e.g., The Lancet require that clinical trials conform to the CONSORT Statement (www.consort-statement.org, Altman et al., 2001; Moher et al., 2001), which is a flow diagram of how a clinical trial should be structured and is worth studying (*Table 4.2*).

The protocol is used as the base document for submission of the study to any "ethics" committee for approval (see below) and, in many centres, for peer review by local research and development (R&D) committees. In most countries the conduct of clinical trials is regulated and audited. Within the European Union (EU), clinical trials are governed by the EU Clinical Trials Directive (http://www.wctn.org.uk/downloads/EU_Directive/Directive.pdf). Particular attention must be paid to data protection and reporting of adverse events. A protocol will be required to state how these requirements will be met.

The final version of the protocol needs to be well written, clear and be in compliance with ethical and regulatory requirements. Many trialists involve patients or members of the public in the design of protocols, as obtaining the view of potential participants may highlight difficulties not appreciated by the trialists; for example, procedures that cause patient discomfort (e.g., colonoscopy), may be off-putting to potential volunteers but may seem perfectly acceptable to a gastroenterologist!

Patient information leaflets and consent forms need to be prepared, which highlight any potential risks or inconveniences to the patient. The text needs to be easily understandable to a lay person. The more involved and complex the study,

Table 4.2

CONSORT Statement 2001 – Checklist Items to include when reporting a randomized trial

(*Cont'd* p. 122)

Paper section and topic	Item	Descriptor	Reported on page #
Title & Abstract	1	How participants were allocated to interventions (e.g., "random allocation," "randomised," or "randomly assigned")	
Introduction	2	Scientific background and explanation of rationale	
Background			
Methods	3	Eligibility criteria for participants and the settings and locations where the data were collected	
Participants			
Interventions	4	Precise details of the interventions intended for each group and how and when they were actually administered	
Objectives	5	Specific objectives and hypotheses	
Outcomes	6	Clearly defined primary and secondary outcome measures and, when applicable, any methods used to enhance the quality of measurements (e.g., multiple observations, training of assessors)	
Sample size	7	How sample size was determined and, when applicable, explanation of any interim analyses and stopping rules	
Randomization – sequence generation	8	Method used to generate the random allocation sequence, including details of any restrictions (e.g., blocking, stratification)	
Randomization – allocation concealment	9	Method used to implement the random allocation sequence (e.g., numbered containers or central telephone), clarifying whether the sequence was concealed until interventions were assigned	
Randomization – implementation	10	Who generated the allocation sequence, who enrolled participants, and who assigned participants to their groups	
Blinding (masking)	11	Whether or not participants, those administering the interventions, and those assessing the outcomes were blinded to group assignment. If done, how the success of blinding was evaluated	
Statistical methods	12	Statistical methods used to compare groups for primary outcome(s); Methods for additional analyses, such as subgroup analyses and adjusted analyses	

Table 4.2

Paper section and topic	Item	Descriptor	Reported on page #
Results Participant flow	13	Flow of participants through each stage (a diagram is strongly recommended). Specifically, for each group report the numbers of participants randomly assigned, receiving intended treatment, completing the study protocol, and analyzed for the primary outcome. Describe protocol deviations from study as planned, together with reasons	
Recruitment	14	Dates defining the periods of recruitment and follow-up	
Baseline data	15	Baseline demographic and clinical characteristics of each group	
Numbers analysed	16	Number of participants (denominator) in each group included in each analysis and whether the analysis was by "intention-to-treat." State the results in absolute numbers when feasible (e.g., 10/20, not 50%)	
Outcomes and estimation	17	For each primary and secondary outcome, a summary of results for each group, and the estimated effect size and its precision (e.g., 95% confidence interval)	
Ancillary analyses	18	Address multiplicity by reporting any other analyses performed, including subgroup analyses and adjusted analyses, indicating those pre-specified and those exploratory	
Adverse events	19	All important adverse events or side effects in each intervention group	
Discussion Interpretation	20	Interpretation of the results, taking into account study hypotheses, sources of potential bias or imprecision and the dangers associated with multiplicity of analyses and outcomes	
Generalisability	21	Generalisability (external validity) of the trial findings	
Overall evidence	22	General interpretation of the results in the context of current evidence	

the less likely patients will volunteer to be part of it. However, the information leaflet and consent form must provide a complete and balanced view of the study to enable the potential study participant to make an informed choice as to whether or not to participate.

4.10 Selection of Target Study Population

For clinical trials to be conducted for the prevention of a given problem, such as *C. difficile* associated diarrhea, then clearly it is clearly important to identify a target population where this is relevant. It may not be appropriate to exclude high-risk populations such as those receiving immunosuppressive drugs or suffering memory impairment, as these are the patients who suffer the majority of the morbidity and mortality associated with this disease. Excluding these patients will require the study to be much larger, be more likely to produce a non-significant result and would not be clinically relevant. Conversely, it may be harder for a clinical trial to show benefit in patients with more severe or end stage disease where there may be less ability to influence the disease process. As such, it may be more appropriate to study patients with milder disease in order to assess any benefits of an intervention. Thus, although it may be more convenient to study for example patients attending hospital clinics, for many conditions such as irritable bowel or asthma, these patients may have more severe problems than patients in the community and demonstration of benefit may be more difficult, and the results of the study may be not be generalisable. However, involving patients in the community rather than for example in a hospital setting, may bring its own set of difficulties that need to be addressed, e.g., low attendance at clinics if they live far away or have poor access to transport.

For the study to be clinically relevant, then the recruited patients should be as similar as possible to those on which the intervention will ultimately be used. Exclusion criteria should be as narrow as possible as the more people that are excluded the harder it will be to recruit and the less generally applicable the results will be. Furthermore, there may be ethical implications of excluding certain populations or groups of people. Thought should be given as to how volunteers will be identified and recruited. This is not always easy, especially if disease-specific databases are not available, and even then potential volunteers may be difficult to recruit if they are not prepared to come to a hospital or clinic, if that is necessary for recruitment purposes. The method of approach may also influence the characteristics of volunteers, e.g., advertisements in magazines or newspapers will only target the readership of those publications. For example, if trying to test whether a probiotic preparation will prevent travellers diarrhea, recruiting volunteers via high street travel agents may produce results that are not relevant to an intended "high-risk" target population (i.e., young backpackers roughing it in hostels with poor sanitation and food hygiene), as the recruited population may

consist primarily of individuals who will be staying in expensive hotels (with hopefully good sanitation and food hygiene).

Clearly considerable thought needs to be spent on identifying a relevant target population of subjects to study, who are easy to recruit and who represent a population to whom the results of the study will be clinically relevant.

4.11 Pilot Studies

Obtaining local data pertinent to the area(s) in which the trial will be run may be informative (e.g., the prevalence of a disease within the local population) as local situations may not reflect national or previously published data. Such information may be useful in terms of assessing the feasibility of running a trial in a particular area.

Where there is little previous data on which to base a trial design a pilot trial will usually be very helpful. The results can provide information that can be used in the calculation of the sample size needed for a definitive study (see below) and the experience gained in running a pilot study may help to iron out any major study design problems prior to starting the main trial. Furthermore, the pilot study may identify any unforeseen ethical problems. A pilot study can quantify likely recruitment rates and enables examination of inclusion and exclusion criteria. They can also give an indication of likely compliance with the protocol. However, whilst doing a pilot study is generally a valuable exercise, many trialists decide not to do them because of the increased effort and expense of doing two studies.

4.12 Statistical Considerations: Power and Sample Size

Obtaining statistical advice when designing a trial is almost an obligatory prerequisite and usually well worth while. Determining the sample size of a study is not an exact science, as one has to make realistic assumptions before doing calculations to decide on an appropriate sample size. It is essential that trials be designed to recruit sufficient numbers of volunteers to avoid Type 2 errors (i.e., where there is a difference between treatments, but the study has failed to detect it), but clearly avoid recruiting too many patients. Type 2 errors occur where the natural variation for the outcome being measured is wider than expected, and the sample size was insufficient to detect any difference. When doing sample size

calculations, consideration needs to be given to the likely "drop-out" rates and potential for poor compliance. Many studies using pre-, pro- or synbiotics are too small to detect benefit even if it occurs. Determining the number of patients required for recruitment will depend on the frequency of occurrence (and standard deviation) of the primary end-point in the study population, and the predicted degree of improvement likely to be seen with the interventional agent. This data may be available from previously published trials or pilot studies, but if not, educated guesses are required. Often trialists overestimate the degree of benefit likely to be seen which may result in an unrealistically small sample size estimate. The smaller the expected difference between treatment groups, the more people will be needed for the trial to be definitive. Another approach is to assume the degree of improvement of the primary outcome measure required from an intervention to be of clinical significance (likely to change clinical practice), e.g., a 20% reduction in length of hospital stay. When planning a large or expensive study, if there are no robust data on which to base sample size calculations it may be desirable to do a pilot study or even schedule an interim analysis to provide information, which will help determine the final size of the study.

Given a fixed sample size, it is nearly always true that simple one to one random allocation (i.e., one person assigned to the intervention for every one person assigned to the placebo/comparison treatment) is statistically the most efficient approach to the randomization process. Placing more participants in one group relative to the other reduces the chance of observing a difference if the sample size is fixed, although the power of the statistical test does not greatly decline unless the ratio exceeds 3–1. If the sample sizes can be increased then unequal distribution of subjects between groups may be beneficial if there are resource constraints or costs (i.e., if the intervention is very expensive or labor intensive), or if a high dropout rate is expected from an intervention because of poor tolerability.

To determine the sample size required for a clinical trial the "power" of a trial needs to be chosen. A trials "power," is the ability to show a significant difference between groups if it exists. The power of a study is calculated as 100%- β, where β is the Type 2 error (chance of arriving at a false negative result). Traditionally β values of 20–10% are used which equates to a power of 80 or 90%. The higher the study's "power" the increased chance the study has of detecting any difference, if a difference between groups exists. The increased confidence in detecting difference between groups is gained with increased study power and requires a larger sample size. Sample size calculations also require assumptions on the Type 1 error rate (detection of a false positive result); this is usually taken as a

1 in 20 chance (5%) and is denoted by α. The motivation behind most clinical trials is to show superiority for an interventional agent over a placebo or other treatment option. Occasionally, trials deliberately set out to show that two interventions are equally effective (called non-inferiority for clinical studies and equivalence for pharmacokinetic studies). In such cases a larger number of patients are usually required than for trials that are designed to show superiority of one agent over another. Calculating the sample size is best done with the aid of a statistician and suitable computer software (e.g., SPSS or STATA).

After obtaining a sample size estimate for the trial size the next step is to assess the likely recruitment rate of patients into a trial. Commonly, trialists overestimate how many patients would be eligible and willing to consent to participate. Once the likely recruitment rate has been established, an estimated time frame for the trial can be calculated. Long accrual periods are associated with failure to complete the trial, as the trialists may lose motivation. The best way to get around this is by getting more investigators and centers to participate. However, the organization of multicentre trials is considerably more complicated and expensive than a single centre trial, which perhaps explains why most studies of pre, pro and synbiotics are done in single centres.

4.13 Randomization Process and Labeling of Packaging

If the trial involves random allocation of one group of patients to a particular intervention (treatment) and another group to a placebo or current/standard treatment, computers are usually used to generate random allocations so that the investigators cannot influence who is given which treatment. Ideally, to avoid bias, the allocations need to be kept secret from the people running the trial and also from the trial subjects themselves (i.e., double-blind). The best way to achieve this is through the use of a separate randomization coordinator or service that can be contacted by telephone or email each time a new randomization code is required. The use of sealed envelopes, randomization lists, etc, are more open to abuse. A master code is often kept locally to enable decoding should there be a need due to, for example, a complication from a trial intervention.

Labeling of the packaging of trial products individually, i.e., 1,2,3,4,5. . ., rather than as "A" or "B" makes it harder for the trialist to predict the content. If all the placebos are labeled as "A" and the active interventions as "B," it is possible that the trialists could eventually work out which was which and potentially introduce bias. Ideally, a person unconnected with data collection and

analysis should supervise the administration of the trial intervention to the study participant. However, this would not completely exclude the possibility of bias if they are not blinded to which treatment the study volunteer is getting. As an estimate of the quality of blinding, when each study participant has completed the study, they and the trialists can be asked whether they thought that they had received the active or placebo treatment.

4.14 Interim Analysis

Interim analysis is generally not encouraged as it may increase the opportunity for bias within the trial, and there are several instances in which early results were published which were later not substantiated by the completion of the whole trial. However, in some cases interim analysis may be appropriate (e.g., where there was little pre-existing data on which to base power calculations prior to the start of the study or if it is important to know if the intervention is providing a beneficial effect or if it is increasing morbidity). If an interim analysis is needed, it should be conducted by people independent from the trialists and if possible, the trialists should be kept blinded from the results if it does not impact on the subsequent running of the trial. Strict criteria for any interim analysis (end-points, safety data, patient accrual rates, quality issues or complaints) should be established in the design stage. The original protocol should contain details of how the trial will be analysed and have predefined criteria for either stopping the study early (either because the effect being studied is too small and extending the study will not detect it, or the effect is so obvious that a larger study is not needed), or allowing it to continue and perhaps increasing the sample size. Interim analysis may also look at recruitment rates as well as safety and compliance issues. Often, after the interim analysis is complete protocol changes are made after discussion with the trialists, interim monitors, trial sponsor and trial financer. Any substantial changes to the protocol would require ethical committee approval.

4.15 Data Analysis

The exact statistical analysis used will depend on the trial design and type of data collected (e.g., quantitative or qualitative). Collecting accurate data and entering it into a spreadsheet in a timely fashion is essential to avoid any potential for later

confusion (e.g., from incorrectly recorded data or if original hard copies of data are lost). Analysis of data from a randomized trial is usually done on an intention to treat basis, where the analysis is based on the initial assigned treatment, not the treatment received. In real life, patients do not take all their medications for a variety of reasons (e.g., side effects), thus it is important to know how well an intervention would be expected to perform and be tolerated if used in a clinical situation. Indeed, one of the "problems" in conducting a clinical trial is that participation is voluntary; thus, the subject may be more or less likely to be compliant with an intervention, than if given to a patient as part of their standard clinical care.

Baseline data particularly on covariates can be used to demonstrate that the random allocation of groups was successful and that both treatment groups were similar at the start of the trial.

4.16 Ethical Considerations

There is a responsibility of the trialist to ensure that the trial design is of the highest quality and that the question being asked is meaningful. It is important for the researcher to be familiar with the ICH-GCP guidelines and any local guidelines that may be in place.

It is vitally important that serious consideration is given before subjecting patients to any risk or inconvenience such that the potential benefits are (1) proportionate to the value of the clinical question being asked, and (2) essential to the correct interpretation of the trial outcome. All clinical research requires approval by a research and ethics committee. These committees will demand that due attention has been paid to the ethical involvement of patients/volunteers, and especially to the involvement of vulnerable patients, such as those with learning difficulties or psychiatric problems. The research trial should be compliant with "The declaration of Helsinki" and subsequent amendments (http://www.wma.net/e/policy/b3.htm). Participants should be informed of what the trial involves and be fully aware of their rights whilst they are taking part. Patients should be given a suitable amount of time to think about their involvement and to ask questions before consenting to take part. They should be aware of the right for them to withdraw from a study at any time, without having to give a reason (and without it affecting their usual care). The involvement of minors or patients who are unable to comprehend the trial design for whatever reason is clearly even more complex. Informed consent needs to be obtained and if dealing with

a patient population who are unable to give this then an ability to obtain appropriate consent from carers should be part of the trial design.

Many trials rely on the motivation of a few individuals and the goodwill of patients. The larger and more complex the study, the more difficult it will be to manage any trial to the required standard without providing incentives for trialists and patients. Financial incentives can be beneficial with respect to improved recruitment rates and motivation of trialists, but ethically these can be problematic (Lexchin et al., 2003). If financial incentives are available to trialists, it is possible that they may also be detrimental, as a trialist may ignore recruitment criteria, or recruit patients without the appropriate motivation, for financial gain. Similarly, if financial incentives are provided to study participants, it is possible that they may be interested only in financial gain and not the collection of reliable data.

All studies should have adequate indemnity insurance. Often the trial sponsor, whether a hospital, university or commercial company will be responsible for arranging this.

Any conflicts of interest must be declared when publishing the findings of a trial and many journals will require a declaration of not only where the monies for the trial came from, but also the sources of any relevant past contributions the trialists may have received.

4.17 Misconduct

Trialists have an ethical and legal responsibility to conduct research and present the results honestly. ICH-GCP framework highlights that all clinical trials have to be conducted to a high standard and that all data are made available for audit, thus ensuring compliance with the approved protocol and honesty in data collection. It is essential to have someone knowledgeable and up to date with this framework who is responsible for collecting trial related data in the appropriate way. Commercially sponsored trials often include continuous audit to ensure completeness in data collection and to highlight any potential problems early. It is important to ensure that data is clear and not open to miss-interpretation.

Data should be kept in a secure environment and if stored on a computer, it should be at least password protected. It is a usual requirement that data is not personalised and is linked only to a named patient/volunteer via a trial specific identification number, with the master key being held elsewhere.

4.18 Recruitment, Consent and Data Collection

Trialists should aim not only gain a legal knowledge of the consenting procedure but also improve their practical skills in this important aspect of a trial. How potential volunteers are approached and encouraged to participate in a trial can have a major influence on the way they are recruited and their subsequent compliance with the trial protocol (Donovan et al., 2002). Potential volunteers should be given background information, a trial specific information sheet, and be encouraged to ask questions. As noted above, potential volunteers should be given sufficient time to make up their minds and ask friends or relatives for advice before signing a consent form and should be aware that they can withdrawal from a trial without having to give a reason. Whilst a trialist should be enthusiastic about recruiting to a trial, a degree of realism is required when enrolling volunteers who are not completely comfortable with being in the study: if a volunteer later withdrawals as a result of, for example not having had a full understanding of the requirements of the trial from the outset, then this is counter productive. Conversely, being too negative about the demands of the trial will result in poor recruitment rates.

It is usual to keep a log not only of those who agree to participate in the trial, but also of those who do not and their given reason(s) for declining participation. Potentially this information can be used to help future recruitment.

Patients in clinical trials are often managed more closely than patients not in a trial. Occasionally this could lead to an overall better outcome in these patients. Conversely the improved data collection and patient management may identify higher than predicted complication rates. In order to reduce bias, individuals blinded to which intervention the patient received should collect and interpret the data.

Compliance with a trial should be recorded if practical (e.g., by recording the amount of remaining tablets at the end of the study, concentrations of an agent in stool or urine, or by direct observation). Information on side effects should also be recorded along with comments on the likelihood that the side effect is due to the interventional agent or not.

Trial data should be collected accurately and anonymously whether on paper or computer, and should always be compliant with any data protection laws. There is a requirement for the original data generated to be stored securely and be available for audit. The exact length of time data is required to be stored will depend on the type of trial and local advice should be taken.

4.19 Monitoring Trial Progress and Protocol Deviations

There is a requirement for trials to have a monitoring committee. It is clearly important to identify and deal with any unpredicted problems that may arise during a trial. This ensures compliance with the protocol and that adverse effects are identified and data is processed appropriately. There is a requirement for adverse event reporting to be done throughout a trial. If serious adverse events occur frequently or unexpectedly they can be investigated in a timely fashion and the trial stopped if it meets the pre-defined criteria for stoppage due to such events.

Maintaining the enthusiasm of the trialists, especially over long recruitment periods can be a challenge, especially if the trial is being conducted in more than one centre. Regular contact, meetings, newsletters and feedback on recruitment rates and problems are essential.

There are numerous reasons why trials do not run according to plan. Planning how protocol deviations, non-compliance, and withdrawal of patients are handled, is ideally done in the trial planning stage. Amendments to the trial protocols are often made after the trial has begun based on unforeseen problems that may have been encountered, newly available information from other published studies, or the results of interim analyses. Clearly identifying any issues with design or recruitment early on in the trial is imperative.

4.20 Dissemination of Research Findings

Even before starting a research project the trialist should ideally have given thought as to how the results will be written up and distributed. If the intention is to publish the trial results in a peer-reviewed journal, then the design and implementation will have to conform to the journal's requirements. Thought should also be given as to how publishable the trial will be if the results were not as expected or "null." Although null results are scientifically important, it is often more difficult to publish the results of a trial if it shows no difference between treatments. Anything that can be done to make a study more attractive even if null should be considered, e.g., producing data on the natural history of a disease process, looking at the underlying mechanism of the disease process, or the mode of action of the intervention. Any trial will be considerably enhanced if it not only answers the question as to whether or not the intervention provided benefit, but also if it sheds light on the mechanism of benefit.

The order in which the names of the trialist are presented on reports or publications should reflect their degree of contribution to the study. It is well to agree this order upfront to avoid later disagreements. It is inappropriate to include the names of people who have not contributed significantly to a trial (Hwang et al., 2003; Slone, 1996). At the time of submission for publication some journals require authors to state their contributions to the trial.

Most trials are written up using a standard format of Abstract, Introduction, Methods, Results and Discussion. If you are submitting your findings to a journal for publication, it is advisable to check the "information for authors" section as individual journals may have slightly different requirements. Generally, the best approach is to keep things as clear and succinct as possible. The title should indicate what the trial is about and possibly its results. The abstract should be brief and give the reader a quick overview of what was done, the main results and their implications. Trialists need to be realistic about their findings and it is advisable to avoid extravagant claims.

Introduction: The introduction should give the reader background information on the topic area, the experimental hypothesis and the importance of the research question being asked.

Methods: This section should include what was done in the trial in enough detail so that others could replicate the study if they so wished. Results should not be presented in this section. This section can be subdivided as relevant, e.g., Design, Participants, Apparatus, Procedures, Laboratory analysis and Data analysis.

Results: Traditionally, this section begins with descriptive statistics such as the age of participants in each group and distributions of other baseline variables, such as sex. The text then goes on to list the main findings of the study in an organized manner. The results should be intelligible to most readers. If results are presented in tables and/or as graphs, they should be understandable without reference to the text. Data should be presented without speculating.

Discussion: This should start with a brief statement of the main results. It is then usual to discuss the implications of the results and how they relate to previous studies and the provisional hypothesis. Comment should be made on any problems that may have occurred during the study, the strength and weaknesses of the study, and other possible interpretations of the results. Comment can then be made on the other findings and ideas for further work.

4.21 Summary

- The results of well-conducted clinical trials provide the evidence base for present day medical practice.
- Setting up and seeing a well-designed clinical trial through to completion and publication requires a considerable amount of energy and enthusiasm from both trialists and study participants.
- Trialists should ensure that they are asking a clear and useful question and seek appropriate advice and support where needed.
- The strict requirements to comply with ICH-GCP guidelines can place a significant burden on the trialists, and may require the employment of persons to help run the trials and ensure compliance with the guidelines.
- The choice of interventional agent and outcome measures needs careful thought.
- Obtaining appropriate advice from other trialists and statisticians is invaluable.
- A multidisciplinary team approach is often required for the successful completion of a clinical trial.
- Ensure an appropriate placebo is used and both trialists and subjects are blinded as to the interventional groups.
- Seeing a clinical trial through to completion and publication is a rewarding experience for those involved and the results may directly improve patient care.
- For further reading consult Field and Hole, 2006; Institute of Clinical Research, 2008; Torgerson and Torgerson, 2008; Wang and Bakhai, 2006.

List of Abbreviations

DBPC	Double Blinded, Placebo Controlled
ICH-GCP	International Conference for Harmonization of Good Clinical Practice

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5 Mechanisms of Prebiotic Impact on Health

H. Steed · S. Macfarlane

5.1 Introduction

Prebiotics were originally defined as non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activities of one or a limited number of bacteria in the colon, thereby improving host health (Gibson and Roberfroid, 1995). However, a more recent definition is that "A prebiotic is a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microbiota that confers benefits upon host wellbeing and health" (Gibson et al., 2004). The principal concept associated with both of these definitions is that the prebiotic has a selective effect on the microbiota that results in an improvement in the health of the host. Common prebiotics in use include inulins, fructo-oligosaccharides (FOS), galacto-oligosaccharides (GOS), soya-oligosaccharides, xylo-oligosaccharides, pyrodextrins, isomalto-oligosaccharides and lactulose. The majority of studies carried out to date have focused on inulin, FOS and GOS (Macfarlane et al., 2008).

To be effective, prebiotics need to reach the large bowel with their chemical and structural properties essentially unchanged. Experimental evidence shows that feeding inulin to ileostomy subjects allows recovery of between 86 and 89% of what is fed. Breath tests after intake of prebiotics show an increase in hydrogen excretion, and no increase in blood glucose or insulin, after feeding healthy individuals 25 g of neosugar (fructo-oligosaccharides). Incubation of fructans in homogenized rat intestinal mucosa show a hydrolysis rate less than 1% of that of sucrose. Prebiotics are able to escape the rigors of digestive processes in the upper gut due to their molecular and structural composition, which renders them essentially resistant to mammalian digestive enzymes. In the main, prebiotics have been considered to be short-chain carbohydrates that

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have a degree of polymerization of two or more, and which are not susceptible to digestion by pancreatic and brush border enzymes.

5.2 Local and Physiological Effects of Prebiotics

5.2.1 Mucosal Structure

Prebiotics can have direct trophic effects on the structure of the colonic mucosa. Studies on rats fed chicory inulin and oligofructose, or pectin for 4 weeks caused an increase in numbers of epithelial cells, and the intensities of their secretory functions (Poldbeltsev et al., 2006). The prebiotic diet also increased the length and width of colonic crypts, which enlarged the available area of nutrient absorption, as well as micronutrients and trace elements in the gut. Studies on the rat caecal and colonic mucosa found that a concentration of 10 g/l of a FOS/inulin mixture (Raftilose® Synergy 1™) increased the macroscopic surface area 2.2-fold, and tissue wall weight increased 2.4-fold (Raschka and Deniel, 2005). Analysis of male Sprague-Dawley rat mucosa in animals fed the indigestible saccharide difructose anhydride III showed significant increases in the outer and inner circumferences of caecal epithelial tissue ($P < 0.005$ and <0.001, respectively), a significant increase in crypt depth ($P < 0.001$), and a significant increase in total cell numbers ($P = 0.001$), particularly goblet cells (Mineo et al., 2006).

The exact mechanisms whereby these morphological changes occur are not known, but butyrate, a major product of bacterial carbohydrate fermentation in the gut, is known to have trophic actions that stimulate mucosal proliferation (Blottiere et al., 2003), while lactic acid stimulates mitosis in the rat caecal epithelium (Ichikawa and Sakata, 1997). It is therefore possible that SCFA are responsible for these adaptive changes. This notion is supported by the fact that these trophic changes do not occur in prebiotic fed germ-free animals, implicating that bacterial metabolic processes are responsible for inducing major architectural alterations in the gut wall.

Administering prebiotics appears to increase the permeability of the paracellular pathway, which is dependent on tight junctions that link adjacent epithelial cells and large multi-protein complexes. Using the Caco-2 intestinal cell line model, the administration of non-digestible oligosaccharides (NDO) was found to rapidly increase paracellular ion transport. Studies are underway to characterize the molecular and physiological changes that occur in these important structures, to see if prebiotics can locally alter epithelial cell junction bonding structures.

5.2.2 Intestinal Mucus

Intestinal mucus is a cysteine-rich glycoprotein made up of high molecular weight mucin (m.w. $> 10^6$) and trefoil factor peptides, produced by goblet cells lining the gut. It is one of the first lines of defense in the intestine, and forms a thick slimy layer on mucosal surfaces. The mucus layer is a dynamic barrier, and the density and number of goblet cells varies throughout the digestive tract, being thickest in the most distal part of the colon. It is also more highly sulfated in the distal bowel, where bacteria are at their highest numbers, giving it a more negative charge to make it less sensitive to bacterial enzyme attack. Mucus is a source of nitrogen and carbon for bacteria; its continuous production by the host makes the gut an inviting niche for microorganisms, despite the fact that very few (culturable) bacteria are able to produce the complete panel of enzymes required to degrade the macromolecule. These organisms include certain species belonging to the genera *Bifidobacterium*, *Bacteroides* and *Ruminococcus*.

Some lactococci can increase the production of trefoil factor peptides in mice, and these substances are able to increase the viscosity of the mucin (Cummings and Macfarlane, 1991). Administration of dietary prebiotics appears to thicken the mucus layer and increase its secretion by goblet cells. The precise mechanism is not well understood, but there is an association with the presence of bacteria and a global increase in the synthesis and secretion of mucin.

5.2.3 Phytic Acid and Mineral Bioavailability

Phytic acid is found mostly in legumes, and possesses apparent anti-carcinogenic properties, providing it reaches the colon without degradation. Phytate is a molecule with six charged phosphate groups, which allows it to bind mineral cations such as zinc, iron and calcium, making them unavailable for absorption by the body. Iron deficiency is associated with anemia, calcium deficiency is associated with osteoporosis, and zinc is needed for skeletal growth and maturation. Research shows that there is a strong inverse relationship between the amount of phytic acid in the diet and iron absorption. Only very small amounts of phytic acid (0.7%) in the diet are needed to halve iron absorption. However, prebiotics are known to have stimulatory effects on iron absorption in the large bowel, by increasing the soluble fraction of iron found in caecal cells, and enhancing absorption by as much as 23% ($P < 0.001$).

The ratio of phytic acid to zinc in the diet is associated with progressive inhibition of metal ion absorption, and in human studies, phytate is strongly associated with decreased zinc uptake in healthy young people, elderly subjects, and in rats. Addition of FOS to the diet restores zinc absorption by enhancing zinc bioavailability ($P < 0.05$).

Studies in the rat caecum have shown that bacterial metabolism results in phytate hydrolysis, and that prebiotic consumption is associated with enhanced breakdown of the molecule (60%), and significantly reduced excretion in feces. This is also linked to greater cation absorption, which is associated with increased bacterial metabolic activities. Organic acids produced during fermentation, particularly SCFA, form soluble ligands with cations to prevent the formation of insoluble mineral phytates, as well as inducing phytase enzymes. The direct effect of SCFA on pH has also been associated with decreased solubility of phytate-mineral complexes.

There have been fewer studies on whether fiber or prebiotics can affect copper bioavailability. Administration of FOS with high and low degrees of polymerization decreased the absorption of copper and other minerals in rats (Delzenne et al., 1995). However, in human studies, administration of FOS increased copper absorption by 45% ($P < 0.05$, Lopez et al., 2000) and 10 g/day short-chain FOS significantly enhanced its absorption in postmenopausal women (Ducros et al., 2005). This increased bioavailability of copper may also depend on the presence of phytic acid in the diet, since this has been shown to decrease total body retention of this metal ion in rats by 57%. However, in one human investigation, feeding high fiber or phytic acid to young men did not have a marked effect on calcium absorption (Turnlund et al., 1985), and phytic acid enhanced copper bioavailability in copper deficient rats (Lee et al., 1988).

5.2.4 Release of Bone-Modulating Factors

FOS increase the bone-preserving effects of phyto-oestrogens in non-ovariectomized mice and rats. Prebiotics also stimulate the growth and metabolic activities of some bifidobacteria and lactobacilli, which can increase synthesis of the luminal bacterial enzyme β-glycosidase, which hydrolyses the glycosidic bond of isoflavone conjugates. This allows flavonoids to be more rapidly absorbed in their free aglycone form, rather than as an intact glycoside. Improvement of

isoflavone bioavailability as an isoform with a higher bone-preserving potential may be an important mechanism that improves bone preservation.

Polyamine production by bacteria in the gut modulates changes in bone structure in rats, in a dose-dependent manner. In ovariectomized rats fed a prebiotic diet, studies using microcomputed tomography to assess bone composition analysis showed that higher levels of polyamines resulted in greater trabecular numbers and areas.

5.3 Modulation of the Gut Microbiota

Evidence from human feeding trials, animal models and *in vitro* modeling systems has shown that prebiotics affect the composition of the gut microbiota, leading to an increase in health-promoting organisms such as bifidobacteria and lactobacilli. These bacteria are generally regarded as safe because they mainly ferment carbohydrates, are not pathogenic and are non-toxigenic, while they have a role in colonization resistance and frequently manifest immunomodulatory properties in the host. Some species are also able to ferment prebiotics to SCFA such as acetate and butyrate, which are important sources of energy for the host. While bifidobacteria do not produce butyrate, they have been shown to stimulate butyrate producing bacterial species such as eubacteria in the gut (Belenguer et al., 2006). SCFA also play a role in regulating growth and cellular differentiation, colonic epithelial cell transport processes, and hepatic control of lipid and carbohydrate metabolism. One advantage that prebiotics have over probiotics is that the target bacteria are already present in the host; however, it should be noted that if the organisms required to promote health are not already present in the gut, due to disease, for example, the prebiotic might manifest no useful effects. Studies with prebiotics have shown that in certain cases, they are able to reduce the numbers of some groups of bacteria in the gut, such as clostridia, bacteroides, enterococci and enterobacteria, some members of which may have a detrimental role in host health. Some of these organisms, particularly the clostridia, are directly toxigenic and are able to breakdown proteins, and ferment their component amino acids, resulting in the production of toxic metabolites such as indoles, phenols, ammonia, thiols, H_2S and amines which may be involved in colorectal cancer (see later).

However, to date the selectivity of most prebiotics has not really been proven since the majority of studies have only looked at changes in a few large groups of bacteria, at group or genus level, which does not really show what is

happening in a global sense. The sugar composition, and degree of polymerization of the prebiotic, together with the availability of other carbohydrates, all affect the way in which bifidobacteria (and other saccharolytic species) are able to grow on these substances, since individual species and strains have specific substrate preferences. Consequently, their utilization can vary markedly in different species. Increases in other groups of bacteria such as eubacteria and roseburia have been found in studies with inulin, while species belonging to other genera such as clostridia and bacteroides have been reported to be able to ferment FOS and GOS, respectively. The increasing use of molecular methods of microbial community analysis, which are able to detect difficult-to-culture species in the gut microbiota, will further highlight the specificities of individual prebiotics. Although much work has been done, there is still a real need for detailed qualitative and quantitative assessments of the gut microbiota to be made, so that the effects that changing microbiota composition has on the nutrition and immunity of the host can be fully understood.

In addition, it may be useful to look at the effects of prebiotics not only on fecal populations, but on mucosal microbiotas in the gut. Recently, a daily combination of 7.5 g inulin and 7.5 g FOS was shown to increase levels of mucosal bifidobacteria and eubacteria (Langlands et al., 2004). This may be useful in the treatment of gastrointestinal diseases such as ulcerative colitis (UC), where decreased levels of bifidobacteria and a general dysbiosis in mucosal bacterial populations have been shown to exist (Macfarlane et al., 2004).

5.4 Immune System

There is increasing interest in modulation of the immune system using prebiotics, which may be particularly useful in inflammatory conditions, or in children and the elderly. Evidence so far suggests that prebiotics can have significant effects on the immune system (› *Table 5.1*). It is however, unknown if these are direct or indirect effects resulting from stimulation by immuno-modulatory bacteria, or production of SCFA, which are known to have immunomodulatory properties, and can bind to SCFA G protein coupled receptors on immune cells within gut-associated lymphoid tissues (Brown et al., 2003).

Addition of FOS and lactulose to the diet has been shown to increase mucosal immunoglobulin production, mesenteric lymph nodes, Peyer's patches and altered cytokine formation in the spleen and intestinal mucosa (Schley and Field, 2002). Investigations on the effects of prebiotics on the immune system

Table 5.1

Modulation of immune function by prebiotics

Beneficial effects on gut associated lymphoid tissue (GALT) and mucosal immune system (MALT)
Increase in mucosal immunoglobulin, altered cytokine and lymphocyte expression, increase in secretory IgA
Indirect effects
Stimulation of immunomodulatory bacteria (bifidobacteria, lactobacilli)
Increased production of SCFA and other fermentation products
Butyrate suppresses cytokine induced and constitutive expression of NF_KB in HT29 cell lines
Propionate is anti-inflammatory to colon cancer cells
Acetate increases peripheral blood antibody production and natural killer cell activity in cancer patients
Pyruvate is anti-inflammatory and decreases NF_KB expression
Through specific G-protein coupled SCFA receptors (GPR41 and GPR43) on immune cells
Direct effects
Little data showing direct effects of prebiotics on immune function

require careful assessments of the choice of markers, which will vary, and be dependent on the condition under study.

Animal studies have indicated that mice fed with FOS and inulin have an improved response to salmonella vaccine. At 1 week post-immunization, splenic cell cultures were shown to have increased production of cytokines such as INF-γ, IL-12 and TNF-α, as well as salmonella-specific blood IgG, and fecal IgA. Overall, the implication is that the FOS/inulin mix stimulated host mucosal immunity to produce a greater response to the salmonella vaccine.

5.5 Lipid Metabolism

Experimental investigations in animals, and a few human studies, have shown interesting cholesterol and/or triglyceride lowering effects, and have raised the question of possible lipidaemia and cardiovascular benefits associated with prebiotic consumption (Jackson et al., 1999; Vigne et al., 1987). Human studies have been small in scope, few and far between, and have focused on the relationship between the intake of prebiotics and serum lipid levels. The results have been inconsistent, and any mechanisms of action unclear.

Rats supplemented with oligofructose have significantly lower serum phospholipid and triacylglycerol levels, and in particular, significantly reduced serum very low density lipoprotein (VLDL), which is produced in the liver by assembling lipids and apoproteins. This effect was caused by a reduction in hepatic synthesis of triacylglycerol, because hepatocytes from these animals have a 40% lower capacity to synthesize this molecule (Fiordaliso et al., 1995; Kok et al., 1996). This is due to a 50% reduction in the activities of key hepatic lipogenesis enzymes; FAS, malic enzyme, ATP citrate lyase, acetyl-coA carboxylase, and glucose-6-phosphate 1-dehydrogenase, as well as a reduction in fatty acid synthase mRNA. The proposed mechanism of action, therefore, is that prebiotics, in some way, modify gene expression of lipogenic enzymes.

In rats fed a high fat, oligofructose-supplemented diet, postprandial triglyceridaemia was halved, and there were no raised free cholesterol levels in the plasma, which would usually be expected after a high fat meal (Kok et al., 1998). These findings implicate an extrahepatic effect of lipid metabolism, which as yet is not fully understood. However, it is postulated that there is a link with prebiotic effects on insulin, which potentiates the gene expression effects. Another possible mechanism is the production of propionate during fermentation, which has been shown to inhibit fatty acid synthesis *in vivo*. When acetate enters the hepatocyte, it is activated by enzymes and then enters the lipogenesis pathways, but propionate competes with the protein that promotes acetate entry to the hepatocytes. Consequently a proposed mechanism of action is the prebiotic's ability to alter the acetate to propionate ratio in the cell.

In obese animals fed a fructan-supplemented diet, no effect was seen on postprandial triglyceridaemia, but there was a reduction in hepatic steatosis. Reductions in fat mass and body weight were also observed, and were presumed to be due to a reduced availability of fatty acids from adipose tissue (Daubioul et al., 2000). Fat digestibility is significantly decreased in dogs receiving an oligosaccharide diet, which may be another mechanism of reducing lipid levels, and the general effects seen in other studies.

A direct effect of alterations in the composition of the microbiota may also be partly responsible for prebiotic manifestations in lipid metabolism. The evidence for this rests with probiotic studies in which lactobacilli and bifidobacteria given in dairy products are known to have a cholesterol-lowering action, which may be due to the production of propionate from lactate, or by enhancement of bile acid deconjugation. However, this notion has to be interpreted with a degree of caution, because in some trials, probiotics have had no demonstrable effects on serum triacylglycerol or cholesterol, while using the same bacteria in synbiotic studies have demonstrated cholesterol-lowering effects.

5.6 Mineral Absorption

One of the most significant health effects of prebiotics on mammalian physiology is their abilities to improve calcium, magnesium, iron and zinc absorption, and the attendant enhancement of bone mineralization and can be seen in *Figure 5.1*, several mechanisms have been proposed for prebiotic action in mineral absorption. Although human studies have been limited and small in scale, this could be beneficial in preventing osteoporosis, a common and often painful disease, as well as in avoiding diet-related anemia and enhancing micronutrient absorption to avoid states of malnutrition.

Intestinal calcium absorption has been observed to increase by up to 20% in animal and human studies, and *in vivo* measurements also show increased calcium retention (Coudray et al., 1997; Griffin et al., 2002). In humans, calcium is mostly absorbed in the small intestine, and prebiotic feeding studies ileostomists have failed to demonstrate increased calcium absorption, suggesting that prebiotics were affecting these processes in the large intestine. Several investigations have confirmed findings suggesting that some calcium is absorbed from the colon, and prebiotic metabolism is thought to increase large intestinal calcium uptake.

There are a number of mechanisms whereby this could occur. Fermentation of prebiotics, as discussed earlier, acts to lower intraluminal pH in the large

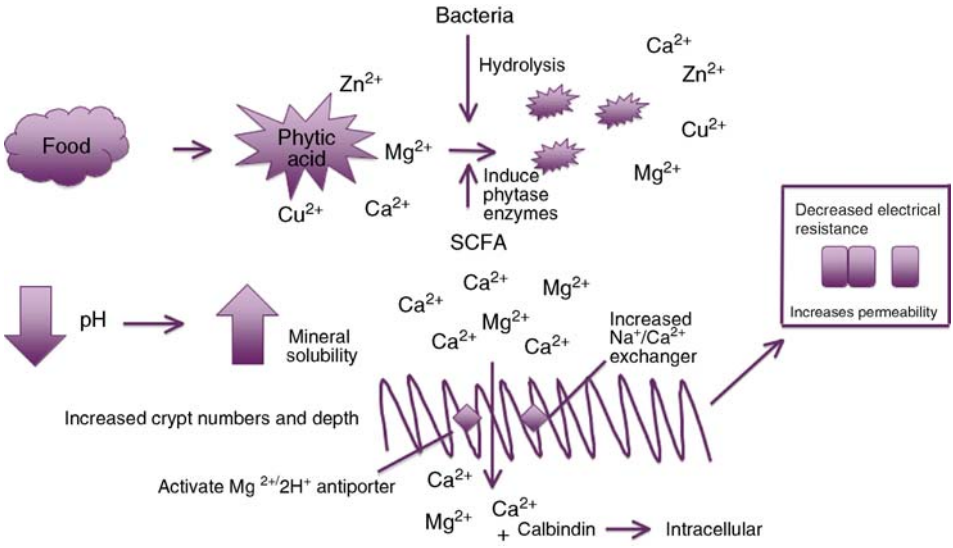

Figure 5.1
Prebiotics mechanisms in mineral absorption.

bowel, thereby enhancing calcium solubility and bioavailability for absorption (Dupuis et al., 1978). Calcium moves across the epithelium via calcium channels, and by ATPase extrusion. Increased calcium solubility increases the gradient across the epithelium and promotes passive uptake, and most studies have shown that net calcium absorption only occurs when a downhill concentration gradient prevails. However, detailed rat experiments have looked at lowering pH, with and without the use of SCFA, and it was found that the calcium flux across the epithelium was only increased in the SCFA experiment, implicating other potential mechanisms of divalent ion transport (Raschka and Deniel, 2005). These authors used paracellular markers, and recorded transepithelial electrical resistance. It was found that the addition of SCFA to rat mucosae increased the permeability of the marker, and decreased electrical resistance. They concluded that some prebiotics interact with the tight epithelial cell junctions, and thereby increase the paracellular permeability of minerals.

Other potential mechanisms that have been proposed for enhanced divalent metal ion uptake have been based on *in vitro* studies on calcium absorption from the ovine rumen. They suggest direct uptake of calcium, with colonic uptake of SCFA, and a direct fermentation effect on gene expression of proteins that are linked to sequestration and mucosal ion binding. This has been confirmed by experiments measuring mRNA levels of mucosal target genes, which found increased transcription of Na+/Ca^{2+} exchanger and calbindin, which would allow increased intracellular binding of calcium, and the basolateral membrane extrusion rate.

Studies looking at the effects of different chain length, and types of branching of inulin-type fructans have found no significant differences in calcium or magnesium absorption, with the exception of the combination of oligofructose and HP-inulin, which appeared to work synergistically to significantly increase the absorption of calcium (Coudray et al., 2003). A mixed diet of inulin and FOS in ovariectomized rats showed a significantly suppressed bone resorption rate relative to the formation rate. However, the mechanism of suppressed resorption is not clear, and requires further investigation.

Magnesium absorption has been specifically linked to the lactate pool in the gut, and low pH, but not the presence of SCFA. Lactic acid is more acidic than SCFA, implying that the mechanism is the act of lowering the pH directly absorption. Despite this, one of the postulated mechanisms of enhanced magnesium absorption with prebiotics is a direct contribution by SCFA via a cation exchange mechanism, where SCFA stimulate the flux of magnesium ions by activating the $Mg^{2+}/2H^{+}$ antiport. This has been demonstrated in

in vitro studies only, using rat hindgut segments. Butyrate was shown to be the most effective SCFA in stimulating the magnesium flux in the mucosal to serosal direction (Kashimura et al., 1996). Absorption of iron from the gut has also been correlated with the presence of SCFA, and to increased weight of the caecal wall, but the precise mechanisms of enhanced iron absorption need to be clarified (Asvarujanon et al., 2005).

Assuming these mechanisms of increasing calcium absorption are operative in humans, prebiotic consumption may be an effective adjuvant to oral calcium for osteopenia and osteoporosis, or they could be administered early to enhance peak one mass prior to the onset of demineralization, which has been observed in rats (Zafar et al., 2004).

5.7 Infants

Oligosaccharides are prebiotic factors in human milk, and increased levels of bifidobacteria in breast-fed babies compared to bottle-fed infants is thought to be due to their ability to utilize these substances. The putative ability of GOS to resemble glycoconjugate receptors on cell surface receptors may also offer protection from pathogenic microorganisms. Prebiotics have been used in infant formulas in Japan over the last 2 decades, and in Europe for the last 5 years. A large number of trials have been carried out in infants, with the majority aimed at determining the ability of the prebiotic to increase levels of fecal bifidobacteria. After repeated trials in babies, the Scientific Committee on Food for the European Commission published a statement to the effect that the addition of 0.8 g/dl of a mixture of 10% short chain FOS and 90% long chain infant formulas was safe to add to infant formula.

5.7.1 Atopic Disease

At birth, babies have acquired high levels of Th2 cytokines from the mother. The Th1/Th2 balance is redressed as the sterile gut becomes colonized after birth. Allergic disease in infants is based on IgE-mediated food allergies, and a Th2-biased response and lower numbers of bifidobacteria are found in allergic infant feces (He et al., 2001; Kalliomaki et al., 2001). Based on this, a logical mechanism of action of prebiotics in allergic disease is their bifidogenic and immune-mediated effects. This idea has been supported by studies administering

raffinose and alginate-based oligosaccharides to allergic infants, which induce a reduced Th2 response (Nagura et al., 2002; Yoshida et al., 2004), presumably by stimulating the Th1-response, and rebalancing the immune response to increase anti-inflammatory cytokines such as IL-10 and TGF-β (Kelly et al., 2005).

Prebiotic feeding studies in allergic infants have demonstrated significant reductions in the incidence of atopic dermatitis, and this was associated with increased numbers of fecal bifidobacteria, although the stools had been frozen prior to analysis, which may have affected bacterial recoveries (Bonten et al., 1997; Moro et al., 2006).

Part of the mucosa-associated immune system (MALT) of the gut includes the large amounts of sIgA, which protect against pathogenic bacteria and viruses adhering to, or invading the intestinal mucosa. IgA secretions are partly degraded by intestinal bacteria, but the antibody coats the organisms, preventing the host having immune reactions against the commensal species i.e., immune tolerance. Formula-fed babies are more prone to allergic disease than those that have been breast-fed, and they are also known to have lower concentrations of sIgA. These levels can be increased by bifidobacteria, lactobacilli and FOS in animals (Moreau and Gaboriau-Routhiau, 2000; Nakamura et al., 2004). A feeding study by Scholtens et al. (2008) involved 215 healthy infants in the first 26 weeks of life, where the infants were randomized to a combination of GOS and FOS or placebo, and used ELISA to measure fecal sIgA, found significantly higher levels in the prebiotic group, at 719 mg/g, compared to 263 mg/g in the placebo group ($P < 0.001$), and higher levels of bifidobacteria ($P < 0.04$, Scholtens et al., 2008). There were also lower numbers of clostridia in the prebiotic group ($p = 0.006$), which was interpreted as evidence of a positive effect on mucosal immunity.

Possible mechanisms of action are for organisms supported by the prebiotic to immunomodulate the host pathway, as described earlier, and INF-γ is known to stimulate the expression of the secretory component of IgA by epithelial cells. However, in prebiotic feeding studies in the mouse, no correlation was found between caecal sIgA concentrations, and changes seen in interferon production, arguing against a potential role for this cytokine.

5.7.2 Necrotising Enterocolitis

In premature infants, bifidobacterial colonization is delayed in favor of high levels of enterobacteria and clostridia, and some premature illnesses, for example necrotising enterocolitis (NEC) are associated with these organisms

e.g., klebsiella, and *E. coli*. Breast-feeding has always been thought to protect against NEC, and in rat models, bifidobacterial colonization reduces the risk of NEC by modulating the inflammatory cascade. In the quail model, use of prebiotics and probiotics reduces caecal lesions in NEC, but there have been no human studies using prebiotics alone. The animal mechanisms have only been studied in so far as to note changes in the bacterial populations, specifically an increase in lactobacilli and bifidobacteria. Presumably, many of the local actions of prebiotics would also be helpful in prevention of this disease, such as the antimicrobial effects, and increased secretory function.

5.7.3 Infection Prevention

Studies in children aimed at prevention of infection have also had mixed results. The addition of 1.1 g of oligofructose daily to cereal in a DBRCT of 123 infants (4–24 months) was associated with reduced episodes of fever and medical visits. The control group had more sick days, and a higher intake of antibiotics. The treatment group had fewer episodes of emesis, regurgitation and perceived discomfort, but there were no changes in growth, constipation and flatulence. However, in a study of 140 children aged 1–2 years, who were receiving antibiotics for bronchitis, the subjects were randomized to a prebiotic or control formula for 3 weeks. Significant increases in fecal bifidobacteria were observed in the prebiotic group, but there were no differences in gastrointestinal side-effects associated with antibiotic use in either group. In another double-blind randomized control trial (DBRCT) involving 134 infants fed prebiotics (8 g/l GOS/FOS) for the first 6 months of life, principally looking at allergic disease, the subjects were followed up until they were 2 years of age. Growth was assessed together with infectious episodes as a secondary endpoint (Arslanoglu et al., 2008). Not only was the incidence of atopic disease reduced in the prebiotic group ($P < 0.05$), but there were fewer episodes of physician diagnosed infections ($P < 0.01$), fewer episodes of fever ($P < 0.00001$), and fewer antibiotic prescriptions ($P < 0.05$). However, the study did not demonstrate mechanisms of action for any of the effects seen, in what was a relatively well-powered investigation.

The main problem with most prebiotic studies in children is that they have largely relied on demonstrating increased levels of bifidobacteria and lactobacilli as evidence of effectiveness. In reality, the bifidogenic effect is well documented. What are now needed are studies linking clinical benefits to more precise mechanisms of action, be they local, systemic, immune or microbiological.

5.8 Gastrointestinal Effects

5.8.1 Irritable Bowel Syndrome

Irritable bowel syndrome (IBS) has been linked to intestinal bacteria in a number of different trials. Culture-based techniques have shown lower levels of bifidobacteria and lactobacilli in IBS patients, but this has not been confirmed in DNA studies. IBS is a difficult disease to investigate, because the symptoms are highly subjective and difficult to quantify, making open label studies inadequate and of limited usefulness.

The main products of prebiotic metabolism in the large bowel are bacterial cell mass, SCFA, and the gases carbon dioxide and hydrogen. Production of gaseous by-products can be a significant disincentive to prebiotic usage, and unwanted gas-related symptoms have been widely reported in prebiotic feeding studies. There have been mixed results in breath hydrogen studies, some demonstrating no change after a 10 g challenge with FOS, and others showing dose-related increases in breath hydrogen, borborygmi and mild flatulence, with intakes ranging from 5 to 20 g/day. Perhaps unsurprisingly, inulin has been reported to have much the same effects as FOS, and doses of 14 g can cause significant increases in flatulence, together with colicky abdominal pain and bloating, which was considered to be unacceptable to 12% of the volunteers taking part in these trials. Undoubtedly there is widespread variation amongst subjects in response to the potential adverse effects of prebiotic fermentation, but there is no question that the production of gas as a major byproduct of fermentation can cause abdominal discomfort, belching, bloating and flatulence. These are amongst the principal symptoms of IBS.

Prebiotic studies in this area have reported symptomatic improvements in general health and nausea ($P = 0.042$), indigestion and flatulence ($P = 0.008$) and diarrhea ($P = 0.003$), but, as yet, there are no major investigations using prebiotics in IBS. It remains likely that there is a subset of patients, possibly those individuals with constipation-predominant IBS, who may benefit from prebiotics, but since many have visceral hypersensitivity, prebiotic fermentation and gas production may exacerbate their symptoms, and have adverse affects on health and wellbeing.

5.8.2 Constipation

Laxative effects have been documented with a number of prebiotics, although as a treatment for constipation, their benefits are limited and unclear. The mechanism

of increasing bowel habit depends on the fermentation of prebiotics to produce SCFA, as well as an increase in bacterial cell mass, leading to stimulation of gastrointestinal peristalsis. The influence of prebiotics on constipation has been assessed in several trials. In two studies by the same group on elderly constipated volunteers, one showed a significant increase in stool weight from 32.4 to 69 g/day with 10 g daily consumption of oligofructose, in the other investigation, a 70% increase in stool weight was found with isomalto-oligosaccharides (Chen et al., 2000, 2001). Gibson et al. (1995) showed that 15 g of FOS could significantly increase stool output from 136 to 154 g/d in a small group of subjects (n = 8). However, this is contrasted by other studies that failed to show any increase in fecal weight when the volunteers were given galacto-oligosaccharides. With the exception of lactulose, the prebiotics studied so far in human trials have been shown to have little effect on managing constipation, and to have only mildly laxative properties. This variability in outcome may be due to the difficulty in measuring daily fecal outputs, while the methods used to measure constipation are mostly qualitative, using bowel habit diaries and patient questionnaires.

5.8.3 Infectious and Antibiotic-Associated Diarrhea

There have been a number of animal studies investigating the efficacy of a prebiotic diet in preventing colonization by pathogenic microorganisms, showing the inhibition of *Salmonella* Typhimurium survival in the gut lumen, and reduced pathogen densities in Peyer's patches. One postulated mechanism involves loss of adhesion sites for pathogenic bacteria, due to the increased number of bifidobacteria in the gut, and by the prebiotics acting directly and blocking adhesion to epithelial cells by functioning as receptor analogues (*Figure 5.2*). This has been translated into human studies looking at the prevention of traveler's diarrhea in a DBRCT, which showed a non-statistically significant reduction (P = 0.08) in the frequency of diarrhea in the prebiotic group (11.2%), compared to the placebos (19.5%) (Cummings et al., 2001). A large study of Peruvian children assessed the frequency and severity of diarrhea in a DBRCT using prebiotic supplementation, and found no benefit in the prebiotic group (Duggan et al., 2003). One reason for the failure to demonstrate any difference in this population is the large numbers of mothers who breast fed, because breast milk contains naturally contains high levels of prebiotics.

Clostridium difficile infections are currently of great interest, and preventative studies for antibiotic-associated diarrhea (AAD) have found that prebiotics

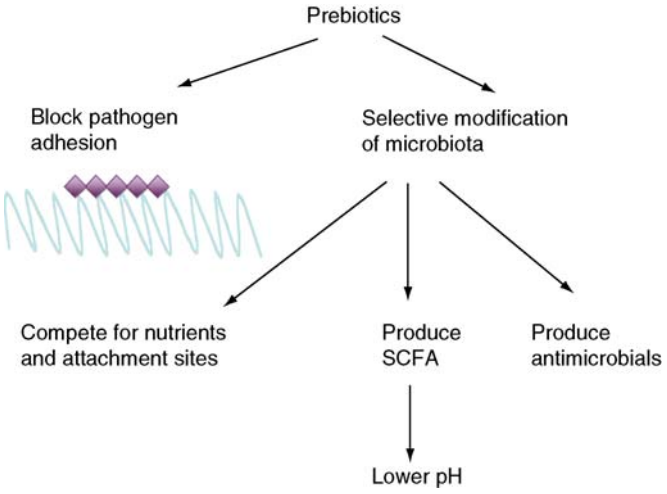

Figure 5.2
Mechanisms of prebiotic action against pathogens.

reduce episodes of AAD and *C. difficile* diarrhea. These investigations have been criticized for excluding the most at risk population groups, and have failed to identify mechanisms of action, but the results are interesting and appear to suggest an ability to prevent relapse, and possibly primary disease, and will inevitably lead to more trials (Lewis et al., 2005).

5.8.4 Inflammatory Bowel Disease

Inflammatory bowel diseases (IBD) such as ulcerative colitis and Crohn's disease are multifactorial disorders associated with reduced levels of bifidobacteria, and increased levels of putatively pathogenic micro-organisms such as *E. coli* and peptostreptococci. Immune tolerance in the bowel mucosa is usually strictly maintained, together with the balance of Th1 and Th2 response profiles. The inflammatory cascade is activated in IBD but then remains inappropriately activated. Therefore, potential mechanisms of prebiotic action in IBD include the bifidogenic effects already described, as well as immunomodulation of the Th1 and Th2 immune responses.

Prebiotic feeding in animal studies with induced colitis, using inulin, FOS or GOS have found improvements in levels of mucosal inflammation. This is associated with reduced inflammatory markers, including TNF-α, tissue

myeloperoxidase activities (an index of neutrophil infiltration), leukotriene B4, IL-1β, thromboxane B2 and prostaglandin E2 ($P < 0.05$, Videla et al., 2001), reduced pH ($P < 0.001$), increased fecal lactate ($P = 0.02$) and butyrate concentrations ($p < 0.001$), and increased proliferation of lactobacilli and bifidobacteria. Murine studies have also demonstrated histological improvements using prebiotics, including reduced severity of crypt damage, that are associated with increased caecal butyrate levels, an attenuation in NFκB activation, serum IL-6 levels and mucosal STAT3 expression (Kanauchi et al., 2003). Treatment of colitis-induced Sprague-Dawley rats with dietary prebiotics have found accelerated colonic epithelial repair and increased caecal butyrate concentrations.

Human prebiotic feeding trials have been limited, and have focused on the outcome rather than the mechanism of action. There are only three reports, one using inulin, one using FOS, and the other employing a combination of the two. They have shown endoscopic and histological improvements in inflammation, which were associated with increased butyrate levels, reduced pH and significantly increased fecal bifidobacterial load ($P < 0.001$). In the mixed prebiotic study, there was an increase in the percentage of IL-10 positive dendritic cells ($P = 0.06$), and of dendritic cells expressing TLR2 and TLR4 ($P = 0.08$ and $P < 0.001$, respectively).

A prebiotic study in 20 patients with pouchitis, which is a clinical condition in patients who have had an ileal pouch-anal anastomosis after total colectomy, used 24 g of inulin per day, for 3 weeks. This study showed improvements in histological and endoscopic scores that were associated with increases in fecal butyrate, and reductions in bacteroides counts ($P < 0.05$) (Welters et al., 2002).

Synbiotic studies have also demonstrated immune changes in IBD, but it is unclear as to what can be attributed to the probiotic, the prebiotic, or to synergy between the two is unclear, and requires delineation. For example, the use of *Bifidobacterium longum* and Synergy I as a synbiotic in a pilot study of UC showed that mucosal TNF-α was significantly reduced, as were inducible human beta defensins 2, 3 and 4, which are specific epithelial markers of inflammation in epithelial cells (Furrie et al., 2005). IgA alterations are discussed in the infants and allergic disease section of this chapter, but there is no record of fecal or MALT sIgA being measured in any IBD studies.

In summary, the use of prebiotics in IBD is a relatively neglected area, in that most studies have tended to focus on the therapeutic benefits of probiotics. As to the mechanistic effects, there are well documented immunological and bifidogenic changes associated with prebiotic use in animal models, and to a lesser extent, in humans. It seems likely that some of the local actions of

prebiotics improve the condition of IBD patients, but as yet, mucosal changes are just referred to as reduced damage, or histological improvements, rather than specific assays of crypt numbers and depth.

5.9 The Elderly

As people get older they often have a greater susceptibility to diseases and suffer from an increase in gastrointestinal infections, malnutrition, constipation and diarrhea. There is evidence for a decrease in immune function and a reduction in numbers and species diversity of beneficial bacteria such as bifidobacteria, along with an increase in potentially harmful organisms such as clostridia, enterococci and enterobacteria in older people (Mitsuoka, 1992; Woodmansey et al., 2004). There have been few studies on the use of prebiotics in the elderly, however, in one investigation involving feeding a synbiotic comprising 6 g of the prebiotic Synergy 1 given in combination with a capsule containing 10^{10} cells each of *Bif. bifidum* and *Bif. lactis* to nine healthy elderly volunteers twice per day, the numbers and diversity of fecal bifidobacterial populations increased significantly in the synbiotic group, compared to the placebos who were given maltodextrin (Bartosch et al., 2005).

5.10 Cancer

Colon cancer is the third most common cancer worldwide, and evidence suggests that along with genetic and lifestyle factors, it is associated with diets high in animal fats and proteins and reduced intakes of fruits and vegetables. Evidence indicates that intestinal bacteria are intimately involved in disease aetiology, and many species have been shown to be able to produce genotoxic and mutagenic products from food components. Thus, supplementing the diet with prebiotics to increase the availability of fermentable carbohydrate, reduce proteolysis and modulate bacterial species to decrease the production of mutagenic and toxic metabolites may protect against large bowel cancer. Stimulation of SCFA production by intestinal bacteria is a mechanism whereby prebiotics could play a role in reducing colorectal cancer (CRC). Butyrate has been shown to stimulate apoptosis, and may be protective in cancer prevention, while propionate has been shown to be anti-inflammatory to colon cancer cells. Prebiotics may also suppress the activities of microbial enzymes involved in genotoxicity and production of cancerogenic metabolites in the large gut. *In vivo* studies administering 4 g of FOS

(Neosugar) per day found that feeding the prebiotic decreased the activities of β-glucuronidase and glycocholic acid hydroxylase (Buddington et al., 1996), which are associated with carcinogen formation. In other fecal studies, 12.5 g of FOS per day had no effect on β-glucuronidase, nitroreductase or azoreductase, although the metabolic significance of fecal enzyme activity measurements is difficult to interpret. But in a larger study involving 53 healthy subjects in a 4 week randomized crossover study, the combination of lactulose and inulin significantly reduced fecal β-glucuronidase activity (De Preter et al., 2008).

Results from several investigations have demonstrated that prebiotics can reduce aberrant crypt foci, decrease cellular proliferation, and lower the incidence of tumors in animal models of colonic cancer. However, to date, there have been no studies on the use of prebiotics alone in human trials, which may be due to the lack of, until recently, suitable markers of disease. In one placebo-controlled study of patients at high risk of colon cancer, a synbiotic combination of 12 g of Synergy 1, and two probiotic bacteria, *Lactobacillus* GG and *Bifidobacterium* BB12, for 3 months. Biomarkers of colon cancer risk and DNA damage in mucosal tissue were reduced by 60% in patients fed the synbiotic, compared to the placebo group (Rafter et al., 2007). Since the distal colon and rectum are the main sites of cancer formation, the use of Synergy 1, which contains rapidly fermentable short-chain FOS and slower fermented long-chain inulin, which prolong the effects of the prebiotic along the length of the gastrointestinal tract, may explain some of the protective effects of the prebiotic in CRC.

5.11 Other Areas

5.11.1 Diabetes

Dietary interventions in diabetes are the first line treatment in type 2 diabetes, aimed at reducing body weight, and altering hyperglycaemia, hyperlipidaemia and insulin resistance, which are the principal mechanisms of pathophysiology in this disease. Because prebiotics are non-digestible low energy bulking ingredients, this makes any beneficial properties they have in diabetic control appealing to a patient group who are often overweight. The proposed mechanisms of action in diabetic disease involve SCFA, particularly acetate and propionate. High concentrations of free fatty acids in plasma lower the use of glucose in tissues, and induce insulin resistance. Acetate has been shown to lower free fatty acids in the plasma, while propionate, as a long-term dietary supplement in rats and humans, has been demonstrated to decrease blood glucose.

Feeding prebiotics to broiler chickens increases glucose absorption in the jejunal mucosa by 70%. In a study with healthy human subjects in which they ingested 20 g of FOS daily for 4 weeks, it was found that the prebiotic reduced hepatic basal glucose production by 6%, but failed to affect insulin-stimulated glucose metabolism or fasting plasma glucose. Studies in type 2 diabetics have variously reported that a FOS diet either failed to change fasting plasma glucose, or lowered it. In a crossover study involving 12 type 2 diabetics taking FOS for 4 weeks, Luo et al. (2000) looked at assessing mechanisms as well as actions, using the glucose tolerance test (GTT) to determine insulin resistance, which was unaffected by the FOS diet. This is contrary to animal studies, where in rats a 3-week 10% FOS diet prevented insulin resistance, and in baboons, a propionic acid supplemented diet caused a lower glycaemic response to the oral GTT. This study also failed to find any difference in basal hepatic glucose production.

From the small number of studies done so far, it is hard to determine whether the apparent differences in animal and human studies are species-related, or are due to the different doses of prebiotic used. What is clear, however, is that more research is needed to clarify the effects and mechanisms of prebiotic action in human type 2 diabetics. These studies will require subjects to take the prebiotic for longer periods of time, and where possible, in higher doses.

5.11.2 Rheumatoid Arthritis

Adjuvant-induced arthritis in Wistar rats and type II collagen-induced arthritis DBA/1J mice were fed galacto-oligosaccharides orally, which reduced erythema, joint swelling and histopathological findings. These changes were directly associated with a reduction in plasma nitrite/nitrate levels in the rats. In cell culture systems using peritoneal rat macrophages, the prebiotic increased IL-1 production. Therefore, by modulating the intestinal microbiota in animals with arthritis, an immunomodulating effect can be achieved in such a way as to alter inflammatory joint symptoms. In a study involving 16 rats, half of which were fed Synergy 1, the prebiotic significantly reduced inflammatory scores, pro-inflammatory cytokines and increased caecal bifidobacteria and lactobacilli (Hoentjen et al., 2005).

5.11.3 Obesity

Prebiotics are currently of particular interest to the food and drink industry because of their low calorific values. GOS are stable at high temperatures and

have low metabolic value at 1.73 kcal/g, while FOS are similar at 1.5 kcal/g. The non-digestibility of prebiotics in the upper GI tract and their long and safe use by the food industry demonstrates that they have excellent potential to act as substitutes for sucrose, and to be used as sweeteners. Feeding studies (Cani et al., 2006) in ten healthy subjects fed 8 g of oligofructose per day showed that they promoted satiety, and reduced food intake ($P < 0.05$). There is also evidence from animal work implicating intestinal bacteria in the pathogenesis of obesity, and theoretically, selective modification of the microbiota may impact on this. In terms of clinical trials in the obese population, this has yet to be investigated, but these early results show promising potential for use in the so-called obesity epidemic that is occurring in Western countries.

5.11.4 General Wellbeing

The promotion of wellbeing is one of the main claims made by manufacturers for many functional foods. However, few studies have been undertaken in humans on the effects of prebiotics on wellbeing or enhancements in quality of life. This may be due to the fact that wellbeing is difficult to measure, largely due to the number of parameters that need to be taken into account to substantiate this, such as those related to energy, mood and cognitive function. In one recent study on 142 healthy volunteers, where patients filled out questionnaires on a range of factors related to wellbeing (Aspiroz, 2005), consumption of 10 g/day inulin was shown to have no effect compared to the placebo group, in mood, sleep quality or bowel function, however, increased wind, bloating and stomach cramps occurred in some subjects.

5.11.5 Other Developing Areas

The mechanism of hepatic encephalopathy is incompletely understood, but it has been attributed to ammonia levels in the plasma, and an altered aromatic amino acid, branched chain amino acid ratio. A longstanding basic treatment of hepatic encephalopathy has been regular and frequent high doses of lactulose, with patients expected to move their bowels at least two or three times per day. Unsurprisingly, the basic mechanisms of action are, as with constipation, an increased bacterial cell mass and SCFA production to cause peristalsis. The act of increased peristalsis itself has been presumed to remove deaminating bacteria,

and reduce intestinal uptake of toxic bacterial metabolites such as ammonia. These suppositions are just that, and have as yet to be followed up by confirmatory research.

There has been some work investigating the possibility of preventing infections in critically ill patients using prebiotics. One trial looked at whether prebiotics affected gastrointestinal permeability, using urinary excretion of sucrose and the lactulose/mannitol ratio in burns patients (Olguin et al., 2005). This group failed to demonstrate any improvement in barrier function, but this method is more useful for assessing upper gut permeability rather than lower that in the large bowel, which is where prebiotics would be expected to have their main effects.

5.12 Summary

- Prebiotics have a long history of safe commercial use, and are consumed on a daily basis by most adults. With the potential for prebiotic use to promote health being so wide, it is inevitable that research in this area will be exponential over the next decade.
- The bifidogenic effects of prebiotics are well documented, and their immunomodulatory properties are becoming better understood. However, what is now clear is that simply documenting a bifidogenic effect is no longer an acceptable way to explain any health benefits that may accrue from prebiotic consumption.
- Well-planned human prebiotic trials are needed in numerous areas, either to clarify conflicting results from previous studies, or to assess the basic health effects of these substances.
- Researchers conducting investigations need to take into account the importance of demonstrating the mechanisms involved, rather than simply reporting phenomenological observations.
- It is becoming increasingly evident that the mechanisms of prebiotic action are more complex than first thought, and involve genetic and systemic effects that we are only just beginning to understand, even with the sophisticated investigative techniques that are now available.
- The reasons for changes in cytokine formation in IBD studies, changes in bacterial cell mass in laxation, or the metabolic activities of prebiotics in lipid and diabetic studies need to be determined.

- In light of animal studies showing the potential for harm by prebiotic consumption by increasing salmonella translocation due to increased intestinal permeability (Bovee-Oudenhoven et al., 2003), it is important that mechanisms of action are understood as completely as possible to enable their safe and appropriate selection for human trials.
- A study looking at the gene expression found that FOS significantly induced the expression of 177 mitochondria-related colonic genes in rats (Rodenburg et al., 2008), emphasizing how much more we have to discover about prebiotic actions in health.
- More clarity is needed concerning the mechanisms of prebiotic impact on health, and it is essential that researchers and industry back up any health claims, with factual scientific evidence of how prebiotics work.

List of Abbreviations

AAD	antibiotic-associated diarrhea
CRC	colorectal cancer
DBRCT	double-blind randomized controlled trial
ELISA	enzyme linked immuno absorbent assay
FOS	fructo-oligosaccharide
GOS	galacto-oligosaccharide
GTT	glucose tolerance test
IBD	inflammatory bowel disease
IBS	irritable bowel syndrome
IL-10	interleukin 10
INF-γ	interferon gamma
MALT	mucosa-associated immune system
NDO	non-digestable oligosaccharides
NEC	necrotising enterocolitis
SCFA	short chain fatty acid
TLR-2	toll receptor 2
TNF-α	tumor necrosis factor alpha
UC	ulcerative colitis
VLDL	very low density lipoprotein

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6 Fructan Prebiotics Derived from Inulin

Douwina Bosscher

6.1 Introduction

Inulin, as well as the shorter form oligofructose, is a nondigestible carbohydrate (fructan) that has been part of the daily food of mankind for centuries. Inulin-type fructans naturally occur in many edible plants as storage carbohydrates. They are present in leek, onion, garlic, wheat, chicory, artichoke, and banana. It is estimated that an average North American consumes about 1–4 g/day of inulin or oligofructose. In Western Europe, the average intake varies between 3 and 10 g/day. Occasionally, people can have higher intakes, e.g., after consuming a bowl of French onion soup, salsify dish, etc., and intakes can then exceed easily 10 g. This illustrates that via the normal diet some, and at certain times, all populations consume relatively high quantities of inulin-type fructans. It also follows that wheat, onion, and banana, and to a lesser extend garlic are the most important sources of inulin-type fructans in the diet. Although inulin-type fructans are nutritive substances and part of our daily diet, these compounds are currently not taken up in food composition tables.

On an in industry scale, inulin-type fructans are obtained from the roots of the chicory plant. After the extraction process, inulin and oligofructose are processed and purified for use as functional food ingredients in a variety of foods. Foods that can contain inulin and oligofructose are bakery products and breakfast cereals, watery systems such as drinks, dairy products and table spreads, and even tablets. Inulin is often used in the manufacture of low-fat dairy products, such as milk drinks, fresh cheeses, yoghurts, creams, dips, and dairy desserts. Other low-fat products in which inulin can be applied are table spreads, butter-like products, dairy spreads, cream cheeses, and processed cheeses. Inulin allows the replacement of significant amounts of fat (up to 100%) and the stabilization of the emulsion while improving the foods' organoleptic characteristics, upgrading both taste and mouthfeel, in a wide range of applications.

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Oligofructose is also often formulated in dairy products through incorporation into the added fruit preparation (e.g., fruit yoghurts). Other (low-calorie) dairy products in which oligofructose can be applied are frozen desserts and meal replacers. In such food products oligofructose is often used as sugar replacer on account of its sweet taste. Its incorporation into baked goods allows, besides sugar replacement, also better moisture retention. The use of oligofructose in recipes is quite straightforward and mostly only requires minor adaptations of the production process. Additionally, new food technical applications of inulin-type fructans are being studied for their potential as carriers for probiotic cultures improving their stability and viability in food products during food processing and storage, but also during gastrointestinal passage and residence in the host microbiota. Probiotic foods are often dairy-based products in which inulin-type fructans can be easily applied. The use of inulin-type fructans together with probiotic cultures is often referred to as "synbiotic," given the combination of probiotics and prebiotics to act in a dual beneficial way. The term "prebiotic" will be explained further in the text.

Inulin-type fructans are nowadays more and more used in foods, and more particularly in the manufacture "functional foods," because of their nutritional advantages. Inulin-type fructans have various physiological, metabolic, hormonal, and immunological effects, being beneficial to the health and well-being of the host. The origins of these effects lie in their fermentation by the endogenous microbiota of the lower intestinal tract and the characterized "prebiotic" effect. They are not digested, nor absorbed, in the upper gastrointestinal tract. In the large intestine, they are selectively fermented by the endogenous microbiota, increasing growth and/or activity of mainly bifidobacteria and lactobacilli, which are bacterial genera that are considered as biomarkers of a healthy colon microbiota. The prebiotic effect of inulin-type fructans in humans has been demonstrated in randomized, double-blind, and placebo-controlled studies in children, adults, and elderly and more recently in neonates. Given the well-described prebiotic effect of inulin-type fructans, these compounds are nowadays considered as the reference prebiotics and, therefore, frequently used in studies to compare the prebiotic effect of potential new compounds with.

There exists a large body of evidence, ranging from *in vitro* work to studies in animal models and humans, not only on the effects of inulin-type fructans on intestinal function, intestinal infection and inflammation, colonic cancers, immuno-modulation activity, and mineral absorption and accretion but also on the effects outside the intestine affecting sugar and lipid metabolism, oxidative stress, appetite, and subsequent energy intake. The systemic effects of inulin-type

fructans have their origins in the intestinal tract, and new insights in the role of the colonic microbiota in overall health and disease (e.g., obesity) explain the potential of compounds modulating the microbiota to have extra-intestinal effects too. In certain fields the evidence on the benefits of prebiotics is well documented, whereas in others still some discrepancy exists. However, it is clear that the role of the colonic microbiota in health and disease, as well as the strategies of modulating it, is a most exciting area of research and that more knowledge can be expected to be generated in the coming years.

6.2 Natural Occurrence

Inulin-type fructans are naturally occurring oligosaccharides that represent the carbohydrate reserve in plants. After starch, fructans are the most abundant nonstructural polysaccharides found in nature. Plants containing inulin-type fructans primarily belong to the Liliales, e.g., leek, onion, garlic, and asparagus; or the Compositae, such as Jerusalem artichoke (*Helianthus tuberosus*), dahlia, and chicory (*Cichorium intybus*). The following text gives some examples of the inulin content and the distribution in chain length (degree of polymerization, DP) of the inulin chains in a selection of food items.

Samples were analyzed by HPLC by Van Loo et al. (1995). For more information about the analytical methodologies to measure the inulin content in foods, the reader is referred to Section 4. The inulin content of commercial onion types (*Allium cepa*) ranges from 1.1 to 7.1 g/100 g (fresh weight) with a range distribution of DP of the inulin chains of 1–12 (most occurring DP is 5). The Jerusalem artichoke has an inulin content ranging from 17 to 20.5 g/100 g (fresh weight) with 74% of the chains of DP 2–19, 20% of DP 19–40, and 6% of DP $>$ 40. Chicory contains 15.2–20.5 g/100 g (fresh weight) inulin with a distribution of 55% of DP 2–19, 28% of DP 19–40, and 17% of DP $>$ 40.

Most of the inulin produced on an industrial scale is nowadays derived from chicory roots. Of interest to the reader is that the chicory used for inulin production (*C. intybus*) is of the same type as the one used for the production of the coffee substitute. The roots look like small, oblong sugar beets. Their inulin content is high and fairly stable over time for a given region, with values ranging from 16.2, 17.0, 16.1, 14.7, and 14.5 g/100 g from August till November, respectively. The distribution in chain lengths, however, is more prone to variation and decreases during harvest from DP of 13.6–9.5 over the same period respectively.

6.3 Chemical Structure

Inulin is a polydisperse carbohydrate material consisting mainly, if not exclusively, of ß (2–1) fructosyl–fructose links. A starting glucose moiety can be present. Fructan is a more general name used for any compound in which one or more fructosyl–fructose links constitute the majority of linkages (e.g., covering both inulin and levan). Inulin-type fructans can be represented as both GF_n and F_m. In chicory inulin, *n*, the number of fructose units linked to a terminal glucose, can vary from 2 to 70 units. This means that inulin is a mixture of oligomers and polymers. The molecular structure of inulin compounds is shown in *Figure 6.1*.

Native inulin is a mixture of oligomers and polymers with a DP ranging from 3 to 70 (average 10). "Native" refers to the inulin as it is extracted from the fresh roots. The lengths of the inulin chains can be reduced by means of an *endo*-inulinase to a DP between 2 and 8 (average DP = 4). The resulting product, called "oligofructose," is a mixture of GF*n* (G = glucose, F = fructose, and *n* = number of fructose monomers) and F*m* fragments, which are respectively, the sucrose endings and the fragmented polymer tail. By physical separation of the longer chains, a long-chain inulin product can be manufactured (DP ranges between

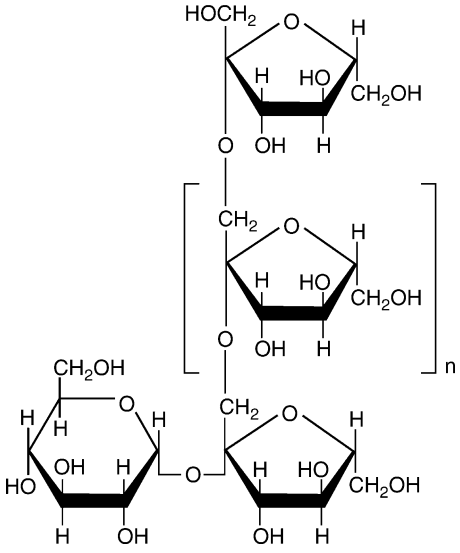

Figure 6.1
Chemical structure of inulin compounds.

12 and 65; average DP = 25). Oligofructose-enriched inulin (a patented mixture which is commercially named Synergy1) is made of a mixture of oligofructose and long-chain inulin and comprises a preparation with a selected range of chain lengths.

6.4 Quantitative Analysis

Several methodologies for the determination of inulin-type fructans have been established. These include HP-anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD), high-temperature gas chromatography (GC), or liquid chromatography (LC). In all these analytical methods, however, other oligo- and polysaccharides present in the sample interfere with the inulin peaks, and therefore allow only partial quantification. Therefore, techniques that hydrolyze the inulin chains prior to analysis of their constituent monosaccharides by GC, LC, HPAEC-PAD, or thin-layer chromatography are most preferred and in common use nowadays.

Inulin-type fructans can be quantitatively determined by the AOAC method no. 997.08 (Hoebregs, 1997). This method is a reliable enzymatic and chromatographic method for the quantification of inulin and oligofructose, but is very labor intensive and requires expensive chromatographic equipments. An outline of the method is given in *Figure 6.2*. If inulin is the only compound present in the sample, the method consists only of steps 1 and 3. The inulin is extracted from a substrate at 85°C for 10 min; part of the extract is kept apart for determination of free fructose, glucose, and sucrose by any reliable chromatographic method available (HPLC, HGC, or HPAEC-PAD) and the other part is submitted to an enzymatic hydrolysis. After the hydrolysis step, the resulting fructose and glucose are determined again by chromatography. By subtracting the initial glucose, fructose, and sucrose content from the final ones, the following formula can be applied: $Inu = k(G_{inu} + F_{inu})$, where k (<1) depends on the DP of the inulin analyzed and corrects for the water gain after hydrolysis. G_{inu} and F_{inu} are, respectively, the glucose and fructose strictly originating from inulin. If a complex sample needs to be analyzed, as is often the case dealing with food products, an amyloglucosidase treatment must be included before step 3 and an extra sugar analysis performed to avoid overestimation of the glucose originating from starch or maltodextrins present.

A faster and easier method is an enzymatic and spectrophotometric analysis (AOAC Method 999.03). This method can be used for a correct quantitative

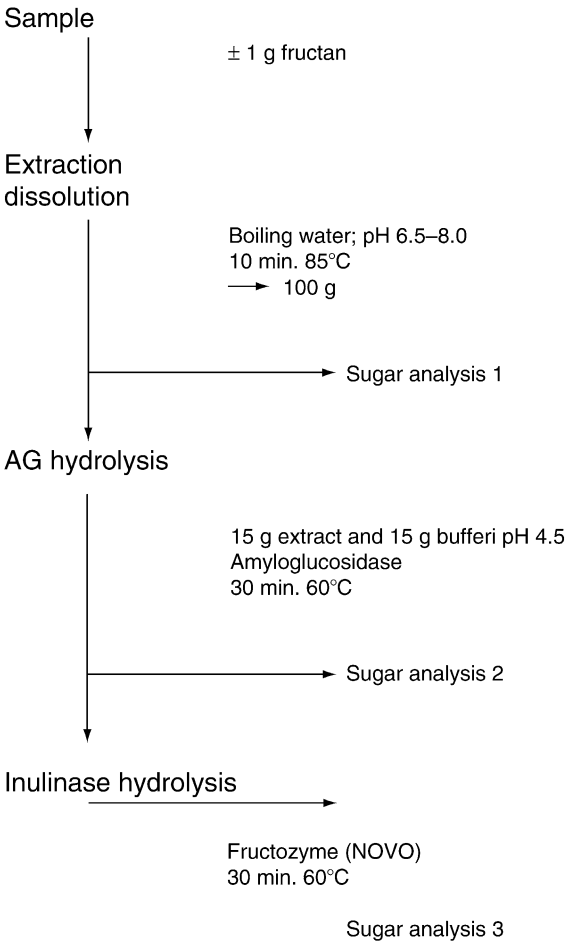

Figure 6.2
Flow diagram of enzymatic fructan method.

determination of the inulin content of foods. In case of oligofructose, however, the technique causes a considerable underestimation of the result (about 20%), and therefore cannot be generally applied. Therefore, a new enzymatic and spectrophotometric method was developed that is simple and accurate and quantifies both inulin and oligofructose from any matrix using only standard laboratory equipment. The principle is an enzymatic hydrolysis of both inulin and oligofructose by a fructanase into glucose and fructose. Sugars are determined in the initial extract and subsequent hydrolysates by a spectrophotometer. Subsequent calculation of the contents of inulin and oligofructose in the matrix

are based "only" on fructose measurements. Extensive validation of the method has yielded high recovery (96.0 ± 5.3%) and good accuracy and precision data (5.9%) (Steegmans et al., 2004).

6.5 Physical and Chemical Properties

Chicory inulins are available as white and odorless powders with different particle distribution and density. The taste is neutral or slightly sweet, without any off-flavor or aftertaste. The shorter chains have about 10% sweetness compared to sugar, whereas long-chain inulin has no sweetness. It behaves like a bulk ingredient, and contributes to body and mouthfeel, provides a better-sustained flavor with reduced aftertaste (e.g., in combination with high-potency sweeteners), and improves stability. Inulin is moderately soluble in water (maximum 10% solubility at room temperature), which allows its incorporation into watery systems (other fibers often precipitate). In very acidic conditions, the β(2-1) bonds between the fructose units in inulin can be (partially) hydrolyzed and fructose formed. At high concentration (>25% in water for native inulin and >15% for long-chain inulin) it has gelling properties and forms a particle gel network after shearing. When thoroughly mixed with water, or another aqueous liquid, a white, creamy structure results, which can easily be incorporated into foods to replace fat by up to 100%. Inulin also improves the stability of foams and emulsions, such as aerated dairy desserts, ice creams, table spreads, and sauces. It can, therefore, replace other stabilizers in different food products (Franck, 2002). Oligofructose is much more soluble than inulin (up to 85% solubility in water at room temperature). It is fairly sweet (35% compared to sucrose) and has a sweetening profile closely approaching that of sugar with a clean taste (no lingering effect). Oligofructose combines with intense sweeteners (such as aspartame and acesulfam K), providing mixtures with rounder mouthfeel and improved flavor with reduced aftertaste. Oligofructose has technological properties close to sucrose and glucose syrups, and, therefore, together with its sweetness profile, is frequently used as a sugar alternative, e.g., in low glycaemic or diabetic foods (Franck, 2002).

Dairy products are frequently used as food vehicles for probiotics. Frequently used probiotics belong to the genera of bifidobacteria and lactobacilli, which most often are able to ferment inulin-type fructans. From this perspective, many food manufacturers have studied the functionality of selected synbiotic combinations in food matrices with respect to probiotic viability not only in the food product itself but also their survivability in the gastrointestinal tract.

In the PROTECH (Nutritional Enhancement of Probiotics and Prebiotics: Technology Aspects on Microbial Viability, Stability, Functionality and Prebiotic Function; QLK1-CT-2000-30042) project, the strategies to address and overcome such specific scientific and technological hurdles that impact on the performance of functional foods based on probiotic–prebiotic interactions were the subject of investigation (Ananta et al., 2004). Increased growth of probiotic bacteria, enhancement in their activity, and good retention of their viability during food storage under different conditions (duration, cooling, pH conditions, etc.) and during passage in the gastrointestinal tract in the presence of inulin or oligofructose have been demonstrated for some strains. In addition, progress in the development of probiotic delivery systems by the use of prebiotic encapsulation materials has also offerred new insights into the ways to maximize probiotic viability and survivability. A good matching of the probiotic with an appropriate prebiotic is a prerequisite to obtain maximum beneficial effect of the synbiotic combination with respect to its nutritional properties as well as technical applications and, therefore, optimal utilization in the respective food matrix.

6.6 Nutritional Properties

6.6.1 Caloric Value

The digestive enzymes in mammals are not able to hydrolyze the inulin polymer (or its oligofructose oligomers), and therefore the compound passes unaltered through the mouth, the stomach, and the small intestine. Studies in ileostomized volunteers have demonstrated that orally ingested inulin enters the colon almost quantitatively (>90%), where is it subsequently completely metabolized by the endogenous colonic microbiota.

In the colon, inulin-type fructans are completely converted by the microbiota into bacterial biomass, organic acids such as lactic acid and short-chain fatty acids (SCFA: acetic, propionic, and butyric acid), and gases (CO_2, H_2, CH_4). SCFA and lactate contribute to the host's energy metabolism. SCFA and lactate are partly used by the bacteria themselves and partly taken up by the host. Still, SCFA and lactate are less effective energy substrates than sugars. These factors together explain the low caloric value of inulin-type fructans (compared to its constituent monosaccharide moieties).

On the basis of ^{14}C studies in humans, a caloric value of 1.5 kcal/g was calculated for short-chain fructo-oligosaccharides. Experimental *in vitro*

(fermentation) and *in vivo* data (rat experiments) allowed Roberfroid et al. (1993) to calculate the caloric value of inulin and oligofructose to be 1.4 kcal/g, according to basic biochemical principles. Other scientific observations have even suggested lower caloric values. Currently, a caloric value between 1 and 1.5 kcal/g inulin-type fructans is being used for food labeling practices.

6.6.2 Acceptability

Because of their indigestibility, nondigestible oligosaccharides that pass into the colon can induce osmotic effects. These are mainly induced by smaller molecules and lead to an increased presence of water in the colon. This is probably the reason why, e.g., lactulose has a higher laxative potential than inulin. Other determinants of acceptability are the production of gases that results from bacterial fermentation in the colon. Slowly fermenting compounds have been shown to be easier to tolerate than more fast fermenting analogs. This can explain why inulin is easier to tolerate than polyols and short-chain oligofructose. Flatulence is a well-known and often accepted side effect of higher intake of dietary fibers in general.

6.6.3 Intestinal Function, Metabolism, and Microbiota

6.6.3.1 Intestinal Function

Inulin-type fructans, through their presence and subsequent fermentation in the large bowel, influence the colonic metabolism in its lumen and the integrity and functioning of the epithelial cell lining. Randomized, double-blind, and placebo-controlled studies in humans have observed a significant increase in weighted stool output (also called the "fecal bulking effect") upon inulin intake with an (approximate) increase in stool weight of 1.5–2.0 g per gram of inulin intake (referred to as "bulking index") (Den Hond et al., 2000; Gibson et al., 1995a). This level of stool bulking is more or less comparable to that of other soluble dietary fibers that are well-known bulking agents, such as pectins and gums. The increase in stool bulking upon inulin and oligofructose consumption was found in randomized, double-blind, and placebo-controlled studies in subjects with low stool frequency patters or in constipated patients to result in a significant increase in the number of stools per week and to exert a laxative effect reducing functional

constipation (Den Hond et al., 2000; Gibson et al., 1995a; Kleessen et al., 1997). In a recent randomized, double-blind, and placebo-controlled multicenter study in patients with minor functional disorders, its was found that oligofructose supplementation significantly decreased the intensity of digestive disorders (Paineau et al., 2008). In the CROWNALIFE ("Crown of Life" Project on Functional Foods, Gut Microflora and Healthy Ageing; QLK1-2000-00067) project, it was demonstrated that the administration of a synbiotic supplement containing a probiotic *Bifidobacterium animalis* and an oligofructose-enriched inulin preparation to elderly subjects significantly increased stool frequency and reported to improve the well-being and the quality of life (Zunft et al., 2004).

6.6.3.2 Prebiotic Effect

The term "prebiotic" was defined by Gibson and Roberfroid (1995b) as "a nondigestible food ingredient that selectively stimulates growth and/or activity of one or a limited number of bacteria in the colon, thereby improving host health." As research progressed, three criteria were accepted that a food ingredient should fulfill before it could be classified as prebiotic: first, it should be nondigestible and resistant to gastric acidity, hydrolysis by intestinal (brush border/pancreatic) digestive enzymes, and gastrointestinal absorption; second, it should be fermentable; and third, it should in a selective way stimulate growth and/or metabolic activity of intestinal bacteria that are associated with health and well-being. Inulin-type fructans do fulfill all the above criteria and are generally accepted prebiotics (Gibson et al., 2004). Indeed, the prebiotic properties of inulin-type fructans are well documented among various age groups and in people living in different regions around the world.

The intestinal microbiota can be considered as a metabolically adaptable and rapidly renewable organ of the body. The induction of a prebiotic effect with inulin-type fructans can, therefore, be rather rapidly achieved within a few days of administration. A prospective, randomized, and double-blind two-center study on the prebiotic effect of an oligofructose-enriched inulin-supplemented infant formula found a significant increase in the levels of bifidobacteria already after two weeks of supplementation and remained over the full 4 weeks of the intervention, whereas numbers of lactobacilli, bacteroides, and clostridia remained stable. Interestingly, the inclusion of a nonrandomized breast-fed group showed that bifidobacteria levels in the colon of neonates supplemented with the inulin milk reached those levels found in breast-fed infants. It has been previously

demonstrated that feeding breast milk creates an environment favoring the development of a simple flora dominated by bifidobacteria to which various health benefits have been ascribed. Formula-fed infants, on the other hand, have a more complex pattern in which bacteroides, clostridia, and streptococci are prevalent with lower levels of bifidobacteria. These observations have led to the current practice of including prebiotics into infant formula milks to more closely resemble the microbiota and intestinal functioning of the breast-fed infant. And, even more, as our general knowledge about the multiple health benefits of bifidobacteria progressed and our belief of the importance of a well-balanced flora for overall health and well-being became confirmed in well-conduced research, high levels of bifidobacteria in the colon are considered favorably at all ages during life.

The administration of oligofructose to infants at older age (after their weaning period) has also been shown to increase the numbers of bifidobacteria (up to 9.5 log of colony-forming units per gram of feces). The increase in bifidobacteria was higher in infants with lower initial colonization levels. This has been demonstrated also in previous studies. Interestingly, the numbers of staphylococci and clostridia in the feces and the number of infants colonized with staphylococci appread to be lower in the oligofructose group. However, any observed difference disappeared after stopping the intervention, which indicates that the supplementation needs to be continued as part of the diet (Waligora-Dupriet et al., 2007).

During childhood, the bacterial populations residing in the microbiota increase in numbers and complexity, a process that has been initiated during weaning as solid foods are introduced in the infant's diet. At that time, numbers of enterobacteria and enterococci have increased sharply with higher levels of colonization by bacteroides, clostridia, and streptococci, resembling more and more the adult-like flora. In adulthood, however, day-to-day variations in microbial populations are less pronounced and the microbiota becomes more stable and constant over time. In healthy adult volunteers, the prebiotic properties of inulin and oligofructose have been demonstrated in numerous well-controlled, randomized human intervention studies using varying doses (ranging from 5 to 40 g/day) of short- and long-chain inulins over various periods of supplementation and by measuring microbial changes in the luminal as well as the mucosa-associated microbiota (Gibson et al., 1995a; Langlands et al., 2004; Rao, 2001; Tuohy, 2001).

In elderly people, the administration of a synbiotic, containing *Bifidobacterium bifidum* and *Bifidobacterium lactis* together with oligofructose-enriched

inulin, in a randomized, double-blind, controlled manner induced significant higher numbers of bifidobacteria (Bartosh et al., 2005). Similar results were also obtained in other studies and accompanied by beneficial effects on intestinal functioning and well-being (Kleessen et al., 1997; Zunft et al., 2004). These changes in microbiota composition toward higher levels of bifidobacteria are of particular interest, as with aging structural changes occur in the intestinal tract and the numbers of putative pathogenic bacteria (e.g., clostridia and enterobacteria) have been found to increase.

Interestingly, the magnitude of the prebiotic effect of inulin-type fructans (increase in log colony-forming units of bifidobacteria per gram colonic content) is dependent on the initial bifidobacteria level of an individual rather than the intake dose.

In vitro studies with three-stage fermentor vessels that mimick the different parts of the colon (ascendents, transversum, and descencents) have demonstrated that depending on the chain length of the inulin-type fructan a prebiotic effect can be induced at different sites along the colon. Oligofructose is more rapidly fermented, and therefore has an impact on the composition of the intestinal flora in the proximal part (vessel 1). The longer inulin chains, being fermented more slowly, have their effect in the mid colon and more distal parts (vessels 2 and 3). In humans, combining short (oligofructose) and long inulin chains (long-chain inulin) has been demonstrated to increase levels of bifidobacteria in the mucosa-associated flora of biopsies taken from proximal and distal colon mucosa (Langlands et al., 2004). Given the so widely studied prebiotic effects of inulin-type fructans, these compounds are nowadays accepted as the gold standard of a prebiotic food ingredient.

6.6.3.3 Barrier Function

In some conditions such as old age, the use of antibiotics, or in case of (critical) illnesses (acute or chronic such as inflammatory bowel diseases and cancer), the intestinal barrier functions less and gastrointestinal dysfunction can occur. As a result, increased bacterial translocation may happen, leading to systemic illness.

The interdigestive intestinal motility (e.g., migrating myoelectric/motor complex) is one physiological mechanism that prevents bacterial overgrowth and translocation in the gut. As there appears to be a relationship between the intestinal motility and the composition of the intestinal microbiota, the effect

of synbiotics on the motility response was tested in rats by implanting electrodes and recording duodenojejunal electromyography. Administration of *Lactobacillus rhamnosus* GG, *Bifidobacterium lactis* Bb12, and oligofructose-enriched inulin to elderly rats regularized the occurrence of intestinal contractions of high amplitude, which are more effective in propelling the residual food, debris, secretions, and bacterial cells (Lesniewska et al., 2006). Other animal experiments to test the potential of modulating the microbiota to efficiently discriminate and eliminate pathogenic organisms showed decreased translocation of bacteria (total aerobic, anaerobic, and the Enterobacteriaceae) to the mesenteric lymph nodes and liver, after oral administration of probiotics (*Bifidobacterium infantis*) and/or prebiotics (oligofructose-enriched inulin) in DSS-colitis-induced rats (Osman et al., 2006). These data are indicative of an improved epithelial barrier function and in agreement with earlier studies. In mice infected (intraperitoneally) with virulent strains of systemic pathogens (*Listeria monocytogenes* and *Salmonella typhimurium*), mortality rates were much lower upon inulin feeding (Buddington et al., 2002). Other studies in rats, also infected with *Salmonella*, showed lower pathogen colonization in the intestines with oligofructose; however, the authors observed an increase in translocation rate to the spleen and liver. These observations can most likely be ascribed to the low calcium diet used in this model, which by itself damaged barrier function, as the authors demonstrated that increasing the calcium level of the diets was accompanied by a decrease in the rate of translocation (Ten Bruggencate et al., 2004).

In humans, no effect on barrier function of (high dose) oligofructose was found in healthy volunteers. Barrier function was measured by the levels of intestinal epitheliolysis and the excretion of O-linked oligosaccharides in stools. The latter refers to the production of glycoproteins which build up the mucus gel layer that covers the intestinal epithelium. The authors, however, did observe a lower level of cytotoxicity of the fecal water with oligofructose (Scholtens et al., 2006). More extensive research has been performed on the cytotoxicity of the fecal water as a biomarker for the risk of colonic cancer in the SYNCAN (Synbiotics and Cancer Prevention; QLK-1999-00346) project, which will be described in Section 10.6.5. In the SYNCAN project, the effect of oligofructose-enriched inulin (as synbiotic) on the epithelial barrier function was studied. The trans-epithelial resistance (*ex vivo*) of cell lines subjected to the fecal water from polypectomized volunteers supplemented with oligofructose-enriched inulin was measured as an indicator of barrier functioning. The fecal water is the fecal fraction in most intimate contact with the colonic epithelium and mediates its functioning. A common observed effect of tumor promoters is the reduction in

barrier function of the epithelium inducing lower protection of the mucosa to carcinogenic substances. Interestingly, the synbiotic intervention increased the barrier function of the epithelium, which was shown by the significantly increased percentage in trans-epithelial resistance of the Caco-2 cell monolayer when subjected to the fecal water of polyp patients receiving the synbiotic (Rafter et al., 2007).

6.6.3.4 Butyrogenic Effect

The link between the consumption of inulin-type fructans, their bifidogenic effect, and the increase in butyrate production in the colon was, until recently, unclear. Inulin-type fructans are prebiotics and selectively fermented by bifidobacteria (and to a minor extend also lactobacilli). This colonic fermentation process generates organic acids to which several beneficial effects linked to the ingestion of inulin-type fructans are (to some extent) attributed. Organic acids that are produced by bifidobacteria are primarily lactate and acetate, and these organisms have not been reported to produce butyrate. Nonetheless, studies (both *in vitro* and *in vivo*) have demonstrated that the colonic fermentation of inulin-type fructans increases the production of butyrate, which is the so-called butyrogenic effect.

Eubacteria (*E. hallii*-like strains) are butyrate-producing bacteria and can account for 4% of the bacterial flora in feces. Recently, some previously unknown butyrate-producing colonic bacteria have been identified. These belong to the clostridia cluster XIVa, which is one of the most abundant bacterial groups in human feces. Contributing species such as *Anaerostipes caccae* and *Roseburia intestinalis* have been shown to be efficient lactate and/or acetate converters, and bacteria related to these species have been reported to compose up to 3% of the colonic microbiota. Cross-feeding between these microorganisms and inulin degraders (e.g., bifidobacteria) might play a key role in the gut ecosystem with important consequences for human health.

Fermentation studies (*in vitro*) using simple and complex bacterial cultures or fecal slurries offer a valuable tool to study individual bacterial metabolism and interspecies interactions. Kinetic analyses of co-cultures with *Bifidobacterium* spp. and butyrate-producing colonic bacteria in the presence of oligofructose revealed distinct types of cross-feeding reactions that were strain dependent. In such studies, butyrate-producing bacteria (e.g., *A. caccae* and *R. intestinalis*)

were unable to degrade oligofructose, whereas in the presence of bifidobacteria and/or fermentation metabolites (acetate) or breakdown products, degradation did occur with corresponding butyrate production (Falony et al., 2006). Studies with stable isotopes, which enable the following of carbon flows, showed that indeed *Bifidobacterium* spp. in the presence of oligofructose produce lactate (and/or breakdown products), which in turn are converted into butyrate in the presence of butyrate-producing bacteria (e.g., *R. intestinalis* and *E. halli*) (Belenguer et al., 2006). Fecal batch cultures found that the addition of oligofructose significantly increased butyrate production. About 80% of the newly synthesized butyrate derived from oligofructose fermentation originated from the interconversion of extracellular acetate and lactate. Also, Duncan et al. (2004) found that the contribution of external acetate to butyrate formation from oligofructose fermentation ranged from 82% (fecal slurry batch culture) to 87% (continuous cultures). The increased flux of extracellular acetate to butyrate upon oligofructose fermentation in mixed (fecal) slurries is in agreement with butyryl CoA:acetyl CoA transferase being the dominant butyrate-producing pathway. It appears that this pathway is selectively activated upon oligofructose fermentation, with concomitant butyrate production. These cross-feeding mechanisms could play an important role in the colonic ecosystem and contribute to the combined bifidogenic and butyrogenic effect observed after addition of inulin-type fructans to the diet.

6.6.4 Intestinal Infection and Inflammation

6.6.4.1 Intestinal Infection

Several mechanisms have been postulated by which inulin-type fructans and their interaction with the resident bacterial flora improve resistance of the intestinal tract against (exogenous) pathogen invasion (called "resistance to pathogen colonization") (Bosscher et al., 2006a). By restricting the availability of substrates within the lumen and adhesion sites on epithelial cells, endogenous bifidobacteria and lactobacilli may inhibit pathogen survival and adherence. Additionally, upon inulin fermentation, lactic acid bacteria produce organic acids (SCFA and lactate), thereby creating an environment unfavorable of pathogen growth. Moreover, butyric acid has been shown to support barrier function of epithelia.

Exploratory *in vitro* work with fecal slurries in the early 1990s indicated that inulin and oligofructose are completely fermented by the colonic microbiota and selectively stimulate bifidobacteria and lactobacilli growth and activity at the expense of pathogenic bacteria (e.g., clostridia). Protective effects of bifidobacteria have been demonstrated in (gnotobiotic) quails against the development of necrotizing enterocolitis (NEC)-like lesions when inoculated with a pathogenic flora (containing *Clostridium butyricum* and *C. perfingens*) from premature newborns. Lesions occurred rapidly after establishment of the NEC flora (e.g., thickening of the caecal wall with gas cysts, hemorrhagic ulcerations, necrotic areas), whereas they were fewer in the presence of *Bifidobacterium infantis* and *B. longum* (Butel et al., 1998). Supplementing the quails' diet with oligofructose induced an increase in the level of bifidobacteria which prevented overgrowth of bacteria implicated in NEC (e.g., *E. coli*, *Clostridium perfringens*., *C. difficile*, and *C. ramosum*) and reduced NEC-like lesions caused by polymicrobial infection (Butel et al., 2001). Other experiments in mice showed that supplementation with inulin-type fructans reduced intestinal yeast densities after oral challenge of mice with *Candida albicans*, resulting in an enhanced survival rate (Buddington et al., 2002). A combination of oligofructose and *Lactobacillus paracasei* also was shown to suppress pathogens (*Clostridium*, enterococci, and enterobacteria) in weaning pigs (Bomba et al., 2002). Furthermore, in pigs with cholera toxin-induced secretory diarrhea, oligofructose suppressed the presence of pathogens and increased the number of lactobacilli (Oli et al., 1998).

Clinical studies in humans have also shown that inulin-type fructans can protect against pathogen colonization and infection. Critically ill patients have a gut microbial ecology that is in dysbalance and is characterized by high numbers of potential pathogens. Such patients, at risk for developing sepsis (at intensive care units) when receiving oligofructose (as a synbiotic), had fewer pathogens in their nasogastric aspirates. Treatment with antibiotics, on the other hand, also changes the gut microbiota and disrupts normal ecological balance, which often leads to antibiotic-associated diarrhea. In the study of Orrhage et al. (2000), antibiotic treatment of patients induced a marked decrease in the anaerobic microbiota, mainly with a loss of bifidobacteria and an overgrowth in enterococci. Oligofructose administration (as synbiotic) in those patients restored their numbers of lactobacilli and bifidobacteria. Also, in patients with *C. difficile*-associated diarrhea, which frequently occurs after antibiotic therapy, oligofructose suppressed colonization with *C. difficile* and increased bifidobacteria levels. These changes were accompanied by a lower rate of relapse of diarrhea and reduced the length of hospital stay (Lewis et al., 2005).

6.6.4.2 Intestinal Inflammation

Chronic inflammatory bowel diseases such as ulcerative colitis, Crohn's disease, and pouchitis are thought to have their etiology to some extent linked to the composition of the colonic microbial community and its activities. Although members of the gut microbiota normally do not induce disease, in genetically susceptible hosts chronic intestinal inflammation can develop in response to commensal bacteria. It appears that bacteria are crucial in the pathogenesis of colitis, but not all bacteria are equal in their capacity to induce chronic intestinal inflammation. An altered immune response toward normal commensal organisms is thought to drive the inflammatory process towards a state of chronic inflammation.

The effect of inulin-type fructans in modulating the disease process has been repeatedly demonstrated in experimental models in which inflammation was induced by chemical agents such as dextran sodium sulphate (DSS) (Videla et al., 2001) or 2,4,6-trinitrobenzenesulfonic acid (TNBS) (Cherbut et al., 2003). In each of these, administration of inulin-type fructans (alone or as synbiotic) to the diets of animals reduced the inflammatory process (e.g., MPO, IF-γ, PGE2) and improved clinical and histological markers with a reduction in corresponding lesions. The HLA-B27 transgenic (TG) rat is a well-characterized model of chronic intestinal inflammation. The model spontaneously develops colitis. Oral administration of oligofructose-enriched inulin to HLA-B27 TG rats decreased gross cecal and inflammatory histological scores in the caecum and colon and altered mucosal cytokine profiles (decreased IL-1β and increased TGF-β levels). Cytokine responses of mesenteric lymph node (MLN) cells were also studied *in vitro* by their response to cecal bacterial lysates (CBL). Stimulation of MLN cells by CBL from oligofructose-enriched inulin-treated TG rats induced a lower IF-γ response (Hoentjen et al., 2005).

Ulcerative colitis (UC) is a relapsing inflammatory disease of the colon whose etiology has not been clarified yet. In patients suffering from ulcerative colitis, it has been found that bifidobacteria populations are about 30-fold lower compared to those in healthy individuals. This led to the hypothesis that restoring bifidobacteria populations in these patients by the use of pre- or synbiotics may influence the disease process. Supplementation of the diet of patients with UC with oligofructose-enriched inulin together with a probiotic (*Bifidobacterium longum*) for 1 month resulted in a 42-fold increase in bifidobacteria numbers in mucosal biopsies. A clinical intervention study in ulcerative patients was performed and patients were supplemened with the same synbiotic as indicated

above. The level of inflammation was assessed both by traditional methods (endoscopy and biopsy examination) and new sensitive methods (changes in gene expression of antimicrobial peptides called human beta defensins, hBD) and cytokine profiles. hBD are uniquely expressed by epithelial cells. hBD2 and 3 are upregulated in UC and their production is positively correlated with the severity of the active disease, making them excellent targets for assessing inflammatory responses in UC epithelia after therapy. Administration of the synbiotic improved the full clinical appearance of chronic inflammation, as evidenced by a reduction in sigmoidoscopy scores, reduction in acute inflammatory activity (cytokines that drive the inflammatory process, e.g., TNF-α and IL1-α), and regeneration of the epithelial tissue. Expression levels of inducible hBD2 and hBD3 in synbiotic patients were lower in the post-treatment period (Furrie et al., 2005). In another placebo-controlled clinical trial in patients with UC, the effects of oligofructose-enriched inulin were measured using noninvasive inflammatory markers. Fecal calprotectin is a quantitative marker of intestinal inflammation (presence of leukocytes) and has been shown to successfully predict relapse of the disease. Patients receiving oligofructose-enriched inulin had lower levels of calprotectin in their feces, thereby improving the patients' response to therapy by mitigating intestinal inflammation (Casellas et al., 2007). A reduction of the inflammation and associated factors was observed also in patients with an ileal pouch-anal anastomosis after therapy with inulin-type fructans (Welters et al., 2002). Moreover, in patients with active ileo-colonic Crohn's disease, dietary intervention with a combination of inulin and oligofructose has been shown to lead towards an improvement of the disease activity (reduction in Harvey Bradshaw Index) as well as enhanced lamina propria denritic cell IL-10 production and TLR2 and TLR4 expression. Strikingly different changes in mucosa microbiota following inulin supplementation were observed between patients who entered remission and those that did not. Patients who entered remission had an increase in mucosal levels of bifidobacteria (Lindsay et al., 2006).

6.6.5 Colonic Cancer

Diet has a strong influence on the etiology of colorectal cancers, and appropriate changes in dietary habits are therefore expected to have a major impact on its prevalence. Evidence pinpoints the role of the colonic microbiota in this process. Intestinal bacterial metabolism can generate substances with genotoxic, carcinogenic, and tumor-promoting potential, and human feces have been shown to be

genotoxic and cytotoxic to the colonic cells. Some bacteria carry specific enzymes that generate carcinogenic substances from food compounds (e.g., clostridia and bacteroides). Studies *in vitro*, animal chemoprevention models, and humans at risk have shown the importance of a balanced gut microbiota in terms of reducing colorectal cancer risk.

Studies in rat models of colonic cancer have demonstrated that inulin and oligofructose reduce the number of aberrant crypt foci (ACF). The increased metabolic activity of bifidobacteria and lactobacilli due to the selective fermentation of inulin and oligofructose is thought to be the basis of their anti-carcinogenic properties. ACF are pre-neoplastic lesions found in the etiology of most colon cancers. ACF show different phenotypic characteristics over normal crypts, e.g., presence of one or more large crypts that appear as a single focus, have thickened epithelia, and appear elevated compared with normal crypts when viewed under a microscope. ACF contain numerous crypts that multiply with time after treatment with a carcinogen. These ACF can develop into polyps and possibly into colon cancer. Injection with azoxymethane (AOM) can induce ACF in rats as early as 2 weeks after the carcinogen injection and mimics the adenoma–carcinoma sequence in humans. Formation of foci of aberrant crypts in the rat colon can be used as a biomarker to evaluate short-term effects of inulin-type fructans and investigate their efficacy as chemopreventive agents. Administration of weanling rats with different types of inulin-type fructans induced a reduction in the number of ACF in the proximal, distal, and total colon. Such reductions in the distal parts of the colon (and the whole colon) were most pronounced when rats were fed oligofructose-enriched inulin, which resulted in the lowest numbers of colonic ACF (Verghese et al., 2005). Long-term studies with probiotics, prebiotics, and synbiotics in rats with AOM-induced colon cancer showed a reduction in the number of colon carcinomas when supplemented with oligofructose-enriched inulin either alone or given as a synbiotic (with *Lactobacillus rhamnosus* GG and *Bifidobacterium lactis* Bb12) (Roller et al., 2004). Treatment with the carcinogen AOM suppressed the rats' natural killer (NK-) cytotoxicity in the Peyer's patches (PP). NK cells are involved in both the recognition and subsequent elimination of tumor cells. Suppression of this NK-cell activity may subsequently contribute to tumor growth. Interestingly, the changes in tumor formation upon the intervention coincided with a stimulation of immune functions within the gut-associated lymphoid tissue (GALT) and that of PP which are the primary lymphoid tissues responsive to oral intake of prebiotics or synbiotics. The supplementation with oligofructose-enriched inulin (alone or as a synbiotic) prevented such carcinogen-induced NK-cell suppression in PP. After 33 weeks of treatment,

immunological investigation of the rat's PP revealed significantly higher NK cell-like activity after the intake of the pre- or synbiotic. Other immunological markers in PP cells that differed upon both interventions were the stimulation in IL-10 production. This increase in IL-10 cytokine production in PP was also found in a previous study by the same authors after short-term exposure of AOM rats to prebiotics, probiotics, and synbiotics (Roller et al., 2004).

The challenges we still face are in the understanding of the mechanism through which prebiotics affect pathways of tumor initiation. For this, the cytotoxicity of the fecal water was measured after fermentation of inulin-type fructans. Rats were supplemented with prebiotics, probiotics, and synbiotics for a given time and aqueous extracts of feces were taken and tested for genotoxicity in colonic cell lines (HT29). Human colon cells were used as target because they are considered to be surrogates of the human colonic epithelium *in vivo*. DNA damage in the human cells was determined by the "comet assay." Damaged DNA is visualized as a "comet" as consequence of the migration of the damaged DNA within an electrical field. Overall, the prebiotic diet yielded fecal water that was less genotoxic, indicating that inulin-based diets reduce exposure to genotoxins in the gut, and therefore could lead to the prevention of tumorogenesis (Klinder et al., 2004). More *in vitro* evidence on the potential of inulin fermentation products in preventing the early stages of cancer onset was in the observations on gene expression profiles in nontransformed human colonocytes. Chirurgically obtained human colonocytes were incubated with oligofructose-enriched inulin fermentation supernatants. This resulted in a 2–3-fold increase of SCFA and induced tropic effects. The supernatants modulated the expression of several glutathione S-transferases (GST) isoforms: GSTM2 (2 fold) and GSTM5 (2.2 fold). GST are phase II enzymes of biotransformation that detoxify many carcinogens and, therefore, could protect the cells from carcinogenicity (Sauer et al., 2007).

Other substances in the diet have also been shown to extert antioxidative and anticarcinogenic functions. Biologically active compounds such as flavonoids, phenolic acids, and phyto-estrogens occur in plants mainly as glycosylated compounds. However, before these compounds can be absorbed by the human body, they need to be deconjugated by the intestinal microbes. The glycosidase activities of bifidobacteria have been demonstrated in some cases to be able to liberate the aglycones from their glycosidic linkages and allow these compounds to be absorbed in the body. Inulin-type fructans therefore could, through their stimulation of bifidobacteria, indirectly improve the absorption and bioavailability of such plant bioactive compounds and/or potentiate or intensify the activity of bioactive products. One example is soy-derived isoflavones (a subclass of the more ubiquitous

flavonoids) that contain genistein and daidzein. Inulin-type fructans have been shown to increase plasma and urinary concentrations of soy-derived genistein and daidzein and their aglycone forms. Another colonic metabolite, which is derived from daidzein, is equol, which has been shown to have enhanced antioxidant activity compared with daidzein. In rats it has been shown that feeding oligofructose-enriched inulin in combination with soy potentiated the chemopreventive effect against AOM-induced aberrant crypt foci development. Concomitently, the combination diet enhanced the activity of antioxidative and detoxifying enzymes, indicating a potential mechanism of the observed reduction in AOM-induced ACF.

In 2001, the SYNCAN project started. SYNCAN was a collaborative European Network involving eight different partners from seven countries. The core feature of the project involved a phase-II anticancer study, randomized, double-blinded, and placebo-controlled, in 80 patients with a history of colon cancer or polyps and supplemented with oligofructose-enriched inulin and *Bifidobacterium lactis* Bb12 and *Lactobacillus rhamnosus* GG for 12 weeks. The synbiotic preparation increased the level of bifidobacteria and lactobacilli. This was accompanied by a decrease in the numbers of pathogens (coliforms and *Clostridium perfringens*). The altered composition of the colonic bacterial ecosystem beneficially affected the metabolic activity in this organ. This was obvious from the decreased DNA damage in the colonic mucosa (measured by the comet assay) and the tendency to lower the level of colorectal proliferation (surrogate biomarker for colon cancer risk) in polyp patients (no measures were taken in cancer patients). Other effects were the decreased cytotoxicity of the fecal water. The fecal water of synbiotic-fed polyp patients also showed a lower level of cell necrosis as demonstrated by the lower cytotoxic potential in HCT116 cell types. This indicates that the synbiotic effectively prevented cell death of the colonic epithelium (Rafter et al., 2007).

6.6.6 Modulation of Immune Function

In the above sections, the potential of inulin-type fructans to modulate immune parameters were described. These studies involved different disease conditions, e.g., chronic inflammation and cancer, and suitable animal models thereof. To study the effect of foods and food ingredients on immunity in healthy conditions, however, vaccination strategies (challenge models) can be applied, or studies in populations at risk (e.g., environments with high burden of infections, malnourished children) can be performed and clinical outcomes (e.g., incidence of illness) investigated.

Inulin and oligofructose have been demonstrated in mice to improve immune responses to oral vaccination with a suboptimal dose of live attenuated *Salmonella* Typhimurium vaccine, thereby contributing to an enhanced oral vaccine efficacy. Specific anti-salmonella antibody responses increased post immunization and were much higher in the inulin and oligofructose mixture as indicated by the high specific blood salmonella immunoglobulin G and fecal immunoglobulin A responses. These changes were concomitant with an increase in the survival rate upon later challenge with a virulent salmonella strain. The vaccination of mice led to a 40% protection, and the rate of protection improved to 73% on the inulin mix feeding regime (Benyacoub et al., 2008).

Infants vaccinated with measles vaccine and fed on weaning foods enriched with a synbiotic, containing inulin and oligofructose, have been shown to improve their vaccination-induced immune response. Post vaccination, specific IgG-antibody levels were higher with the synbiotic, indicating an enhanced immune response to vaccination (Firmansyah et al., 2001). Further investigations in elderly persons confirmed that adequate nutritional supplements can modulate immune responses. Aging is associated with alterations in the immune responsiveness that increase susceptibility to infections, reduce the response to immunization, and increase the incidence of autoimmune diseases. The preservation of an adequate NK cell function is considered part of successful aging. Healthy elderly volunteers received a nutritional supplement containing proteins, vitamin E, vitamin B12, folic acid, *Lactobacillus paracasei*, as well as a mixture of oligofructose and inulin for 4 months prior to vaccination against influenza and pneumococcus. After 4 months, the nutritional supplement increased the NK cell activity. NK cell activity is one of the first-line defense mechanisms against viral infections. Cells with NK activity are a special subset of T cells that respond to glycolipid antigens, are capable of promoting T helper 1 response, and have a role in regulating autoimmunity and rejecting tumor cells. At 4 months, all persons were vaccinated. After vaccination, the production of IL-2 by peripheral blood mononuclear cells (PBMC) remained in the supplemented individuals and did not lower as seen in those not receiving the supplement. One of the functions of IL-2 is to stimulate NK cell activity and to elicit a T-helper 1 immune response. Most persons had a positive antibody response to the vaccine, but no additional effect of the supplement was observed. Interestingly, during the 1-year follow-up, the volunteers that took the supplement reported fewer infections (Bunout et al., 2004).

Infants too are very vulnerable to infections and gastrointestinal tract disturbances. The likelihood of these occurring is ever increasing when attending

daycare centers, where infectious diseases are frequent and easily disseminate from one child to another. Saavedra and Tschernia (2002) examined the long-term (6 months) effect of daily intake of oligofructose (supplemented to cereal) in infants (4–24 months of age) attending daycare centers. The consumption of the prebiotic cereal was associated with a decrease in the severity of diarrhea disease. General gut status was improved with decreased bowel movement discomfort, vomiting, and regurgitation. Furthermore, consumption of the prebiotic cereal resulted in adequate growth and led to a reduction in the number of febrile events and cold symptoms, antibiotic prescription (associated with respiratory illness), and daycare absenteeism. Such observations were later confirmed in a smaller scale study in infants with nearly similar age. Oligofructose intake improved the intestinal bacterial colonization, as characterized by higher levels of bifidobacteria, especially in those infants with lower baseline levels, and a decrease in clostridia. Daily oligofructose administration was well tolerated. Additionally, number of febrile events and gastrointestinal illness symptoms, such as flatulence, diarrhea, and vomiting, were less often observed (Waligora-Dupriet et al., 2007). Fisberg et al. (2000) evaluated the incidence and duration of sickness in 626 mild to moderately malnourished children (1–6 years of age) who received a nutritional supplement with a synbiotic (oligofructose together with *Lactobacillus acidophilus* and *Bifidobacterium infantis*). In a subgroup of children (aged 3–5 years), the number of sick days were fewer with symbiotic administration as were days of constipation.

6.6.7 Absorption and Accretion of Minerals

6.6.7.1 Influence on Calcium Metabolism

Calcium is an integral part of the skeleton, and increasing the amount of calcium absorbed from the diet is an important strategy to improve bone metabolism at all ages. Over the past 10 years, much research has been performed on the effects of inulin and oligofructose on calcium absorption as well as bone metabolism and its mineralization. This extensive amount of research done in animals and humans has repeatedly shown that inulin-type fructans increase calcium absorption from the diet, modulate makers of bone metabolism, and improve calcium sequestration in the bone (Bosscher et al., 2006b).

Two key animal models have been used to determine the impact of prebiotics on calcium absorption and bone mineral density: (1) young growing animals that

represent the adolescent phase in humans, and (2) adult ovariectomized rats that represent the postmenopausal phase in humans. The "apparent calcium absorption" is then measured by subtracting the fecal calcium content from the actual calcium intakes of the animals. First experiments were performed in growing rats fed on inulin-type fructans in the diet, which found increased apparent calcium absorption (Delzenne et al., 1995). Later on, similar results were seen with oligofructose-enriched inulin in ovariectomized rats. Moreover, kinetic data on bone metabolism showed improved bone balance and suppression of bone turnover (Zafar et al., 2004). It is clear that inulin-type fructans improve calcium absorption; but does this translate to advances in bone mineralization and strength? This was evaluated in the two animal models described above. Roberfroid et al. (2002) showed that feeding growing rats with inulin resulted in an increase in whole-body bone mineral content (BMC) and whole-body bone mineral density (BMD). In this rat model, long-term inulin administration appeared to impact mainly on the trabecular bone network. In adult ovariectomized rats, oligofructose increased BMC in the femur and lumbar vertebra and prevented the ovariectomy-induced loss of bone in the trabecular structure (Scholz-Ahrens et al., 2002).

Following this, a number of intervention studies in adolescents were carried out. The best method for determining calcium absorption is believed to be the use of stable isotopes, as this excludes the confounding variable of endogenous calcium excretion. A measure of "true calcium absorption" is then provided, which cannot be obtained by the balance method. If isotopes are administered as part of a metabolic study, one can also see the individual components of calcium metabolism: i.e., absorption, excretion, endogenous secretion, bone formation rates, and bone resorption rates. Only studies in which stable isotopes and supplementation with oligofructose-enriched inulin were used will be described here. In adolescent girls (mean age 11.8 years), supplementation with oligofructose-enriched inulin increased true calcium absorption, whereas urinary excretion of calcium remained unaffected. As the response to treatment differed between subjects, further study was initiated to evaluate the subject's characteristics (Griffin et al., 2003). The new study had a protocol similar to that carried out before in order to merge the two sample sizes. An extra 25 adolescent girls were recruited, bringing the overall sample to 54 (mean age 12.4 years) across two centers in Texas and Nebraska. As with the previous study, there was a significant increase in calcium absorption with the oligofructose-enriched inulin. A range of characteristics was studied: e.g., age, weight, height, Tanner stage, and ethnicity. However, the most consistent determinant of a beneficial effect of the

oligofructose-enriched inulin on calcium absorption was the fractional calcium absorption of the girls at baseline. Thus, girls with lower baseline levels of calcium absorption responded with the greatest increase when supplemented with oligofructose-enriched inulin. This finding is important, as these adolescents are most likely to benefit from the higher amount of calcium absorbed from the diet. Since the all subjects received a similar calcium load from the test meals, the variation cannot be explained by the presence of enhancers or inhibitors of calcium absorption in the diet. Rather, it is likely that the subjects' genotypes governed both their baseline calcium absorption and their response to the oligofructose-enriched inulin consumption. Interestingly, the subjects' polymorphisms of the Fok1 vitamin D receptor gene were determined, and an interaction with Fok1 genotype was present such that ff genotype subjects had the least initial response to the inulin supplementation. This shows that effects of dietary factors on calcium absorption may be modulated by genetic factors including ethnicity and specific vitamin D receptor gene polymorphisms (Abrams et al., 2005).

In a subsequent long-term (1 year) intervention study in 100 pubertal girls and boys in early puberty (9–12 years of age), the impact of oligofructose-enriched inulin on bone accretion was studied. The participating subjects were selected for Tanner stage 2 or 3. This increased the likelihood that calcium accretion was at its highest during the study when it would have the capacity to determine bone health at a later age. Against a background of normal calcium intakes (900–1000 mg/d), subjects received 8 g/day of oligofructose-enriched inulin added to milk or a calcium-fortified orange juice. As *Figure 6.3* shows, 2 months into the intervention, subjects receiving oligofructose-enriched inulin had higher true calcium absorption. The enhanced calcium absorption was maintained throughout the intervention so that at 1 year, the differences were maintained. Correspondingly, subjects receiving oligofructose-enriched inulin had greater change in whole-body BMC at 1 year (see *Figure 6.4*). Assuming that the fraction of calcium in BMC is 32%, these values correspond to a daily skeletal calcium accretion of 218 mg/d for the oligofructose-enriched inulin group (and 189 mg/d for the controls). This can be estimated as an additional net accretion of 30 mg/d. The change in whole body BMD was also greater in subjects receiving oligofructose-enriched inulin. This corresponded to an increment in whole-body BMD after 1 year of 15 $\mu g/cm^2$/year (see *Figure 6.5*). The interpretation of this study is that oligofructose-enriched inulin increased calcium absorption during pubertal growth and enhanced bone mineralization, leading to a greater bone mass accretion during adolescence (Abrams et al., 2005).

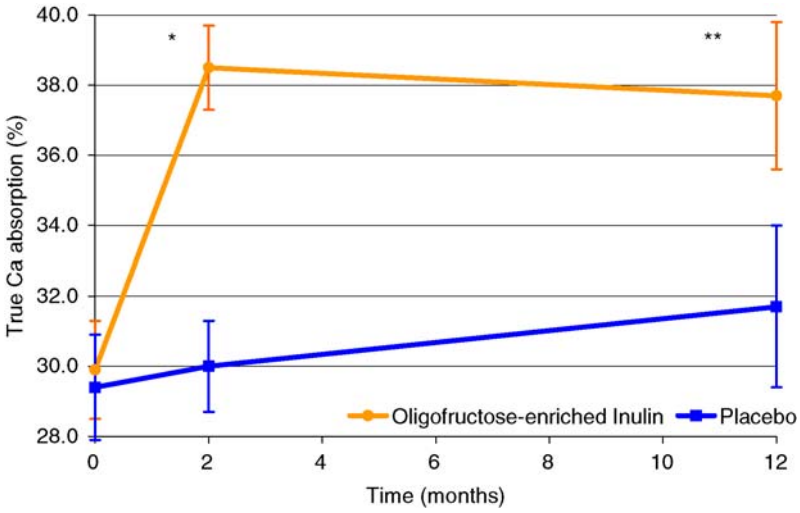

Figure 6.3
True calcium absorption (%) of the subjects (mean ± SD) after supplementation of the diets with oligofructose-enriched inulin or placebo (maltodextrin) for 8 weeks and 1 year (asterisks represent significant difference compared to the placebo * $P < 0.001$, ** $P = 0.04$).

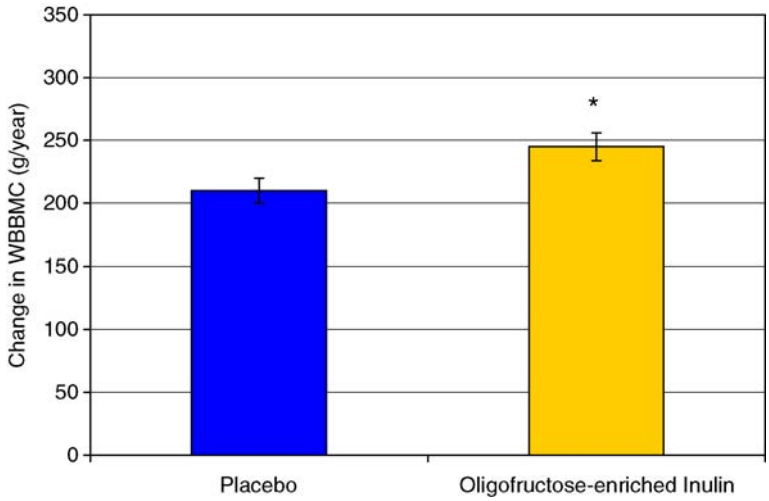

Figure 6.4
Change in whole-body bone mineral content (WBBMC) (g/year) of the subjects (mean ± SD) after supplementation of the diets with either oligofructose-enriched inulin or placebo (maltodextrin) for 1 year (asterisks represent significant difference compared to the placebo $P = 0.03$).

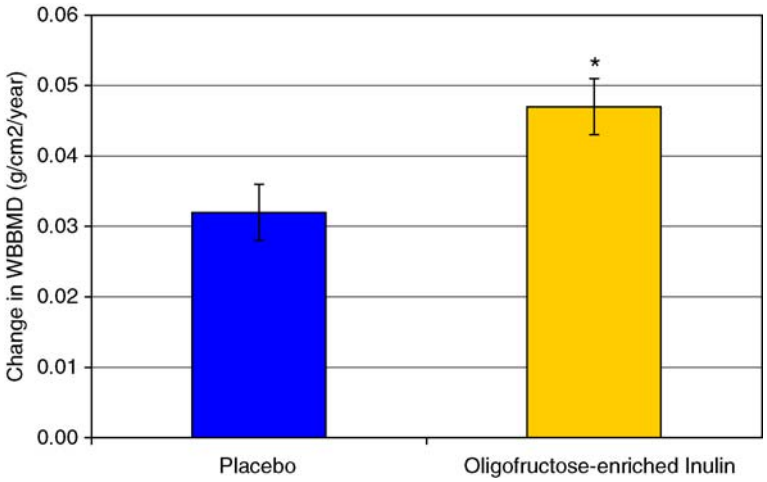

Figure 6.5
Change in whole-body bone mineral density (WBBMD) (g/cm^2/year) of the subjects (mean ± SD) after supplementation of the diets with either oligofrucrose-enriched inulin or placebo (maltodextrin) for 1 year (asterisks represent significant difference compared to the placebo $P = 0.01$).

Other studies on the impact of oligofructose-enriched inulin were performed in elderly women. In women, the menopause is a time when estrogen deficiency leads to accelerated bone resorption and negative bone balance. A subsequent intervention trial with oligofructose-enriched inulin in postmenopausal women (mean 72 years of age) was carried out studying markers of bone turnover. The supplementation improved calcium and magnesium absorption, even when vitamin D status was adequate and calcium intake was good. Markers of bone turnover showed a short-term decrease (urinary deoxypyridinoline crosslinks) in bone resorption and a clear increase in bone formation (osteocalcin), and this was most pronounced in those women with lower initial spine BMD (Holloway et al., 2007).

Another interesting feature of inulin-type fructans that may affect bone spearing in elderly women is their effects on the intestinal metabolism of isoflavones. Isoflavones are found in soybeans and behave as estrogen mimics and, therefore, are implicated for use in the prevention of gonadal-induced osteopenia at the time of menopause. Isoflavones mainly occur in plants as glycosides, which have to be hydrolyzed before they can be absorbed. Glucosidases of intestinal bacteria, such as lactobacilli and bifidobacteria, are estimated to be involved in this process. In ovariectomized rats, fructo-oligosaccharide supplementation to a

soy-based diet was shown to increase plasma levels of genistein, daidzein, and equol and improved the protective effect of isoflavones against bone loss secondary to castration (Mathey et al., 2004). In postmenopausal women, inulin has indeed been demonstrated to enhance blood levels of isoflavones (daidzein and genistein) after soy intake. However, whether this results in improved bone protection (less bone resorption) in this age group is unclear and needs further study.

The underlying mechanisms of the effect of inulin-type fructans on calcium absorption seem to be diverse and affect the different stages of calcium absorption. Calcium moves across the intestinal epithelium in two different ways. The trans-cellular route comprises apical calcium entry via calcium channels, calcium binding and sequestration through the cytosol, mostly by calbindin, and basolateral calcium extrusion mostly by an ATPase. In the para-cellular route, calcium moves through tight junctions where claudins are thought to form channel-like structures. The major fraction of calcium is absorbed in the small intestine; however, part of the dietary calcium is also absorbed in the large intestine. The fermentation of inulin-type fructans and the corresponding acid formation are thought to play a role essentially on the para-cellular route in the colon. This has been shown to lead to a reduced luminal pH with a marked increase in the luminal pools for total, soluble, and ionized calcium.

By the use of Ussing chambers (*in vitro*) of the rats' large intestine, the effect of oligofructose-enriched inulin and its fermentation products was studied on trans-epithelial calcium transport. The presence of SCFA increased trans-epithelial calcium transport independently of the luminal pH. It might be hypothesized that increased protonation of SCFA anions in a more or less acidic environment allows their permeation into the epithelium by non-ionic diffusion. Once in the cell, dissociation might occur, and due to the higher pH release their protons that can then be exchanged with luminal calcium. Alternatively, SCFA anions could also be absorbed in exchange with HCO_3^-, produced by carbonic anhydrase (CA) from H_2CO_3, and with apical secretion of the proton that can be exchanged for calcium. Alternatively, it was hypothesized that inulin-type fructans and/or their fermentation products could also directly interact with the tight junctions. All will ultimately result in an increase in net calcium uptake. The observed changes in the mRNA level of genes linked to trans- and para-cellular calcium transport support the above hypotheses. The upregulation of the CA transcript levels in the proximal colon supports the coupling theory of SCFA movement and calcium flux. CA could provide more intracellular HCO_3^- anions for exchange with SCFA anions, while the remaining H^+ could be extruded by $Ca^{2+}/2H^+$ and Na/H^+ exchangers. Interestingly, and indicative of an

involvement of the para-cellular routs as well was the high expression level of claudin, is a tight junction protein that acts as a calcium channel (Raschka and Daniel, 2005). An upregulation of the calbindin-D9k protein and its associated transcription factors VDR and cdx-2 in the rats' colorectal segment upon oligofructose feeding has been demonstrated by others (Fukushima et al., 2005).

Apart from such molecular alterations, dietary administration of inulin-type fructans has also been found in animal models to lead to a net enlargement in total tissue surface (but not thickness) of the intestinal wall. The possible cause for such tissue enlargement might be the trophic actions of the butyrate formed during the fermentation process.

6.6.7.2 Influence on Iron Metabolism

While the effects of inulin-type fructans on calcium absorption and metabolism are well documented, recent studies also pinpoint to the effects on iron absorption, particularly in a state of iron deficiency. Young weaning piglets are considered a suitable model for studying human iron nutrition because of their similarities in the anatomy of the gastrointestinal tract and digestive physiology. Addition of oligofructose-enriched inulin to the diet of iron-deficient and anemic pigs has been found to dose-dependently increase soluble levels of iron in the colon as well as improve blood hemoglobin levels and hemoglobin repletion efficiency. Sulfide levels were found to be lower. High levels of sulfide occur mostly as a result of protein fermentation and can bind iron in the intestinal tract, rendering it unavailable for absorption. Attenuated levels of sulfide in the colon might be suggestive of a potential mechanism (Yasuda et al., 2006).

6.6.8 Body Weight, Appetite, Energy Intake, and Metabolism

6.6.8.1 Body Weight Modulation

Obesity is one of the greatest public-health challenges of the 21st century. The condition is caused by a persistent imbalance between (high) energy intake and (low) energy expenditure and the development of fat mass as storage of the excess in energy. Changes in lifestyles are seen as the major cause, as diets high in nutrients and complex carbohydrates gave way to energy-dense and high-fat diets.

The long-term effects of oligofructose-enriched inulin on the maintenance of body weight were observed in (primarily nonobese) 100 pubertal adolescents (9.0–13.0 years of age). Those findings were part of a bigger study which also included measurements of calcium absorption and bone mineralization. The bone-related findings are discussed in Section 10.6.7.1. Adolescents that received oligofructose-enriched inulin (8 g/day) over a period of 12 months had lower increments in body mass index (BMI), body weight, and fat mass after one year. In other words, those adolescents that received oligofructose-enriched inulin benefited in the maintenance of an appropriate BMI during their pubertal growth. They had an increase in BMI of about 0.7 kg/m^2 during the supplementation year, which is consistent with expected increases during puberty. Subjects not receiving the prebiotic (placebo) had a higher increase (of 1.2 kg/m^2). Interestingly, the effect of oligofructose-enriched inulin on BMI was in a nonlinear fashion modified by dietary intake of calcium, such that the benefit of oligofructose-enriched inulin was greater when low calcium intakes were avoided. Follow-up data were available for 89 subjects. Interestingly, BMI values for the prebiotic supplemented subjects remained lower after stopping the supplement for one year, indicating that the effect was persistent after discontinuation of the supplementation. The lower increase in BMI in the prebiotic group implies an overall regulatory effect on energy intake associated with the oligofructose-enriched inulin supplementation (Abrams et al., 2007). The overall greater increase in BMI during the year in the nonsupplemented group is likely to be nonideal and is related to the overall trend toward increased BMI during adolescence currently.

6.6.8.2 Appetite Regulation and Energy Intake

Our body is equipped with powerful and complex interacting pathways (often mediated by hormones) that can initiate and terminate food intake and reflect body adiposity and body energy balance by integrating signals to the hypothalamus and brain stems. Several of such hormones have their origins in the gut, and this is often referred to as the "brain-to-gut axis."

Studies starting in the late 1990s using various animal models have consistently shown that the fermentation of inulin-type fructans in the colon can modulate the expression of hormones involved in appetite and having their origins in the gut (e.g., proglucagon mRNA). This in turn can modulate their levels in the blood, affecting appetite and food intake. This has been demonstrated in normal

rats when fed on a diet supplemented with inulin-type fructans. The levels of GLP-1 (7–36) amide in plasma were higher, and proglucagon mRNA in the proximal colonic mucosa showed a marked upregulation in case oligofructose or oligofructose-enriched inulin was added. The expression of proglucagon takes place in the large intestine where it is cleaved into peptides (besides glucagon) such as GLP-1. GLP-1 is a biologically active peptide secreted into the portal circulation with anorexigenic properties. The opposite was true for the plasma levels of the hormone ghrelin, which remained significantly lower in the oligofructose and oligofructose-enriched inulin fed animals (Cani et al., 2004). Energy intake was consequently lower. This led to a decrease in (epidydimal) fat mass after several weeks of supplementation in the oligofructose and oligofructose-enriched supplemented animals. In a subsequent study undertaken in rats put on a diet high in fat (i.e., to mimic the Western diet), oligofructose lowered energy intake and reduced high-fat induced hyperphagia. Consequently, body weight gain on the high-fat diet was lower and the total weight of the adipose tissue was twofold lower than in the animals' not receiving oligofructose. In addition, the plasma triglyceride levels were reduced, and hepatic triglyceride levels were about 30% lower in those receiving oligofructose than those that did not receive it. The levels of proglucagon (mRNA) and GLP-1 in cecum and colonic tissues and GLP-1 in the portal vein were higher with the oligofructose diet (Cani et al., 2005a). Also in other models of obesity, such as genetically obese (fa/fa Zucker) rats, the same effects of oligofructose on body weight (wt) and steatosis were observed (Daubioul et al., 2002). This was found to be linked to a decrease in hepatic de novo fatty acid synthesis, which is thought to be due to the inhibition of the fatty acid synthase activity (Kok et al., 1998). When other dietary fibers, e.g., nonfermentable fibers, were added, those effects were not observed. No protection against the development of steatosis was observed when cellulose was added to the diet. Also, obese rats fed on cellulose had significantly higher food intake when compared to those receiving oligofructose (Daubioul et al., 2002).

A pilot study in healthy men and women (aged 21–39 years) with normal BMI values showed that oligofructose (2 times 8 g/day) given at beatkast and dinner affected satiety scores and energy intakes. Appetite ratings of hunger, satiety, and fullness after each meal were recorded by visual analog scales. Visual analog scales are standard 100-mm lines anchored at each end by a phrase denoting the most extreme appetite sensations. The visual analog scales are a well-accepted tool to assess satiety in its intensity and duration. Oligofructose-supplemented volunteers experienced higher levels of satiety, reduced hunger, and prospective food consumption (see *Figure 6.6*). Subjects were also invited to a day of

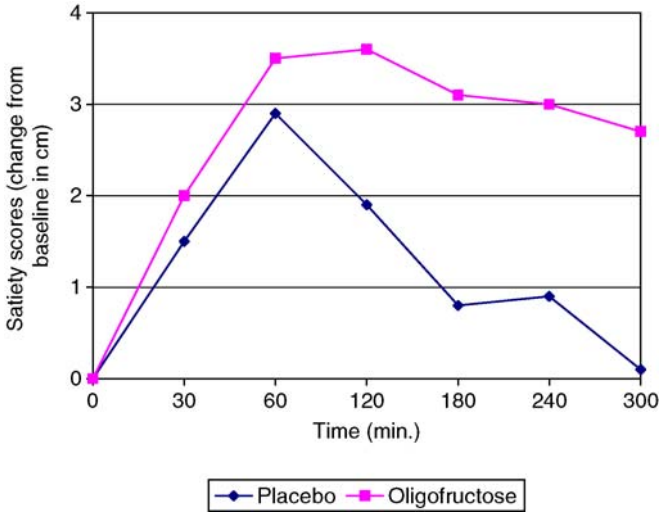

Figure 6.6
Satiety scores after dinner (measured by Visual Analogue Scale) in healthy subjects after 2 weeks of supplementation with oligofructose (8 g twice daily) or placebo. The results are presented as change from baseline scores and are means ± SEM, *n* = 10 subject for each intervention phase.

free-choice buffets during which their food and drink intakes were monitored. The energy intake at breakfast and lunch was lower with oligofructose, which resulted in a significant reduction in the total energy intake during the day (see *Figure 6.7*) (Cani et al., 2006a). Also in other human studies the effects of oligofructose, either alone or in combination with other dietary fibers (pea fiber), on satiety and food intake have been documented. Addition of oligofructose to enteral formula also containing pea fiber was shown to result into higher subjective measures Visual Analogue Scale, (VAS) of fullness and satiety feelings (Whelan et al., 2006).

The satiating power of inulin-type fructans together with their technological benefits to replace fat, while remaining the palatability of foods, could be a dual way to manufacture foods with high "satiety power" while at the same time having a low energy density. In fact, strategies focused on lowering fat content (and energy density) of individual foods have failed in some cases, as poor palatability and the lack of satiating power of such low-fat versions led to the (over)compensation for the reduced energy intake during the rest of the day. Replacing half of the fat by inulin in foods (e.g., reduced-fat patty instead

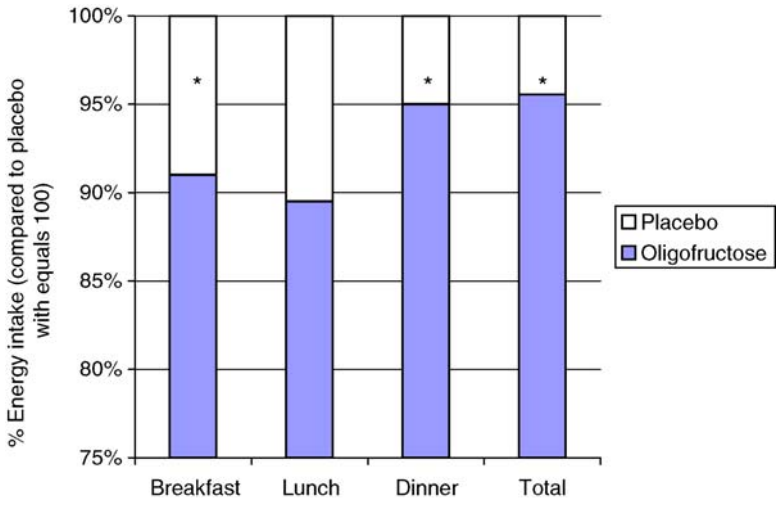

Figure 6.7

The percentage energy intake relative to placebo at breakfast, lunch, dinner, and over the day in healthy subjects after 2 weeks of oligofructose supplementation (8 g twice daily). Values are means ± SEM, $n = 10$ subjects (asterisk Indicates a significant difference from the placebo treatment $P < 0.05$ **, $P < 0.01$).

of full-fat sausage patty), while otherwise remaining similar in protein and carbohydrate content, reduced the fat content by 36% and energy content by 15% and showed good sensorial acceptance of the breakfast patties among healthy men. Although satiety scores of the men participating in the study did not differ after consumption of the low-fat inulin version compared with the full-fat version, the authors were suggestive of an effect of inulin on post-meal satiety given that the inulin breakfast provided less energy than the full-fat breakfast. Moreover, energy intakes during the whole day were lower with the inulin breakfasts, indicating that full compensation for the lower energy content did not occur (Archer et al., 2004).

Nowadays, more evidence has become available about the role of fermentable dietary fibers in enhancing satiety and the relationship with high blood levels of glucagon-like peptide 1 (7–36) amide (GLP-1) with a corresponding upregulation of proglucagon expression in the (proximal) colonic mucosa (the latter coming from animal studies). GLP-1 is a peptide produced by intestinal endocrine L cells through a specific post-translational processing of the proglucagon gene. Feeding rats with oligofructose has been shown to almost double the numbers of GLP-1 positive L cells in the proximal colon, which was associated

with the differentiation of cell precursors (stem cells) into mature L cells (Cani et al., 2007a). GLP-1 is considered a key peptide in the control of glucose tolerance, insulin secretion by pancreatic β-cells, and the transmission of satiety signals to the brain. The current available evidence indicates that the effect of oligofructose is largely dependent on GLP-1 and that the GLP-1 peptide appears to constitute the link between the fermentation of inulin-type fructans in the colon and their modulation of lipid and glucose metabolism, as well as appetite regulation and food intake.

6.6.8.3 Adipose Tissue and Inflammation

Obesity and metabolic disorders (insulin resistance, hyperlipaemia) are tightly linked to a chronic low-grade state of inflammation (elevated levels of circulating inflammatory markers such as IL-6, and C-reactive protein). The adipose tissue is an endocrine organ actively releasing a number of immune active factors (TNF-α, IFN-γ, IL1-β, IL-6, IL-8, IL-10, acute-phase proteins, etc.). Inflammatory factors are known to be involved in insulin resistance, favoring hyperinsulinemia and excessive hepatic and adipose tissue lipid storage. It appears that an altered gut microbiota in the obese state could contribute towards low-grade inflammation resulting in the development of metabolic diseases associated with the condition (e.g., diabetes, cardiovascular disease, etc.). However, the factors triggering such metabolic alterations remain to be determined.

In the obese, lower levels of Bacteroidetes and higher levels of the phylum Firmicutes in the colonic microbiota as compared to lean counterparts are found (Ley et al., 2006). These observations have been associated with increased gut fermentation and calorific bioavailability to the host. Moreover, feeding high-fat diets have been demonstrated to alter dramatically the microbiota composition in mice with reduction in the quantities of dominant Gram-positive groups, e.g., *Bifidobacterium* spp. and *E. rectale, C. coccoides* groups, and the murine Gram-negative group, *Bacteroides MB*.

Recent studies in animal models have shown that such changes within the microbial ecology or functional activities of the gut microbiota can induce a metabolic shift toward a proinflammatory phenotype, whole-body, liver and adipose tissue weight gain, and impaired glucose metabolism. Factors of microbial origins (e.g., bacterial lipopolysaccharides) are hypothesized to be basis of such effects. In mice, high-fat feeding led to (low level of) metabolic endotoxemia, low inflammatory tone, increasing macrophage infiltration in adipose tissue, and

dysregulating lipid and glucose metabolism. Multiple correlation analyses showed that the level of endotoxaemia was negatively correlated with *Bifidobacterium* spp., but no relationship was seen between any other bacterial groups. On the other hand, restoration of the levels of bifidobacteria in the intestine of mice upon oligofructose supplementation lowered endotoxaemia and the level of microbial toxins and improved mucosal barrier function. Interestingly, the lower body weight and visceral adipose tissue mass in the oligofructose group (compared with the non-supplemented high-fat fed mice) showed a positive correlation with the endotoxin plasma levels and a negative one with the levels of bifidobacteria. Moreover, levels of mRNA of IL-1, TNF-α, and plasminogen activator inhibitor type-1 (Pai-1, or Serpine-1) in adipose tissue were increased in high-fat fed mice, whereas the levels were blunted with oligofructose feeding. In addition, a normalization of IL-1α and IL-6 cytokines was observed upon oligofructose feeding. These data indicate that a lower fat mass and body weight "only" are not a prerequisite for a lower inflammatory tone and that this effect is accompanied by prebiotic changes in the microbiota. Plasma cytokines were positively correlated with plasma endotoxin levels and negatively with bifidobacteria levels (Cani et al., 2007b). In diabetic mice, feeding oligofructose reduced hepatic levels of phosphorylate IKK-β and NF-κB, suggestive of a reduction in the hepatic inflammatory status which might relate to an improvement of the insulin sensitivity (Cani et al., 2005b).

6.6.8.4 Glucose and Lipid Metabolism

High glucose and blood cholesterol levels and hyperlipaemia are major risk factors for development of the metabole syndrome, associated diabetes, and cardiovascular diseases, and are most often linked to wrong food choices (e.g., high-fat and low-fiber diets). In the above sections, the beneficial effects of inulin-type fructans on lipid and glucose metabolism have already been indicated briefly; however, more evidence is currently available, especially from animal models.

The effects of oligofructose on satiety and energy intake have been clearly related to the levels of GLP-1. However, as GLP-1 is also a key peptide in the control of glucose tolerance and insulin secretion, effects of oligofructose supplementation on glucose metabolism can be estimated. Indeed, further research in diabetic rats (treated iv with streptozotocin to induce postprandial hyperglycemia) showed that oligofructose supplementation reduced high blood glucose (due to the diabetic condition) and even normalized glycaemia to normal levels. Alternatively, low plasma insulin levels (due to the diabetic condition) were increased and also

normalized to normal (nondiabetic) levels. Moreover, oligofructose supplementation elevated pancreatic insulin levels and beta cell mass (as these were drastically reduced because of the diabetic condition), indicating improved beta-cell mass and function. Consistent with earlier reports, these physiological changes were linked with higher levels of GLP-1 in the portal blood of the rats. Oral glucose tolerance tests (OGTT) in these diabetic animals revealed a lower area under the curve (AUC) in rats fed with oligofructose. The plasma insulin response to glucose during the OGTT, on the other hand, was markedly increased. Interestingly, the AUC for insulin was similar for diabetic rats fed the oligofructose as with normal (nondiabetic) rats (Cani et al., 2005b). The effects of oligofructose are largely dependent on the action of GLP-1. Extensive investigations in diabetic rats indeed showed that the disruption of the GLP-1 receptor function, either by infusing Ex-9 (GLP-1 R antagonist) or by using GLP-1 receptor−/− mice, completely prevented the majority of the beneficial metabolic effects observed following oligofructose supplementation. In the presence of the antagonist, the effect of oligofructose on body weight, glucose tolerance, fasting blood glucose, glucose-stimulated insulin secretion, and insulin-sensitive hepatic glucose production disappeared. Also, GLP-1R −/− mice appeared to be totally insensitive to the systemic effects of oligofructose (Cani et al., 2006b).

Various animal models (hamsters, mice, and rats) have consistently shown the lipid and cholesterol lowering actions of inulin-type fructans. In rats fed on a high-fat diet, oligofructose suppressed the postprandial increase in triglyceride levels and hepatic triacylglycerol load (originating from the diet) (Kok et al., 1998). In genetically obese (fa/fa Zucker) rats, a similar pattern was observed, with lower body weight, fat mass, and steatosis development when inulin-type fructans were part of the diet. Detailed biochemical studies in isolated hepatocytes demonstrated that inulin-type fructans reduce the activities of key hepatic enzymes related to lipogenesis (e.g., de novo synthesis of fatty acid synthesis). Further research revealed that an altered gene expression was the cause of the downregulation, which might have been in response to hormonal changes induced by inulin-type fructans (Kok et al., 1998). More recent evidence comes from studies in ApoE-deficient mice, a genetically modified mice model that spontaneously develops atherosclerosis. Feeding mice with inulin reduced plasma levels of cholesterol, triacylglycerols, hepatic cholesterol, and triacylglycerols. Histo-morphometry of the aortic sinus showed less plaque formation in mice receiving long-chain inulin or oligofructose-enriched inulin, with a mean reduction in the lesion area of 35% and 25%, respectively, when compared to mice not receiving the supplements. It is most likely that the inhibition of atherosclerotic

plaque formation was related to the changes observed in lipid and cholesterol metabolism upon inulin intake (Rault-Nania et al., 2006).

Also, in humans inulin-type fructans have been shown to affect lipid metabolism, although the data are less consistent compared to the results from animal studies. This might be due to differences in methodological setup, type of subjects, as well as duration of the intervention, dose, and type (short or long chain) of the inulin-type fructan used in the study. It has been reported that the consumption of inulin-type fructans reduced serum triglycerides and in some cases also cholesterol (mostly LDL fraction) in healthy volunteers who are (slightly) hyperlipidemic. Lipid parameters in healthy (normolipidemic) young adults, on the contrary, appear to be unaffected. Also, studies indicate that such effects, if any, take some time to become established, urging for a longer term intervention (Brighenti et al., 1999; Davidson and Maki, 1999; Jackson et al., 1999). Although much uncertainty still exists about the mechanisms that are responsible for those effects, it is already clear that those include various interdependent biochemical pathways that may take place in the liver, pancreas, intestine, and peripheral tissues (e.g., adipose tissue). Research in this field has evolved with primary focus on the endocrine activity in the gut, which has been described in earlier sections.

6.7 Outlook and Perspectives

The number of publications on prebiotics and inulin-type fructans has increased dramatically once the concept was established in 1995. Especially, the number of publications demonstrating the nutritional effects of inulin-type fructans is overwhelming. This is expected to continue since the importance of a well-balanced colonic microbiota as being key in the modulation of human immunity, metabolism, and endocrine activities is being more and more recognized among the general scientific population. As new insights are being elucidated about the composition of the microbiota and its species diversity, the role the microbiota in the origins of disease, and the mechanisms of action, interest will continue to rise. Together with this, it is of paramount importance to develop strategies to modulate this microbiota in a way to reduce the risk of developing disease through dietary means. Dietary strategies require a combination of technological means and well-established nutritional evidence as well as legal responsibility to benefit health and well-being of the population. Functional foods with prebiotics and probiotics offer great value in this regard and, although many health benefits have been demonstrated today, more can be expected in the near future.

List of Abbreviations

ACF	aberrant crypt foci
AOM	azoxymethane
AUC	area under the curve
BMC	bone mineral content
BMD	bone mineral density
BMI	body mass index
CA	carbonic anhydrase
CBL	cecal bacterial lysates
DP	degree of polymerization
GALT	gut-associated lymphoid tissue
GC	gas chromatography
GST	glutathione S-transferases
hBD	human beta defensins
HPAEC-PAD	HP-anion exchange chromatography with pulsed amperometric detection
LC	liquid chromatography
MLN	mesenteric lymph node
NEC	necrotizing enterocolitis
NK	natural killer
OGTT	oral glucose tolerance tests
PP	Peyer's patches
PBMC	peripheral blood mononuclear cells
SCFA	short-chain fatty acids
TG	transgenic
UC	ulcerative colitis
WBBMC	whole-body bone mineral content
WBBMD	whole-body bone mineral density

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7 Galacto-Oligosaccharide Prebiotics

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7.1 Introduction

The wide recognition of bifidobacteria as health promoting bacteria (Boesten and de Vos, 2008) has attracted a lot of interest in identifying substances that can selectively promote their growth. Many studies using conventional culture and molecular techniques for bacterial identification have shown that breast-fed infants are characterized by an intestinal microbiota that is dominated by bifidobacteria (Benno et al., 1984), which is different from that of infants fed on cow's milk in that their microbiotas are characterized by lower counts of bifidobacteria, with greater numbers of more potentially harmful organisms such as clostridia and enterococci (Lunderquist et al., 1985). As a result of this difference in the microbiota composition, higher levels of ammonia, amines and phenols and other potentially harmful substances have also been found in infants fed cow's milk products (Lunderquist et al., 1985).

This has led, at the beginning of the last century, to the belief that there are molecules in the human milk that can promote the growth of this specific type of intestinal bacteria, leading to attempts to isolate and characterize those bifidogenic factors (Hamosh, 2001). Although bifidogenic nucleotides have been detected in human milk the predominance of bifidobacteria in breast-fed babies is thought to result from their abilities to utilize the oligosaccharides fraction of breast milk (Sela et al., 2008). Oligosaccharides are the third largest component of human milk and high levels are found in the colostrum where these substances constitute up to 24% of total colostrum carbohydrates (Bode, 2006). Total oligosaccharides in breast milk can reach concentrations as high as 8–12 g/l, which is 100 times greater than in cow's milk, and their concentrations steadily decrease to between 19% and 15% in the first 2 months after birth (Kunz et al., 1999). Milk contains a greater proportion of neutral compared to acidic oligosaccharides and the principal sugar components of oligosaccharides are sialic

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acid, N-acetylglucosamine, L-fucose, D-glucose and D-galactose, resulting in a complex mix of over 130 different oligosaccharides, due to the great variety of different sugar combinations that are possible (Bode, 2006). Amongst those oligosaccharides, human milk contains a large amount of galactose with the backbone structure based on lactose (galactose–glucose) plus a further external galactose residue that leads to the formation of three galactosyl-lactoses, 1→3, 1→4 and 1→6-galactosyl-lactose, with 1→6 galactosyl-lactose being found in amounts ranging between 2.0 and 3.9 mg/l. The total concentration of lactose-derived oligosaccharides in human milk has been estimated to approximately 1 g/l (Boehm et al., 2005).

The ability of those oligosaccharides to replicate the bifidogenic properties of breast milk (Knol et al., 2005) and to resemble glycoconjugate structures on cell surface receptors used by pathogens for adherence in the gut (Rudloff et al., 2002, Morrow et al., 2005) has attracted a lot of interest in further studying their physiological properties and production. As a result, galacto-oligosaccharides have attracted significant interest for their inclusion in various foods as health promoting ingredient, particularly in Japan and Europe.

7.2 Production Process

7.2.1 Transgalactosylation Reaction

Galacto-oligosaccharides are defined as a mixture of those substances produced from lactose, comprising of between two and eight saccharide units, with one of these units being a terminal glucose and the remaining saccharide units being galactose, and disaccharides comprising of two units of galactose.

They can be synthesized by classical chemical synthesis methods from simple sugars, but the preferred mode for their synthesis is by enzymatic catalysis from lactose using an appropriate β-galactosidase enzyme. The two types of enzyme that can be used in the preparation of GOS are the glycosyltransferases (EC 2.4) and the glycohydrolases (EC 3.2.1).

Galactosyltransferases catalyze the transfer of sugar moieties from activated donor molecules to specific acceptor molecules, forming glycosidic bonds. These enzymes are highly regio-and stereo-selective and can produce high yield of GOS, but the fact that they are not readily available and their requirement for sugar nucleotides, make their use for industrial GOS production prohibiting due to the cost involved.

Galactohydrolases are much more readily available than glycosyltransferases but are generally less stereo-selective. They transfer the galactosyl moiety of a substrate to hydroxyl acceptors and are able to catalyze hydrolysis or transgalactosidation depending on the relative concentration of hydroxyl acceptors from water or other carbohydrates respectively. Synthesis of GOS with β-galactosidases is a general characteristic of retaining galactohydrolases that during lactose hydrolysis use a double-displacement mechanism to form a covalent glycosyl-enzyme intermediate, which is subsequently hydrolyzed via oxocarbenium ion-like states. The active site of the enzyme contains a pair of carboxylic acids, serving as a proton donor and a nucleophilic base, with an average distance of ≈5.5 Å apart. Acid base catalysis is important for this enzyme class and is provided by a single carboxyl group at the active site, which is functioning as the acid catalyst for the first glycosylation step and as the base catalyst for the second deglycosylation step. During the glycosylation step one carboxyl acid, the acid catalyst, protonates the glycosidic oxygen, whereas the other carboxyl acid mediates the aglycon departure by acting as the nucleophile. During the deglycosylation step, the produced glycosyl-enzyme intermediate is hydrolyzed by a water molecule activated by the first carboxyl acid which now behaves as the base catalyst. However, in the presence of other carbohydrates in the reaction mixture, especially at elevated concentrations, they can act as acceptors for the glycosyl moiety resulting in the elongation of the carbohydrate acceptor to a higher degree of polymerization.

The enzymatic conversion of lactose into GOS by β-galactosidases is thus a kinetically controlled reaction during which the thermodynamically favored hydrolysis of the substrate generates D-galactose and D-glucose in competition to the transferase activity that generates a complex mixture of various galactose based di- and oligosaccharides of different structures.

In addition to this transferase activity, another mechanism of GOS synthesis that leads directly to formation of the disaccharide allolactose is by the direct internal transfer of galactose from the position 4, found in lactose, to the position 6 of the glucose moiety without the release of the glucose moiety from the active site. Quantitatively, allolactose is one of the major oligosaccharides formed by neutral pH β-galactosidases and although this mechanism has been demonstrated only for β-galactosidase enzymes from *E. coli*, it has been proposed also for other β-galactosidases with similarities to the LacZ enzyme.

During this enzymatic reaction, the amount and nature of the formed oligosaccharide mixture is affected by the ratio of hydrolytic and transferase activities of the enzyme. This ratio depends on the enzyme source, the concentration and

nature of the substrate and the reaction conditions (pH, temperature and time) following the general principle that the transferase activity is favored at high lactose concentration, elevated reaction temperature and lower water activity. The source of the enzyme is directly influencing the type of glycoside bond formed between the galactose moieties of the produced GOS, and is also setting the range of pH and temperature conditions available for the synthesis reaction.

7.2.2 Microbial β-Galactosidase

The most extensively studied β-galactosidases for GOS synthesis are of microbial origin (β-galactohydrolase, EC 3.2.1.23). Enzymes from species belonging to *Kluyveromyces*, *Aspergillus*, *Bacillus*, *Streptococcus* and *Cryptococcus* have been used for the synthesis of GOS from lactose showing differing requirements for reaction conditions in terms of pH and temperature and differing product formation in terms of the glycoside bonds formed between the galactose moieties and the degree of polymerization (DP) of the synthesized oligosaccharides. Usually 55% of the initial lactose is converted into a mixture of products containing glucose and galactose due to the hydrolytic activity of the enzyme, un-reacted lactose, disaccharides of galactose and glucose with different β-glycoside bonds from lactose due to direct internal transfer, and trans-galactosylation products such as galactobiose, galactotriose, galactosyl lactose, tetra- to octasaccharides of similar rearrangement and/or side chain formations (Playne, 2002).

6′-galactosyl lactose is the main product when the yeast β-galactosidase from *Kluyveromyces* (*K. marxianus* subsp. *lactis*, *K. fragilis*) is used, whilst 3′- and 6′-galactosyl lactose are formed when the fungal lactase of *Aspergillus oryzae* is used. Enzymes from *Bacillus circulans* or *Cryptococcus laurentii* form mainly 4′-galactosyl lactose and enzymes from *Streprococcus thermophilus* 3′-galactosyl lactose. Another interesting approach for sourcing microbial β-galactosidase, has been explored in species of probiotic bacteria. The rationale behind the use of β-galactosidases from probiotic bacteria is that since the origin of the enzyme used in this type of manufacturing is important in the final GOS mixture composition and therefore functionality, the use of enzymes originating from probiotic bacteria as synthetic catalyst will produce oligosaccharide mixtures that will be more readily metabolized by the producing organism, resulting so in higher selectivity towards that organism. Following this approach, enzymes from various *Bifidobacterium bifidum* strains have been used to produce mixtures of linear 3′-galactosyl lactose as well as branched oligosaccharides.

The pH conditions of the reaction vary from very acidic to neutral with the fungal β-galactosidase from *A. oryzae* showing optimum GOS formation at pH as low as 3.5, whilst the enzymes from yeast or bacterial origin have more neutral pH optima between 6 and 7.5.

The origin of β-galactosidase is also influencing the temperature tolerability of the enzyme. Generally high temperatures are preferred in order to speed the reaction, to increase the solubility of the lactose substrate and prevent its crystallization and also to reduce the viscosity of the reaction mixture so that the transferase activity is favored over the hydrolytic activity. At the same time, the higher lactose concentration is reducing the water activity of the reaction solution which in turn is influencing the degree of polymerization of the formed GOS products, since lower water activity conditions are favoring the production of trisaccharides and higher water activity is required to synthesize GOS of greater length. Therefore, considerable efforts have been focused on sourcing thermostable β-galactosidases as a mean for the improvement of the reaction yields.

Another approach to improve the reaction yields through improving the transferase/hydrolytic ratio of β-galactosidases has been attempted through genetic engineering. Jørgensen et al. (2001) investigated the functional importance of the C-terminal part of BIF3 β-galactosidase by deletion mutagenesis and expression of truncated variants using *E. coli* cells as the host. Deletion of approximately 580 amino acid residues from the C-terminal part converted the enzyme from a normal hydrolytic β-galactosidase into a highly efficient transgalactosylating enzyme. Quantitative analysis showed that the truncated β-galactosidase utilized approximately 90% of the reacted lactose for production of GOS while hydrolysis constituted a 10% side reaction. This 9:1 ratio of transgalactosylation to hydrolysis was maintained at lactose concentrations ranging from 10% to 40% (w/w), suggesting that the truncated β-galactosidase is behaving as a true transgalactosylase even at low lactose concentrations.

7.2.3 Production Process

Various reactor designs and configurations have been reported for GOS synthesis, including the batch reactor, continuous stirred-tank reactor (CSTR), CSTR coupled with crossflow filtration, hollow fiber membrane reactor, fixed-bed and fluidized-bed reactor (Boon et al., 2000). The batch modes of operation prevail so far in the scientific literature on trans-galactosylation processes, mainly because of their ease of operation, but also the reduction in the risk of possible microbial

contaminations that long-term continuous processes, especially under realistic reaction conditions of moderate temperatures, have or fouling that can occur in systems where membranes are used for the retention of the enzyme.

Continuous systems, however, have been proposed as a way for reducing the process cost by offering a more efficient usage of the enzymes that can be very expensive. In the batch process, the initially added to the reaction mixture enzyme is usually lost at the end of the reaction. In the continuous process re-usage of the enzyme can be achieved by immobilizing it on a carrier and thus limiting the loss of enzyme activity or by retaining the soluble enzyme in the reactor with the use of an ultra-filtration membrane.

Another very interesting aspect, regarding the mode of operation, is the effect on the composition of the produced mixture. In batch systems, the composition and concentration of possible galactosyl acceptors are changing constantly over the reaction time whilst in the steady state of a continuous system the concentrations of possible galactosyl acceptors stay constant over the entire reaction time. This leads to more defined GOS mixtures being expected in a continuous process system and has been suggested as the reason why a larger fraction of trisaccharides is formed in batch production compared to continuous ones. Once the conversion of lactose to GOS mixture has been completed, the efficient removal/inactivation of the β-galactosidase is an important factor in order to prevent product hydrolysis that takes place if the reaction is continued beyond the peak of oligosaccharide formation.

For the commercialization of GOS-based products the purification of the produced oligosaccharides from the reaction mixture is a significant and challenging step. On a larger scale, monosaccharides can be separated using chromatographic applications such as ion-exchange resins or activated carbon. In the case of ion exchange chromatography, cation-exchange resins are mainly used since they have the highest affinity for monosaccharides and therefore oligosaccharides are the first to elute from the column. Activated carbon has a higher affinity for oligosaccharides, compared to mono- and disaccharides, which makes their operation at industrial level more preferable, since regeneration can take place off-line without large substrate losses. The separation of lactose from the disaccharide fraction of the GOS products has been proven to be extremely difficult and usually results to large losses of GOS products. Lactose-free GOS mixtures are of great interest considering that 70% of the world population lack β-galactosidase in the small intestine and are therefore sensitive to lactose. An approach based on the selective enzymatic oxidation of lactose into lactobionic acid using a fungal cellobiose dehydrogenase has been described

(Splechtna et al., 2001). The produced lactobionic acid can then be easily separated from the non-ionic sugars of the mixture with the use of anion exchange chromatography, whilst the monosaccharides are subsequently removed in a single chromatographic step, yielding to purified GOS mixture with only minor losses of the main components of interest.

7.2.4 Commercially Available GOS

GOS have been used as food ingredients in Japan and Europe for at least 30 years and their application is currently expanding rapidly. At present, Japanese companies still dominate worldwide galacto-oligosaccharide production and development activity, although, European interest in GOS based products is increasing with several companies currently producing or planning to produce GOS mixtures. In contrast, GOS production in the USA at present remains negligible. The major companies manufacturing GOS are still located in Japan. Yakult Honsha (Tokyo, Japan), Nissin Sugar Manufacturing Company (Tokyo, Japan) and Snow Brand Milk Products (Tokyo, Japan) together with Friesland Foods Domo (ex Borculo Domo ingredients) in the Netherlands and Clasado Ltd in the UK are the main manufacturers. Most of the manufacturers produce several classes of products in terms of GOS purity in either syrup and/or powder format.

Yakult is producing three GOS product: Oligomate 55 in syrup form, Oligomate 55P in powder form and TOS-100 a purified version of 99% oligosaccharide content. Nissin is producing Cup-Oligo in syrup (Cup-Oligo H70) and powder format (Cup-Oligo P) and Snow Brand produces GOS that is incorporated into its infant milk formula P7L, without offering sales outside its organization.

In Europe, Friesland Food Domo is offering Vivinal GOS in a syrup format containing 57% oligosaccharides on dry matter and in a powder format containing 29% oligosaccharides on dry matter. Clasado Ltd is offering mainly a powder GOS product, Bimuno, with 52% galacto-oligosaccharide content on dry matter, as well as a syrup version of that product.

Besides the differences in the purity amongst the commercially offered products, there are differences also in the linkages of the oligosaccharide chain due to the different enzymes used in their production. The Oligomate range is produced with enzymes originating from *Aspergillus oryzae* offering mainly β 1–6 linkages, the Bimuno product is produced using enzymes from *Bifidobacterium bifidum* and contains mainly β 1–3 linkages whilst Cup-Oligo and Vivinal offer

mainly β 1–4 linkages as a result of the activity of enzymes from *Bacillus circulans* for the latter and *Cryptococcus laurentii* for the former GOS product. Yakult is also considering dual enzymes systems combining the activity of enzymes from *A. oryzae* and *B. circulans* to produce GOS mixtures of β 1–4 and β 1–6 linkages.

Although different enzymes are used in the production of the various commercially available GOS products the overall process flow chart of them is very similar (*Figure 7.1*). 20–40% (w/v) lactose solution is incubated with the β-galactosidase enzyme in either batch or continuous reactor until the optimum of lactose conversion into oligosaccharides has been reached. The solution is then decolorized and demineralized before being further processed for removal of

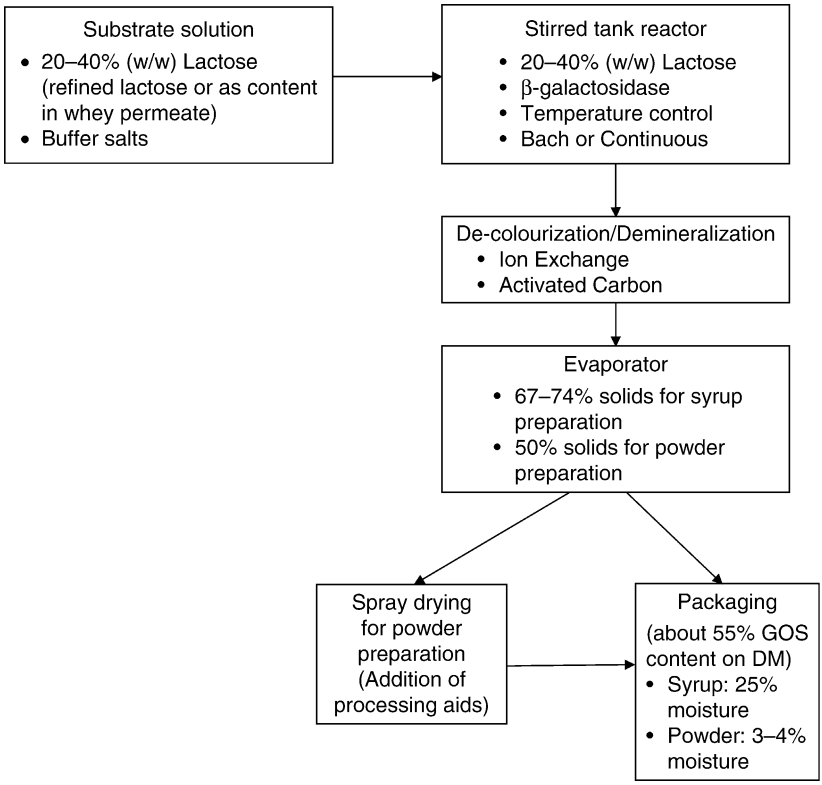

Figure 7.1
Process flow schematic for the manufacturing of GOS from lactose.

monosaccharides, either by chromatographic separation or nanofiltration, to increase the oligosaccharide purity. The resultant solution is concentrated by evaporation usually to 67–75% total solids to produce the syrup format or spray dried to produce the powder format.

A wide range of granted patents or patent applications related to the conditions as well as problem solving during the production of GOS have taken place and are available on-line (www.uspto.gov, www.epo.org).

7.3 Safety-Toxicity of GOS

In terms of safety and toxicity, GOS has been evaluated in both acute and chronic toxicity tests in rats. Oral administration of GOS did not show any toxicity as a single dose of 20 g/kg of body weight and a daily dose of 1.5 g/kg of body weight for 6 months. Using the Ames' test and the Rec assay no mutagenicity was found and the only known adverse effect of GOS is transient diarrhea due to osmotic pressure, which occurs when excess oligosaccharides are consumed. In the case of GOS, the amount of oligosaccharides which does not induce osmotic diarrhea is estimated to be approximately 0.3–0.4 g/kg body weight, or about 20 g/human body. When GOS syrup was administered to rats by gavage at 2,500 or 5,000 mg/kg of body weight daily for 90 days, no significant adverse toxicological effects attributable to treatment were noted. Clinical signs were unremarkable, and there were no ocular findings in any of the animals. Statistical analysis of clinical pathologies, including blood biochemistries, hematology, urinalysis and coagulation did not reveal any significant effects (Anthony et al., 2006).

Galacto-oligosaccharides have a generally regarded as safe (GRAS) status in the USA, a non Novel Food status in the EU and are regarded as foods of specific health use (FOSHU) in Japan.

7.4 Physicochemical Properties

GOS provide several physicochemical and health benefits, which make their use as food ingredients particularly attractive. Since commercially available food grade GOS are mixtures their specific physicochemical and physiological properties will to some extend vary depending on the mixture of oligosaccharides of different degree of polymerization, the un-reacted lactose and the generated

monosaccharides. Overall, galacto-oligosaccharides are colorless, water soluble with a viscosity similar to that of high fructose syrup leading to improved body and mouth-feel. They are stable to pH 2 at 37°C for several months making their application in non-refrigerated fruit juice matrices possible, whilst the presence of β type linkages makes them ingredients of increased resistance to high temperature in acidic medium. They remain unchanged after treatment at 160°C for 10 min at neutral pH and after treatment at 120°C for 10 min at pH 3 or 100°C for 10 min at pH 2, offering potential for a wide range of food applications. They are mildly sweet, typically 0.3–0.6 times the sweetness of sucrose and can be used in very sweet foods as bulking agent to enhance other food flavors. GOS are resistant to salivary degradation and are not utilized by the oral microbiota and can therefore be used as low cariogenic sugar substitutes in chewing gums and confectionary. They are not hydrolyzed by pancreatic enzymes and gastric juice passing the small intestine offering reduced glycemic index and a calorific value lower than 50% that of sucrose, which makes them suitable for low-calorie diet food and for consumption by individuals with diabetes. Galacto-oligosaccharides have a high moisture retaining capacity preventing excessive drying that can be useful in baked goods especially bread where GOS are not broken down during fermentation with yeast and the baking process, providing the bread with excellent taste and texture. Their low water activity can help in controlling microbial contamination and depending on the molecular weight of the oligosaccharide content, they can alter the freezing temperature of frozen foods and reduce the amount of coloring, due to Maillard reactions, in heat processed food as relatively fewer reducing moieties are available.

Analysis of galacto-oligosaccharides in different foods matrices is of high significance in terms of inclusion in various food products and a method, based on the principle of the quantification of FOS in food matrices, is available (AOAC 2001.02). This two stage method relies on the enzymatic treatment of a test solution with a β-galactosidase enzyme, followed by the quantitative determination of galactose by high-performance anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD), in order to overcome the lack of sensitivity and selectivity that HPLC suffers. In the first stage, the free galactose and lactose are determined in the initial test solution and in the second stage, the total amount of galactose released from GOS and lactose is determined in the β-galactosidase treated solution. The GOS content is then calculated from the concentrations of lactose and galactose before and after the enzymatic treatment. The method has been tested and evaluated in dairy, fruit juice, pet candy, biscuit and infant formula matrices.

7.5 Functional Properties

Apart from the advantages provided to food manufacturers by the physicochemical properties of galacto-oligosaccharides, GOS offer a number of health promoting properties mainly due to their ability to modulate the balance of the colonic microbiota and promote the proliferation of intestinal bifidobacteria. Especially this bifidogenicity is making the inclusion of GOS on its own or as part of a mixture with FOS in infant milk formula, follow on formula and infant food the major application of GOS at the current time.

7.5.1 GOS Bifidogenicity

From the early steps of research into bifidobacteria, it has been recognized that those bacteria express higher activity of β-galactosidase than many other members of the colonic microbiota (Desjardins and Roy, 1990). This has been further supported by the isolation and characterization of various β-galactosidases from different *Bifidobacterium* species and subsequently confirmed at the genome level through the sequencing and annotation of numerous β-galactosidase encoding genes present in many *Bifidobacterium* strains of human origin. Within the available literature it is apparent that most bifidobacterial strains make use of more than one β-galactosidase isoenzyme for their growth. Tochikura et al. (1986) isolated and characterized two different β-galactosidases from *B. longum* biovar *longum* 401, whereas the genome sequence of *B. longum* biovar *longum* NCC2705 (Schell et al., 2002) revealed the existence of four different isoenzymes belonging to either GH 2 or GH 42 family. Furthermore, the cloning and sequencing of three genes from *B. bifidum* DSM20215 (Møller et al., 2001) and two genes from *B. longum* biovar *infantis* HL96 (Hung et al., 2001) confirm the ability of bifidobacteria to make extensive use of these enzymes for their growth. Amongst those bifidobacterial β-galactosidases many have been proven to be lactases, hydrolyzing mainly lactose into its simpler glucose and galactose moieties, but some of them, the ones belonging to the GH 42 family seem to preferentially hydrolyze galacto-oligosaccharides other than lactose, as it has been demonstrated with a β-galactosidase extracted from *B. adolescentis* DSM20083. This enzyme was shown to preferentially hydrolyze mainly β-D-(1→4) linked di- and galacto-oligosaccharides with reasonable Km values (2.2–6.4 mM) and be able to liberate galactose from β-D-(1→3), β-D-(1→6) and β-D-(1→1) galactobiose, but not from lactose. The presence of β-galactosidases with different

activity within the same cell of *Bifidobacterium* strains enables them to better utilize GOS mixtures by working in a synergistic fashion. Enzymes belonging to the GH 42 family liberate galactose moieties from the galacto-oligosaccharide molecule whereas enzymes belonging to the GHF 2 family act on lactose backbone of the molecule. The ability of bifidobacteria to utilize GOS molecules if further supported by the presence of appropriate membrane transport mechanisms which facilitate the internalization of the released carbohydrates into the cell. Although, not much research has been carried out, the presence of glucose and/or galactose transport systems has been identified in many species of bifidobacteria. In *B. bifidum* DSM20082, galactose crosses through the membrane by diffusion, whereas glucose is incorporated by a cation symport which is regulated by K^+ ions. Two glucose transport systems have been identified in *B. longum* biovar *longum* NCC2705, whereby one of them additionally participates in the incorporation of galactose even though it is repressed by the presence of lactose. Those transport systems are rapidly fed with simple carbohydrate moieties that exist in the environment of the cells through the action of extracellular enzymes that are able to degrade non-digestible galacto-oligosaccharides. *B. bifidum* DSM20215 has a putative extracellular β-galactosidase (GHF 2) which contains at their N-terminal part signal peptides that enable it to be extracellularly translocated by the cells, whereas the C-terminal part consists of domains that most probably mediate its attachment to the cell wall. Another simpler isoenzyme, in terms of protein domain structure (GHF 42), has been identified as being extracellular in *B. adolescentis* (van Laere et al., 2000). Extracellular location of the different β-galactosidase isoenzymes allows the cells to have better access and ability to degrade galacto-oligosaccharides into their simpler moieties (galactose and glucose) and subsequently internalize them via the described transport mechanisms.

However, many studies have demonstrated the preference of bifidobacteria towards di- or even oligosaccharides over their simpler moieties, suggesting that they have developed a mechanism for internalizing complex oligosaccharides into the cell and thus complement the extracellular degradation. Kim et al. (2005) and Parche et al. (2006) demonstrated the preference of *B. longum* biovar *longum* for lactose over glucose in growth culture experiments containing both carbohydrates. They showed that lactose was consumed first whereas assimilation of glucose was repressed until all lactose disappeared from the growth medium. This mechanism involves a transcriptional down-regulation of the glucose transport system in the presence of lactose that is most probably internalized by a lactose transferase. Gopal et al. (2001) and Amaretti et al. (2007) also

demonstrated the preference of *B. animalis* biovar *lactis* and *B. adolescentis* for oligosaccharides over glucose, suggesting the induction and expression of permeases specific for tri- and tetrasaccharides. Lactose transport systems have been investigated in *B. bifidum* DSM20082 and proved to be most probably based on a proton symporter. Moreover, using proteome analysis of *B. longum* biovar *longum* NCC2705 it was revealed the involvement of 19 permeases for diverse carbohydrates uptake (Parche et al., 2007). Amongst them, three putative lactose transport systems were identified. Through the function of these transporter mechanisms bifidobacteria can internalize a variety of galactooligosaccharide molecules and subsequently degrade them in the cell, avoiding thus the competition of nutrients and cross feeding of other bacteria that can occur when glucose and galactose moieties are liberated in the extracellular cell environment.

Although little data are available, the expression of β-galactosidases, as well as, galactoside transporters seems to occur in a controlled fashion in the *Bifidobacterium* cell. Growth of *B. adolescentis* DSM20083 on galacto-oligosaccharides results in the induction of β-galactosidase activity. When glucose, galactose or lactose were present, the expression of a lactose hydrolyzing enzyme was induced, whereas in the presence of galacto-oligosaccharides *B. adolescentis* DSM20083 expressed a galacto-oligosaccharide hydrolyzing isoenzyme (van Laere et al., 2000). In *B. bifidum* DSM20082, the presence of lactose slightly stimulated the β-galactosidase activity (1.5 times) that was accompanied by higher lactose incorporation into the cell, suggesting higher expression of a lactose permease. Induction of β-galactosidase activity by the substrate has also been reported for *B. longum* biovar *longum* CCRC15708 which contains four putative lactose operons in its genome (Schell et al., 2002). Two of those operons consist of a β-galactosidase gene, an LacI-type transcriptional regulator and ABC-type oligosaccharide transporters (BL0258-BL0260 and BL1167-BL1169). The third operon consists of a putative lactose permease (BL0976) in the opposite direction to a β-galactosidase gene (BL0978; *lacZ*), whereas the fourth, which is probably not functional, consists of a *lac*I-type repressor gene (BL1774) and a cryptic β-galactosidase-like gene (BL1775) (Parche et al., 2006; Schell et al., 2002). Microarray data analysis has indicated that those genes are either constitutively expressed or induced by the presence of lactose but none of them is significantly repressed by glucose (Parche et al., 2006). However, contrary to the above observations which indicate constitutive or inducible expression of β-galactosidases, the gene annotation of *B. longum* biovar *longum* NCC2705 has shown that this strain predominantly uses repressors for the negative

transcriptional regulation of β-galactosidase gene expression, something that is in contrast to most prokaryotes, but has been justified a way to allow a quicker and higher stringent response to environmental changes.

7.5.2 Non-digestibility of GOS

To exert an effect and to come into contact with bacteria growing in the colon, any prebiotic, by its definition, must escape digestive processes in the stomach and small intestine. The non-digestibility of prebiotics can be demonstrated *in vitro* by subjecting them to treatment with pancreatic and other gastrointestinal digestive enzymes. In the case of GOS, several *in vitro* experiments have shown its non-digestibility and stability to hydrolysis by such enzymes. A European consortium studied the effects of various non-digestible oligosaccharides and concluded that more than 90% of GOS arrives into the colon (van Loo et al., 1999).

The non-digestibility of prebiotics *in vivo* for human subjects can be demonstrated with ileostomized volunteers, because the digestion in these individuals is limited to the small intestine and the remainder of the food bolus can be collected from the pouch. However, very few prebiotics have been tested in this way and most of *in vivo* data is available by means of the hydrogen (H_2) breath tests. In general, studies report increased breath H_2 excretion following ingestion of GOS (Tanaka et al., 1983). These studies, therefore, give an indication that GOS is fermented by the colonic bacteria. However, the measurements of breath H_2 excretion do not provide information on the amount of GOS that actually escapes digestion and arrives into the colon intact. In the study by Bouhnik et al. (1997), reduced breath H_2 excretion was observed after daily dose of 10 g of GOS was administered for 21 days to eight volunteers. Nevertheless, the bifidobacterial numbers were increased, demonstrating that GOS was fermented by bacteria in the colon.

The calorific value of prebiotics, such as GOS, has been estimated to be 1–2 kcal/g (Cummings et al., 1997). According to the Japanese standard methods, calorific value of GOS has been calculated to be 1.73 kcal/g.

7.5.3 Prebiotic Properties of GOS

Along with inulin, FOS and lactulose, GOS is one of the prebiotics that has been the most thoroughly investigated and its prebiotic effect has been proven.

At present, this prebiotic effect is defined as the selective stimulation of the growth of bifidobacteria in complex microbial communities that exist in the large intestine. Bifidobacteria and lactobacilli are recognized as health-promoting organisms and are widely used as probiotics. Although, their specific health promoting effects are yet to be fully explained, no adverse effects related to their consumption have been reported.

7.5.3.1 *In Vitro* Effects

Methods for the assessment of prebiotic fermentation *in vitro*, range from simple static batch cultures to the multiple-stage continuous cultures inoculated with either single/mixed bacterial strains or fecal homogenates. Such fermentation studies describe the prebiotic capabilities through switches in the composition of purportedly beneficial and detrimental microbiota of fecal homogenates and the fermentation of prebiotics in pure cultures. Summary of *in vitro* data for the prebiotic effect of GOS is shown in *Table 7.1*.

Early studies used pure cultures and showed that GOS was selectively metabolized by all the *Bifidobacterium* strains tested compared to lactulose and FOS whose specificity was less remarkable (Ohtsuka et al., 1989). However, Hopkins et al. (1998) tested abilities of various bifidobacterial isolates to utilize various prebiotics and showed that substrate utilization was highly variable with considerable interspecies and interstrain differences. This finding was confirmed in a study by Gopal et al. (2001), where various strains of lactic acid bacteria were tested for their abilities to utilize GOS, and in addition to bifidobacterial species, some lactobacilli and pediococci strains were also able to utilize these substrates. Authors showed a perfect correlation between the ability of strains to utilize GOS and the presence of β-galactosidase, as well as the ability of *B. lactis* to preferentially utilize tri- and tetrasaccharides whereas *L. rhamnosus* preferred di- and monosaccharides when grown with GOS as growth substrate. Similar finding was observed in another study where *B. adolescentis* was able to degrade tri- and tetrasaccharides, while *B. longum* bv. *infantis* and *L. acidophilus* could only utilize those with a DP < 3 (van Laere et al., 2000). The ability of *B. adolescentis* to utilize GOS more efficiently was attributed to the presence of a novel β-galactosidase with activity towards GOS but not lactose. Indeed, strains belonging to bacteroides and clostridia have also been shown to utilize GOS in pure cultures (Tanaka et al., 1983).

While pure culture studies can offer an insight into various mechanisms involved in the utilization or growth processes, they can not be used to show a

Table 7.1

Effect of GOS on the colonic microbiota *in vitro* (*Cont'd* p. 223)

Method	Substrate	Effect	Reference
Test tubes, pure cultures (various strains)	GOS, glucose, inulin, lactose, lactulose	GOS selectively utilized by all *Bifidobacterium* strains tested and to a lesser extent by *L. acidophilus* but not by other bacteria, compared with lactulose and inulin whose specificity was less remarkable	Ohtsuka et al. (1989)
Static, temperature controlled batch culture (pure *Bifidobacterium* strains)	FOS, GOS, inulin, pyrodextrin, SOS, XOS	Substrate utilization highly variable between species	Hopkins et al. (1998)
Test tubes, pure cultures (various strains of lactic acid bacteria and bifidobacteria)	GOS	Other than bifidobacteria, some lactobacilli and pediococci strains able to utilize GOS. Perfect correlation observed between the ability of strains to utilize GOS and presence of β-galactosidase. *B. lactis* utilizes tri- and tetrasaccharides, and *L. rhamnosus* prefers mono- and disaccharides	Gopal et al. (2001)
Stirred, pH/temperature controlled, anaerobic batch cultures, fecal homogenates	FOS, GOS, IMO, inulin, lactulose, SOS, XOS	All prebiotics increased bifidobacteria. XOS and lactulose produced the highest population of bifidobacteria and FOS resulted in the largest increase in lactobacilli. GOS resulted in the largest decrease in clostridia	Rycroft et al. (2001)
Stirred, pH/temperature controlled, anaerobic batch cultures, fecal homogenates	FOS, fructose, GOS	No differences in the number of bifidobacteria between substrates. *B. adolescentis*, *B. longum* and *B. angulatum* comprised the dominant bifidobacterial species	Sharp et al. (2001)

Table 7.1

Method	Substrate	Effect	Reference
MPE (measurement of changes in dominant bacteria, substrate assimilation and SCFA), using batch cultures and fecal homogenates	FOS, V-GOS, IMO, SOS, ChOS, Psyllium, Benefiber, Sunfiber, FOS:V-GOS (50:50), FOS: Benefiber (90:10), V-GOS:Benefiber (90:10)	V-GOS in combination with FOS and alone resulted in highest MPE, suggesting the best prebiotic effect of the tested substrates	Vulevic et al. (2004)
Three-stage gut model, pH/temperature controlled to represent proximal, transverse and distal colons	B-GOS	In vessels corresponding to proximal and transverse colons, strong bifidogenic effect was observed. There was no effect on bifidobacterial population in the third vessel, due to complete utilization of substrate in previous vessels	Tzortzis et al. (2005)

prebiotic effect. As mentioned above, under these conditions GOS, as indeed most oligosaccharides, can support the growth of various bacteria and thus the selectivity can not be demonstrated. Batch and continuous culture studies using fecal homogenates offer a better *in vitro* assessment of a prebiotic potential.

Rycroft et al. (2001) used various prebiotics as substrates, in pH/temperature controlled anaerobic batch cultures inoculated with fecal homogenates, and showed that all prebiotics increased the numbers of bifidobacteria but GOS resulted in the largest decrease of clostridial population. In another study, fecal homogenates were grown in batch cultures in the presence of FOS, fructose or GOS and no differences were observed in the number of bifidobacteria between the treatments (Sharp et al., 2001). However, quantitative analysis, termed a measure of the prebiotic effect (MPE), which takes into an account a number of dominant bacterial groups, fermentation end products such as short-chain fatty acids (SCFA) and substrate assimilation was developed by Vulevic et al. (2004). Here it was shown that out of 11 substrates tested, V-GOS on its own or in combination with FOS (V-GOS:FOS, 50:50) resulted in the highest MPE suggesting the best prebiotic effect of the tested substrates. Recently, B-GOS mixture (1%, w/v) was shown to have a strong bifidogenic effect in a three-stage gut model, in vessels corresponding to the proximal and transverse colons but not in the third vessel (Tzortzis et al., 2005). The lack of the effect in the last gut model vessel, which represents distal colon, was attributed to low molecular weight and complete assimilation of B-GOS by the bacteria in previous vessels.

The results obtained from *in vitro* fermentation with fecal homogenates are important in prebiotic investigations, particularly when choosing or evaluating substrates to be used *in vivo*. However, *in vitro* models do not provide a full picture of the complex ecosystem that exists within the large intestine.

7.5.3.2 *In Vivo* Human Studies

Table 7.2 summarizes *in vivo* human studies investigating the prebiotic effect of GOS. Several studies showed that administration of various GOS mixtures to healthy humans result in significant increases in the population numbers of bifidobacteria following ingestion (Bouhnik et al., 1997; Gopal et al., 2003; Ito et al., 1993; Tanaka et al., 1983). This increase in bifidobacteria was sometimes accompanied by increased lactobacilli numbers (Gopal et al., 2003; Tanaka et al., 1983) and decreased bacteroides numbers (Tanaka et al., 1983) without any significant effect on the other bacterial groups assessed. Initially higher doses (10 g/day) of GOS were used (Bouhnik et al., 1997; Tanaka et al., 1983), however Ito et al. (1993) showed that lower dose of GOS (2.5 g/day) was sufficient to observe a bifidogenic effect when the initial number of the bifidobacteria population is low. This was confirmed in a study by Gopal et al. (2003), where it was shown that 4 weeks of supplementation with 2.4 g of GOS per day resulted in increased numbers of both bifidobacteria and lactobacilli. However, Bouhnik et al. (2004) studied the effect of 1 week intake of C-GOS (Cup-oligo) at concentrations of 0, 2.5, 5.0, 7.5 and 10.0 g/day and observed no dose-dependent effect, although the low initial numbers of bifidobacteria were associated with overall better prebiotic effect compared to the other oligosaccharides tested. In addition, this study also showed that the effect of GOS on bifidobacterial population level was higher than that observed with other prebiotics (i.e., inulin, FOS, lactulose) used in the study (Bouhnik et al., 2004). In a recent study, the administration of B-GOS mixture (3.6 g/day) was recently shown to result in a better bifidogenic effect than V-GOS (4.9 g/day) after 1 week of intake by healthy humans (Depeint et al., 2008). Moreover in the same study, a different composition of bifidobacteria was suggested after the two GOS treatments which was indicated through the isolation of different fecal *Bifidobacterium* species The difference in the magnitude and quality of the effect on bifidobacteria between the two GOS mixture was attributed to the difference in structures of the oligosaccharide present in the mixtures due to the different enzymes used for their production. Similar effect on bifidobacterial population of B-GOS was

Table 7.2

Bacteriological changes after GOS administration in human volunteer studies *in vivo*

Dose per day	Number of subjects	Treatment period	Effect	Method	Reference
3 or 10 g Oligomate	5	3 weeks	Increased bifidobacteria and lactobacilli. Decreased bacteroides	Agar plate counts	Tanaka et al. (1983)
2.5 g Oligomate	12	3 weeks	Increased bifidobacteria	Agar plate counts	Ito et al. (1993)
8.1 g V-GOS	10	2 weeks	No effect on endogenous bifidobacterial population	Qualitative DGGE analysis and sequencing	Satakori et al. (2001)
2.4 g BPOligo	10	4 weeks	Increased bifidobacteria and lactobacilli. No effect on clostridia and enterobacteria	Agar plate counts	Gopal et al. (2003)
2.5, 5.0, 7.5, 10.0 g Cup-oligo, FOS, IMOS, inulin, lactulose, SOS	8	1 week	10 g of GOS resulted in a best effect on bifidobacterial numbers. No dose-dependent effect was observed, but lower initial bifidobacterial numbers associated with better prebiotic effect	Agar plate counts	Bouhnik et al. (2004)
4.9 g V-GOS	29	1 week	Increased bifidobacteria with both but B-GOS significantly higher than V-GOS. Dose-dependent effect observed in bifidobacterial numbers with B-GOS	FISH	Depeint et al. (2008)
1.9, 3.6 g B-GOS	30				

observed even at a lower dose of 1.9 g/day in this study, and the effect was found to be dose dependent in the type of microbiota with the initial numbers of bifidobacteria being within normal range. It is clear that not all GOS mixtures necessarily result in the same effect on the microbiota, since differences in their degree of polymerization as well as structure composition can become significant when it comes to their assimilation by bifidobacteria in the complex colonic ecosystem.

Additionally, attempts have been made to perform the qualitative analysis (using DGGE and subsequent DNA sequencing) of bifidobacterial population following human intake of GOS. Satokari et al. (2001) showed that 2 weeks of supplementation with a relatively high dose (8.1 g/day) of V-GOS did not affect the qualitative composition of the endogenous bifidobacteria.

7.5.4 Metabolism in the Colon

The major end-products of carbohydrate fermentation are SCFAs, of which acetate, propionate and butyrate are quantitatively the most important in the human colon. All SCFAs are rapidly absorbed from the large intestine and stimulate salt and water absorption: principally, the gut epithelium, liver and muscle metabolize them, with virtually none appearing in the urine and only small amounts appearing in the feces. The three major SCFAs are trophic when infused into the colon, and these trophic properties have important physiological implications in addition to maintaining the mucosal defense barrier against invading organisms. The amount and type of SCFA produced in the colon will depend on the type of substrate as well as the composition of the microbiota. Because SCFA are rapidly absorbed from the gut, measurements in feces do not provide useful information about the fermentative abilities of different substrates. This has been demonstrated in human trials where the administration of GOS resulted in increased bifidobacteria but with no effect on fecal SCFA concentrations (Ito et al., 1993). However, *in vitro* studies, using fecal homogenates, can be useful models for studying the fermentation profiles. Bouhnik et al. (1997) showed that the addition of 10 g of GOS to batch cultures inoculated with human fecal homogenates resulted in increased acetic and lactic acid productions which were not observed in control fermenters. The authors suggested the change of microbiota composition (i.e., increase in the population numbers of bifidobacteria) was responsible for this. Similar findings were reported in other studies using batch cultures. For example, at concentrations of 10 g/l, FOS and GOS were shown to increase acetate and butyrate formation, with transient accumulation of lactic and succinic acids (Hopkins and Macfarlane, 2003). Studies with rats inoculated with human fecal microbiota show clear demonstration of decreased pH and increased SCFA and other organic acids in the caecal contents following consumption of GOS (Kikuchi et al., 1996). In these models, the major increases are observed in the production of lactic and succinic acids suggesting that these may be responsible for the lowering of the pH. In a study with piglets

(Tzortzis et al., 2005), inclusion of 4% (w/w) B-GOS in the diet resulted to the significant decrease of pH in the proximal colonic content, which was attributed to the significant increase of acetic acid and total SCFA concentration. This effect though could not be seen in the distal colonic content as well.

7.5.5 Physiological Effects of GOS

7.5.5.1 Stool Improvement

GOS has been shown to beneficially affect fecal nature and improve some parameters related to constipation. Deguchi et al. (1997) studied the effect of administering 2.5 and 5 g of GOS (Oligomate) daily for 1 week to 75 women who had a tendency to be constipated. Higher dose of GOS was found to improve the defecation frequency significantly. Similar findings were reported by Korpela and colleagues who performed series of studies in both healthy adults and constipated elderly (Sairanen et al., 2007; Teuri et al., 1998). This research group showed that consumption of yoghurt containing high doses (9–15 g) of V-GOS daily for 2–3 weeks, can increase the defecation frequency, however in younger adult subjects gastrointestinal symptoms, such as flatulence, also increased (Teuri et al., 1998).

The frequency of defecation does not provide information on the improvement of stool weight, however, and studies that have examined the effect of GOS on mean daily stool weight did not report any significant changes regardless of the dose (Bouhnik et al., 1997). Furthermore, no differences between of inulin, FOS and V-GOS at an intake of 15 g on mean daily stool weight were also reported (van Dokkum et al., 1999). It is worth noting here that both studies examining the effect of GOS on mean daily stool weight have used healthy adults and not constipated subjects who should provide a better group to study the laxative effect of any prebiotic. The possibility that GOS may have an improving effect in the treatment of constipation may, therefore, not be excluded and further trials are necessary to fully answer this question.

7.5.5.2 Mineral Absorption

Calcium and magnesium can be absorbed from both small and large intestines and their adequate supply and bioavailability are important factors determining the healthy bone structure. Deficiency of these minerals can lead to problems,

such as osteoporosis, later in life and especially in postmenopausal women. It has been noted that these minerals when present in the large intestine exist in forms that are poorly absorbable and that acidification caused by bacterial fermentation increases their solubility and thus absorption. Since prebiotics, including GOS, have an effect on reducing the pH and supporting the growth of lactic acid-producing bacteria, it has been shown that this types of substrate have an enhancing effect on the metabolism of calcium and magnesium as well.

Current works involving GOS are summarized in *Table 7.3*, and as shown the majority of evidence comes from animal models. Chonan and colleagues conducted series of experiments in rats and showed that calcium absorption was stimulated by ingestion of GOS when normal but not low dietary concentrations were used. This calcium absorption in turn increased bone mass and calcium content (Chonan and Watanuki, 1996; Chonan et al., 1995, 1996). Chonan and colleagues have also investigated and showed increased magnesium absorption

Table 7.3

Effect of GOS supplementation on mineral metabolism and bone mineralization

Dose per day	Study	Aim/method	Result	Reference
0.5, 5.0 g per 100 g	Rats	Ca absorption with normal and high dietary levels of GOS	Ca absorption stimulated when normal but not low dietary concentrations were used	Chonan and Watanaki (1996)
5 g per 100 g	Rats	Effect of GOS in overiectomized rats on Ca absorption and bone weight	Ca absorption stimulated and bone mass and Ca content increased	Chonan et al. (1995)
5 g per 100 g	Rats	Effect in Mg deficient rats on Mg absorption, concentration of Mg in bones and accumulation of Ca in the kidney and heart	Mg absorption and bone concentration increased and Ca accumulation in the kidney and heart reduced	Chonan et al. (1996)
15 g V-GOS, FOS, inulin	Humans (n = 12) 3 weeks	Effect on Ca and Fe absorption in healthy man using 24 h urine collections for Ca isotopes measurements	No effect on Ca and Fe absorption	van den Heuvel et al. (1998)
20 g V-GOS	Humans (n = 12) 9 days	Effect on Ca absorption in menopausal women using urine collections for administered Ca isotopes	Increased Ca absorption	van den Heuvel et al. (2000)

and bone concentration in rats fed GOS which in turn suppressed the calcification of the heart and kidney under conditions of high phosphorus and calcium dietary conditions (Chonan et al., 1996).

Although the rat models show clear beneficial effects of GOS on mineral absorption, human studies are scarce and with inconsistent results. In one study, male volunteers were fed 15 g/day of FOS, inulin and V-GOS for 3 weeks and no effect on iron and calcium absorption were observed with any of the tested prebiotics (van den Heuvel et al., 1998). In another study, postmenopausal women received 15 g/day of V-GOS for 9 days and calcium absorption was significantly increased (van den Heuvel et al., 2000). Therefore, current results offer promise, especially in relation to osteoporosis, however more human trials are needed to offer definitive answers. It is worth noting that FOS has been used more widely in human studies with good results.

7.5.5.3 Lipid Metabolism

Several animal studies have shown that administration of prebiotics, namely inulin and FOS or fermented milk products is effective in lowering blood cholesterol levels. However, *in vivo* results are variable, with some studies reporting lowering effects and others no effect on total serum cholesterol levels. Thus far, GOS has been used in two trials where serum cholesterol levels were investigated and in both no significant effect could be seen. In one trial, the effects of administration of 15 g/day of V-GOS, FOS and inulin were compared in healthy humans but no significant changes in serum lipids or glucose absorption were observed (van Dokkum et al., 1999). Recently, it was shown that 5.5 g of a GOS mixture administration to healthy elderly had no effect upon total serum cholesterol and HDL cholesterol levels (Vulevic et al., 2008). However, most studies reporting a decrease in the levels of serum cholesterol and/or increase in the levels of HDL cholesterol, following either pro- or prebiotic administration, have used subjects with initial elevated serum cholesterol levels. To common knowledge, GOS has not been used in this context and it is, therefore, not excluded that it may have an effect in these subjects.

7.5.5.4 Carcinogenesis

It has long been suggested that the human gut microbiota plays an important role in the metabolism and toxicity of both dietary and endogenous substrates.

In many cases the products of bacterial metabolism in the large intestine are associated with detrimental effects on the host and could lead to initiation and/or promotion of colon carcinogenesis. In particular, bacterial enzymes such as β-glucosidase, β-glucuronidase and nitroreductase have been associated with production of carcinogens. Since prebiotics are known to support the growth of probiotic bacteria and lower the pH in the colon it is to be expected that they would have an effect on the production of genotoxic enzymes. However, there are very few studies with GOS that have looked at these effects and most data originates from *in vitro* and animal models (› *Table 7.4*).

An *in vitro* study using a three-stage continuous gut model system showed that 5% (w/v) V-GOS was only weakly bifidogenic, but it strongly supported the growth of lactobacilli in the vessel corresponding to the proximal colon (McBain and Macfarlane, 2001), without any significant effects being observed in the other two vessels corresponding to transverse and distal colons. Fermentation of V-GOS decreased the activities of β-glucosidase and β-glucuronidase in all three vessels, however nitroreductase and azoreductase activities were increased. At the same time, this study showed that inulin exerted similar effects on the beneficial microbiota, nitroreductase and azoreductase activities, but inulin increased enterobacteria and *C. perfringens* and had no significant effect on β-glucosidase and β-glucuronidase (McBain and Macfarlane, 2001).

Studies in human-microbiota associated rats have shown that GOS (5%, w/w) administration for 4 weeks, increases caecal bifidobacteria and lactobacilli and decreases enterobacteria and the pH (Rowland and Tanaka, 1993). This change in the microbiota was followed by significant decreases in the activities of β-glucuronidase and nitroreductase, but not of β-glucosidase activity that showed to increase during GOS fermentation. Kikuchi et al. (1996) showed increased levels of β-galactosidase, lactic acid and caecal pH in human-microbiota associated rats fed GOS (10%, w/w) accompanied by a decrease in the levels of ammonia and β-glucuronidase activity.

The above results from *in vitro* and animal models suggest that GOS may have reducing effect upon the production and activities of some genotoxic enzymes. However, to date, there is only one human *in vivo* study that has looked at the effect of feeding healthy humans with 15 g/day of inulin, FOS or V-GOS on β-glucuronidase production (van Dokkum et al., 1999). Indeed, inulin and V-GOS were found to have a reducing effect upon β-glucuronidase concentration, whereas FOS had no effect in this study.

The effect of V-GOS was also assessed on the development of aberrant crypt foci in rats and it was found that at increased intake levels of V-GOS (10%, w/w),

Table 7.4

Studies on the effect of GOS on colon carcinogenesis

Dose	Study	Aim/method	Result	Reference
5% (w/v) V-GOS, inulin	*In vitro*	Effect on microbiota and activities of genotoxic enzymes in three-stage gut model	V-GOS and inulin weakly bifidogenic but increased lactobacilli. Inulin also increased enterococci and *C. perfringens*. V-GOS decreased β-glucosidase and β-glucoronidase, inulin had no effect. Both substrates increased azoreductase and nitroreductase	McBain and Macfarlane (2001)
5% (w/w) Oligomate	Rats	Effect of feeding human-microbiota colonized rats for 4 weeks on microbiota and genotoxic enzymes	Increased bifidobacteria, lactobacilli and β-glucosidase. Decreased enterobacteria, cecal pH, β-glucoronidase and nitroreductase	Rowland and Tanaka (1993)
10% (w/w) Oligomate	Rats	Effect of feeding human-microbiota colonized rats for 4 weeks on fermentation metabolites and genotoxic enzymes	Increased β-galactosidase and lactic acid. Decreased cecal pH, β-glucoronidase and ammonia	Kikuchi et al. (1996)
5, 10% (w/w) V-GOS	Rats	Fischer rats injected twice with AOM to induce colorectal tumors and fed low or high concentration V-GOS. At 4 weeks, 18 rats in each group were killed and scored, half of the remaining group swapped to the other diet. Six weeks later 9 rats in each group were killed, the rest were killed after 10 months	At first count, number of aberrant crypt foci reduced with higher compared to low V-GOS. After diet change total number of aberrant crypt foci increased, but less with animals swapped onto high V-GOS diet	Wijnands et al. (2001)
15 g per day V-GOS, FOS, inulin	Humans (n = 12) 3 weeks	Originally aimed at assessing the effect of prebiotic administration to healthy young men on serum lipids	Decreased β-glucoronidase with inulin and V-GOS but not FOS	van Dokkum et al. (1999)

it had a reducing effect, thus suggesting its potential as a protective agent against the development of colorectal tumors (Wijnands et al., 2001). However, human studies are still lacking in this respect and indeed in respect of identifying the specific anti-cancer effects of GOS and prebiotics in general, in order to draw any definite conclusions at the present time. It is worth mentioning that one of the possible and very important effects of GOS, in respect of anti-cancer as well as anti-inflammatory potential, may be its immunomodulatory properties.

7.5.5.5 Immunomodulation

The immune system protects the body against foreign agents and invasion by pathogens. It can be divided into the innate or non-specific and adaptive or acquired (specific) immune system. The innate immune system acts as first line of defence and it comprises physical barriers such as skin, and phagocytic, dendritic, inflammatory and natural killer (NK) cells as well as soluble mediators such as complement proteins and cytokines. The adaptive immune system becomes active in response to a challenge to the innate immune system. This system is more antigen-specific and it consists of two major cell types, T- and B-lymphocytes. T-lymphocytes develop into functionally different cell types with specific cytokine patterns, whereas B-lymphocytes are a part of the memory of the immune system and they produce only one type of antibody matching a specific type of antigen. T-lymphocytes can further be divided into those who mediate immunity to intracellular pathogens (Th1 cells) and those responsible for extracellular pathogens (Th2 cells).

The largest component of the immune system is situated in the gut. It is called the gut-associated lymphoid tissue (GALT), and it contains about 60% of all lymphocytes in the body. It also contains large amounts of secretory immunoglobulin A (sIgA), which plays a key role in the defence of the gut against adherence and invasion of pathogenic bacteria and viruses. GALT is constantly in contact with the microbiota and their metabolic by-products, thus dietary substrates reaching the large intestine that are able to influence the microbiota should affect the GALT.

7.5.5.6 GOS and the Immune System

The idea that prebiotics could help the intestinal defence system originated from the observations that newborn babies, who have an underdeveloped intestinal

host defence system, lack an appropriate capacity to defend themselves against intestinal infections. Furthermore, infants consuming their mother's milk were found to have a greatly reduced risk of diarrhea diseases, and a lower risk of respiratory and other infections. Human milk contains various protective components and active ingredients, including non-digestible oligosaccharides, which represent the third largest component of human milk and have been identified as the main factors involved in the development of an appropriate colonization process in infants, which in turn stimulates the maturation of intestinal host defenses.

Although it is known that human milk oligosaccharides can exert a prebiotic effect (Sela et al., 2008), research into the immunomodulatory actions of prebiotics is very recent, with most data originating from animal models and in relation to FOS and its prebiotic effect (i.e., bifidogenicity and SCFA production). However, there are few studies and lines of evidence that either suggest or demonstrate the effect of GOS on the immune system. This effect could either be direct in the form of interactions with immune, mucosal or epithelial cells and/or indirect through the species or even strain selective modulation of the microbiota and their metabolic products.

As outlined earlier, several studies have shown that GOS increases the population numbers of bifidobacteria, lactobacilli and subsequent SCFA production what are known to have immunomodulatory effect. Butyric acid is known to suppress lymphocyte proliferation, inhibit cytokine production of Th1-lymphocytes and upregulate IL-10 production, to suppress the expression of the transcription factor NF-κB and upregulate Toll-like receptors (TLR) expression, as well as to protect against colon cancer as it inhibits DNA synthesis and induces cell differentiation (Hoyles and Vulevic, 2008). Pharmacological doses of acetic acid when administered intravenously to healthy individuals and cancer patients increase NK cell activity and peripheral blood antibody production (Ishizaka et al., 1993).

Specific probiotic species belonging to either bifidobacteria or lactobacilli, when administered orally, are known to increase the secretion of sIgA in the small intestine and the feces, and to stimulate Peyer's patches (PP) B lymphocyte IgA production (Hoyles and Vulevic, 2008). In one recent study 57 infants, split into three groups, were fed either standard formula alone, standard formula containing a probiotic (*B. animalis* – Bb12) or formula containing a prebiotic mixture (V-GOS:inulin; 90:10) for 8 months (Bakker-Zierikzee et al., 2006). Measurements of fecal sIgA were made at regular intervals during the course of the study, and it was shown that babies fed the probiotic formula had variable levels of sIgA,

whereas babies receiving the prebiotic mixture demonstrated a trend towards higher levels of sIgA compared to the controls. Apart from formula fed infants, another group that could potentially benefit from the immunomodulatory effects of GOS is the elderly who are known to generally have reduced levels of beneficial bacteria and impaired immune system. A very recent study investigated the effect of feeding 5.5 g of GOS (B-GOS) to 44 healthy elderly volunteers on the microbiota composition and immune function (NK cells, phagocytosis and cytokines) (Vulevic et al., 2008). This study showed that B-GOS administration led to a significant decrease in the numbers of the less beneficial bacteria (i.e., bacteroides, *C. perfringens*, *Desulfovibrio* spp., *E. coli*) and a significant increase in the numbers of the beneficial bacteria, especially bifidobacteria. The study also found significant positive effects upon the immune response, evidenced by an improvement in NK cell activity and phagocytosis, increased secretion of the anti-inflammatory cytokine, IL-10, and decreased secretion of pro-inflammatory (IL-6, IL-1β and TNF-α) cytokines by stimulated PBMC. Additionally, a clear positive correlation between the number of bifidobacteria and both NK cell activity and phagocytosis was demonstrated. This was the first time that the immunomodulatory effect of GOS has been demonstrated in humans and it was shown that dietary intervention, such as B-GOS, can be an effective and attractive option for the enhancement of both gastrointestinal tract function and immune system (innate and adaptive) function.

7.5.5.7 Inflammatory Bowel Disease (IBD)

IBD principally includes Crohn's disease (CD) and ulcerative colitis (UC). Although, the cause of IBD is not yet known, it is generally accepted that a combination of factors such as genetic susceptibility, priming by enteric pathogens and immune-mediated tissue injury, result in its pathogenesis. Reduced numbers of beneficial bacteria accompanied by increases in the numbers of other less beneficial bacteria, such as *E. coli*, have been reported in both feces and mucosa microbiota of IBD patients. In patients with IBD Th1 and Th2 pattern of cytokine formation seem to be modified or increased in comparison to healthy individuals. For example, IBD patients seem to have increased production of tumor necrosis factor α (TNF-α) which triggers inflammation via the transcription factor NF-κB. There are very few studies with prebiotics in general, that have looked at their effect in human IBD. The use of V-GOS, however, as a therapy for immunomodulation in IBD was tested in one animal study.

The trinitrobenzene sulphonic acid (TNBS)-induced colitis rat model was used to test the effect of feeding rats 4 g/kg of body mass per day either whey or V-GOS, 10 days before the induction of colitis, or dexamethasone at colitis induction, as a control (Holma et al., 2002). Fecal bifidobacteria numbers, myeloperoxidase activity and macroscopic damage were assessed 72 h after the induction of colitis, and it was found that the bifidobacterial numbers increased with the V-GOS administration, but inflammation was not reduced. Although, this study did not show any effect from the V-GOS administration on IBD, this one animal model is not enough to draw any conclusions and more studies are needed to fully determine the potential of GOS in preventing or treating IBD.

7.5.6 Allergy

Improved hygiene and reduced exposure of infants to microorganisms are one of the suggested reasons for the observed increases in the incidence rates of allergic diseases in developed countries during recent decades. Studies indicate that there may be a link between the colonic microbiota and allergy, since reduced numbers of bifidobacteria have been found in the feces of allergic infants (Kirjavainen et al., 2002). These infants have IgE-mediated food allergies, and a Th2-biased immune response. Studies have found that FOS can reduce Th2 response in children, however GOS has not been used in this context and only indirect evidence for the effect of GOS currently exists.

Recently, 1,223 pregnant women carrying children at high risk of allergy, were divided into two groups, and fed for 2–4 weeks, before delivery, either a placebo or a mixture of 4 probiotics (*L. rhamnosus* GG, *L. rhamnosus* LC705, *B. breve*, *Propionbacterium freudenreichii* subsp. *shermanii* JS) (Kukkonen et al., 2007). Their infants received the same probiotic mix combined with 0.9 g V-GOS or a placebo for 6 months after birth, and at 2 years of age their IgE levels and the incidence of allergic diseases were evaluated. It was found that the synbiotic preparation had no effect on the incidence rates of allergy, however, incidences of eczema and atopic eczema were reduced, whilst a tendency towards the reduction of other atopic (IgE-associated) diseases was noted (Kukkonen et al., 2007).

In another study, the effect of a prebiotic mixture (V-GOS: inulin; 90:10) was assessed on the development of atopic dermatitis (AD), which is usually the first manifestation of allergy development during early infancy. Infants (259) at risk of atopy were fed a prebiotic mixture, or a placebo, from birth and for 6 months and

they were examined for clinical evidence of AD (Moro et al., 2006). Additionally, a smaller group of infants (98) was selected to assess the effect of the treatments on the fecal microbiots. The results showed a significant decrease in the incidence of AD in the V-GOS:Inulin group with 10 infants developing AD compared to the 24 infant that developed AD in the placebo group. In terms of the effect on the fecal microbiota, a significantly higher, compared to placebo, number of bifidobacteria was observed in the prebiotic group without any significant effect on lactobacilli.

7.5.7 Anti-pathogenic Activity of GOS

Studies have suggested that prebiotics could directly be involved in protecting the gut from infection and inflammation through the inhibition of the attachment and/or invasion of pathogenic bacteria or their toxins to the colonic epithelium. This attachment is necessary before pathogens can colonize and cause disease, and it is mediated by glycoconjugates on glycoproteins and lipids present on the microvillus membrane. Certain prebiotic oligosaccharides contain structures, similar to those found on the microvillus membrane, that interfere with the bacterial receptors by binding to them and thus preventing bacterial attachment to the same sugar moiety of the microvillus glycoconjugates. For example, α-linked GOS, present in human milk, are known to have anti-adhesive properties and be capable of toxin neutralization (Newburg et al., 2005, Morrow et al., 2005). B-GOS contains an oligosaccharide in alpha anomeric configuration, and it was shown to significantly decrease the attachment of enteropathogenic *E. coli* (EPEC) and *Salmonella enterica* serovar Typhimurium to HT-29 epithelial cell line (Tzortzis et al., 2005). The same GOS mixture was further studied in an oral challenge experiment, during which BalbC mice were fed either a placebo or B-GOS prior to *Salmonella enterica* serovar Typhimurium infection (Searle et al., 2009). It was shown that the animals fed the GOS mixture did not develop clinical symptoms of salmonellosis, even though the pathogen could be recovered in the feces. Furthermore the histopathology and structure of the epithelium were completely protected and translocation of the pathogen to other organs was limited compared to the placebo. In another study, GOS (Oligomate) was shown to inhibit the adhesion of EPEC to Hep-2 and Caco-2 epithelial cell lines more effectively than inulin, FOS, lactulose or raffinose (Shoaf et al., 2006). However, the anti-adhesive properties may be a result of GOS binding to pathogens and not a direct modulation of host immune system.

7.5.8 Neonates and Infants

The significance of a bifidobacteria predominant microbiota in healthy breast fed infant is well accepted. Infants become progressively colonized over a series of weeks and months by different bacteria, and by 2 years of age, the establishment of a more adult microbiota composition begins. Until then, however, bifidobacteria predominate in breast-fed infants, while formula fed infants have a more diverse microbiota composition. As mentioned previously, human milk oligosaccharides are thought to be responsible for this pattern of colonization in breast-fed infants (Hamosh, 2001; Sela et al., 2008), and therefore attempts have been made to develop infant formulas containing GOS mixture that will promote a microbiota pattern similar to that of breast feeding. Thus far, the most studied formulation is the combination of GOS and inulin at a ratio 9:1.

Preterm infants of about 31 weeks' gestational age and about 1 week old, were studied to compare standard formula with formula containing 1% (w/v) of the GOS-inulin mixture whilst a separate group fed fortified human milk was studied in parallel. Within 1 month of feeding the number of fecal bifidobacteria and lactobacilli in the GOS-inulin formula group increased to levels similar to the breast-fed group. In addition to this, the difference in the composition of the fecal flora between the standard formula and the GOS containing formula group was highly significant, with the latter being closer to the breast fed one. Moreover, stool consistency and stool frequency were also found to be similar between the GOS fed group and breast-fed infants (Boehm et al., 2002).

A number of similar studies, using the same prebiotic mixture, were performed in term infants and even toddlers showing similar results on the fecal microbiota (Veereman-Wauters, 2005). Interestingly, FOS was also tested in term infants at concentrations of 200, 400 and 600 mg per bottle for 2 weeks and it was found to exert no effect (Veereman-Wauters, 2005). It is generally accepted by the Scientific Committee on Food of the European Commission that the addition of V-GOS:inulin prebiotic mixture at a concentration of 0.8 g/dl to infant formula is considered safe, and this prebiotic mixture has been widely used in the last few years in Europe.

Although, a vast number of studies suggest the ability of a prebiotic mixture containing inulin and V-GOS to increase fecal bifidobacterial populations in infants, relatively few studies have looked at other possible effects, such as disease prevention. Atopy, as explained earlier, is an area that has been studied in some details and an area that offers a promise for use of GOS-based prebiotic mixtures. However, further studies are required in order to identify

the mechanism of action of these substrates, if GOS-based prebiotic mixtures are to be considered and used in a treatment strategy.

7.6 Under-researched and Possible Beneficial Properties of GOS

The full potential of health related and health promoting benefits of GOS is yet to be fully ascertained. As the interactions of the colonic microbiota, especially the beneficial effects of *Bifidobacterium* spp and *Lactobacillus* spp with the host are elucidated, the increased bifidogenicity of GOS is becoming a significant property for its application as a health promoting food ingredient. Consequently there are many potential areas of research where GOS has not been used and where, indeed, the prebiotics in general have not been studied.

Irritable bowel syndrome (IBS) is a common chronic functional gastrointestinal disorder that exhibits a broad spectrum of severity, ranging from mild symptoms to severe and intractable symptoms. IBS is characterized by recurrent abdominal pain and discomfort associated with alterations in bowel habit. The etiology and physiology of IBS are not fully understood, but it is most likely multifactorial. Alterations in gastrointestinal motility, visceral perception, and psychosocial factors contribute to overall symptom expression. Currently there is no single therapeutic modality of proven benefit to all IBS patients and treatment is based on the physician's understanding of the individual patient's symptom pattern and the associated psychosocial factors. Mixed probiotic combinations, mainly using specific *Bifidobacterium* species, have been used successfully in the treatment of IBS symptoms, however prebiotics have not. The possibility that GOS, through its increased bifidogenicity, could help ameliorate some of the symptoms associated with IBS as has been shown by Silk and colleagues (2009) could not be excluded and this should definitely create one area for the future research.

A common side effect of the use of antibiotics is antibiotic-associated diarrhea (AAD), which presents a particular problem in hospitalized, and especially vulnerable elderly, patients. Diarrhea associated with *Clostridium difficile* is a leading cause of hospital outbreaks of diarrhea and it considerably increases mortality and healthcare costs for inpatients. The condition occurs when patients are treated with antibiotics, for an underlying infection, which result in the disruption of the barrier of normally protective colonic microbiota. Probiotics have been successfully used to reduce the incidence of AAD. Lewis et al. (2005)

investigated the effect of 12 g of FOS on the incidence and relapse of *C. difficile* in elderly patients. Although differences between the prebiotic and placebo group were not observed on the incidence rate, there was a significant reduction in the rate of relapse in the prebiotic group accompanied by a significant increase in bifidobacteria numbers. It is clear that more prebiotic trials and involving of GOS are needed to fully explain the potential in treating or preventing AAD, as well as other GI pathogen related conditions such as travellers' diarrhea and these also offer potential areas for the future research.

Arthritis is associated with a broad spectrum of clinical and experimental intestinal inflammation, ranging from infections with intestinal pathogens, overgrowth and changes to the normal commensal colonic microbiota, injection of purified bacterial cell wall components and dietary manipulation to chronic idiopathic IBD. The common feature of all those conditions is increased exposure of the lamina propria and systemic circulation to colonic microbiota and their products, either through increased proliferation or mucosal permeability, pathogenic invasion, or immune modulation. Experimental data assessing the potential of prebiotics in arthritis is still lacking and limited to animal models. However, α-GOS has been used in adjuvant-induced arthritis Wistar rats, and type II collagen-induced arthritis in DBA/1 J mice. The dose-dependent beneficial effect was observed in erythema, swelling of limbs and on histological findings in the hind paw joints (Abe et al., 2004). This study also showed reduced levels of nitrite and nitrate in blood, although the production of IL-1 by macrophages was increased. The results indicate the potential of using GOS to immunomodulate the inflammation seen in arthritis through either direct effect or via the modulation of the colonic microbiota. However, more research and involving human subjects is needed to clarify the potential.

7.7 Summary

- Commercially available GOS products are mixtures of galactose based oligosaccharides of varying DP and linkage configuration with glucose, galactose and lactose.
- The oligosaccharide composition varies amongst GOS mixtures depending on the origin of the β-galactosidase enzyme used as well as the mode of production. Developments in the production of GOS aim to delivering purer and more efficient mixtures.
- GOS consist of oligosaccharides that are pH and heat stable offering a wide range of food application potential.

- GOS are safe well tolerated ingredients up to levels of 20 g intake per day, have a GRAS status in the USA and FOSHU status in Japan and could be included in the dietary fiber content of foods.
- GOS mixtures are well established prebiotic ingredients with increased selectivity towards *Bifidobacterium* species. Depending on their oligosaccharide composition, GOS products vary in terms of quantitative and qualitative bifidogenic properties.
- Besides through their bifidogenic effect, GOS contribute to the health of the host through their direct interaction, leading to increased immunomodulatory and anti-pathogenic properties.
- Infant and elderly nutrition offer the highest opportunity for GOS applications based on their functional properties (bifidogencicty, protection from pathogens, regulation of the immune function) and the host's requirements.

List of Abbreviations

AD	atopic dermatitis
CSTR	continuous stirred-tank reactor
DP	degree of polymerization
EPEC	enteropathogenic *Escherichia coli*
FOS	fructooligosaccharides
GALT	gut associated lymphoid tissue
GOS	galacto-oligosaccharides
NF κB	nuclear factor κB
NK cell	natural killer cells
SCFA	short chain fatty acids
TLR	toll like receptors
TNF-α	tumor necrosis factor α

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8 Prebiotic Potential of Xylo-Oligosaccharides

H. Mäkeläinen · M. Juntunen · O. Hasselwander

8.1 Introduction

Xylo-oligosaccharides (XOS) are chains of xylose molecules linked with β1–4 bonds (*Figure 8.1*) with degree of polymerization ranging from 2 to 10.

XOS are naturally present in fruits, vegetables, bamboo, honey and milk, and can be produced at industrial scale by enzymatic hydrolysis from xylan, which is the major component of plant hemicelluloses and therefore readily available in nature (Alonso et al., 2003; Vázquez et al., 2000).

XOS are non-digestible carbohydrates and have been suggested to exert prebiotic activity. They were hence first used as food ingredient for gastrointestinal health in the 1990s in Japan. This chapter provides an overview of XOS with a particular focus on the prebiotic potential.

8.2 Manufacture of XOS

XOS can be produced commercially by hydrolysis of xylan, the most abundant hemicellulosic polymer. Possible lignocellulosic raw materials for XOS production include corn cobs, hardwoods, straws, bagasses, hulls, malt cakes and bran. Xylan can either be hydrolyzed enzymatically, by chemical methods (hydrothermal treatments) or a combination of both. The resulting crude XOS solutions require a sequence of purification steps to yield high purity XOS containing at least 70–95% XOS (Moure et al., 2006; Vázquez et al., 2000). Depending on the source of raw material, XOS may be branched and contain arabinose units or carry acetyl or uronic acid residues (Vázquez et al., 2000).

XOS have been used as food ingredient predominantly in Asia, particularly Japan and Suntory Limited (Japan) was the first commercial-scale producer

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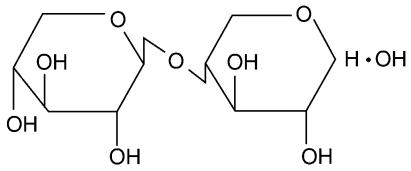

Figure 8.1
Disaccharide Xylobiose (β-D-xylopyranosyl (1→4)- D-xylopyranose).

applying enzymatic hydrolysis of xylan. The production volume of XOS was estimated at 650 tons annually in 2004 and the XOS price was 2,500 Yen/kg (Taniguchi, 2004). More recently, XOS were also offered by the Chinese producer Shandong Longlive Bio-technology co. In 2003, XOS represented only a small proportion (less than 3%) of the total Asian oligosaccharide market (Nakakuki, 2003).

The commercial XOS products are available in syrup or powder form and are predominantly composed of the disaccharide xylobiose and the trisaccharide xylotriose with small amounts of higher oligosaccharides also present.

8.3 XOS as Prebiotics

8.3.1 Resistance to Digestion

XOS are relatively stable in acidic conditions due to structural properties. This may endow protection from decomposition when passing through the stomach (Imaizumi et al., 1991). The degradation of XOS (xylobiose) in the gastrointestinal tract has been studied *in vitro* with an artificial model of digestive enzymes (α-amylase, pancreatin, gastric juice and intestinal brush border enzymes) and no hydrolysis of xylobiose was observed (Koga and Fujikawa, 1993; Okazaki et al., 1991).

The fate of xylobiose was also studied in humans after oral administration. Xylobiose was not excreted into feces or urine during 24-h following the ingestion, thus, supporting the fact that xylobiose is degraded *in vivo* not by the action of digestive enzymes, but by the gastrointestinal microbiota (Okazaki et al., 1991).

8.3.2 Fermentation by the Gastrointestinal Microbiota and Selective Stimulation of Growth and/or Activity of Intestinal Bacteria Associated with Health and Well-Being

8.3.2.1 Pure Culture Studies

Pure culture fermentation studies with single microbes and substrates can be used to identify bacterial strains that are able to degrade oligosaccharides. Since these fermentations do not resemble the competitive environment of the colon, the results can be used to gain knowledge of the fermentative capacity of individual strains within the intestinal microbial population, but not to study the effects that oligosaccharides have on the whole microbiota.

XOS are reported to be preferentially fermented by a relatively limited number of intestinal microbes *in vitro*. Several pure culture studies have indicated that XOS are well utilized by *Bifidobacterium* species, namely some strains of *B. bifidum*, *B. catenulatum*, *B. longum*, *B. animalis* and *B. adolescentis* (Crittenden et al., 2002; Jaskari et al., 1998; Moura et al., 2007; Palframan et al., 2003; Yamada et al., 1993). Furthermore, utilization of XOS seems to be strain-dependent, since not all studies have shown enhancement of for example all *B. longum* and *B. adolescentis* strains (Hopkins et al., 1998). Lactobacilli are not able to utilize XOS as a sole carbon source (Jaskari et al., 1998; Kontula et al., 1998), with the exception of *Lactobacillus brevis*, which growth was enhanced moderately by XOS (Crittenden et al., 2002; Moura et al., 2007). Some other intestinal microbes are also able to utilize XOS, but not to the same extent as bifidobacteria. Crittenden and co-workers studied the fermentation of a wide group of numerically dominant saccharolytic intestinal bacterial species and demonstrated that besides many *Bifidobacterium* strains only some *Bacteroides* isolates were efficiently fermenting XOS. *Escherichia coli*, enterococci, *Clostridium difficile* and *Clostridium perfringens* were not able to ferment XOS (Crittenden et al., 2002). Jaskari et al. found that XOS was metabolized by bifidobacteria, but also moderately by *Bacteroides thetaiotaomicron, Bacteroides vulgatus* and *Clostridium difficile*. However, these strains mainly utilized the monosaccharide xylose fraction of the XOS mixture, which in humans does not reach the colon (Jaskari et al., 1998).

Unpublished research (carried out by M. Juntunen at Danisco's Health and Nutrition Center in Kantvik, Finland) suggests a selective utilization of XOS by *Bifidobacterium lactis* strains (*Figure 8.2*). Other tested microbes showed poor growth on commercial (XOS Longlive 95P, Shandong Longlive Bio-technology

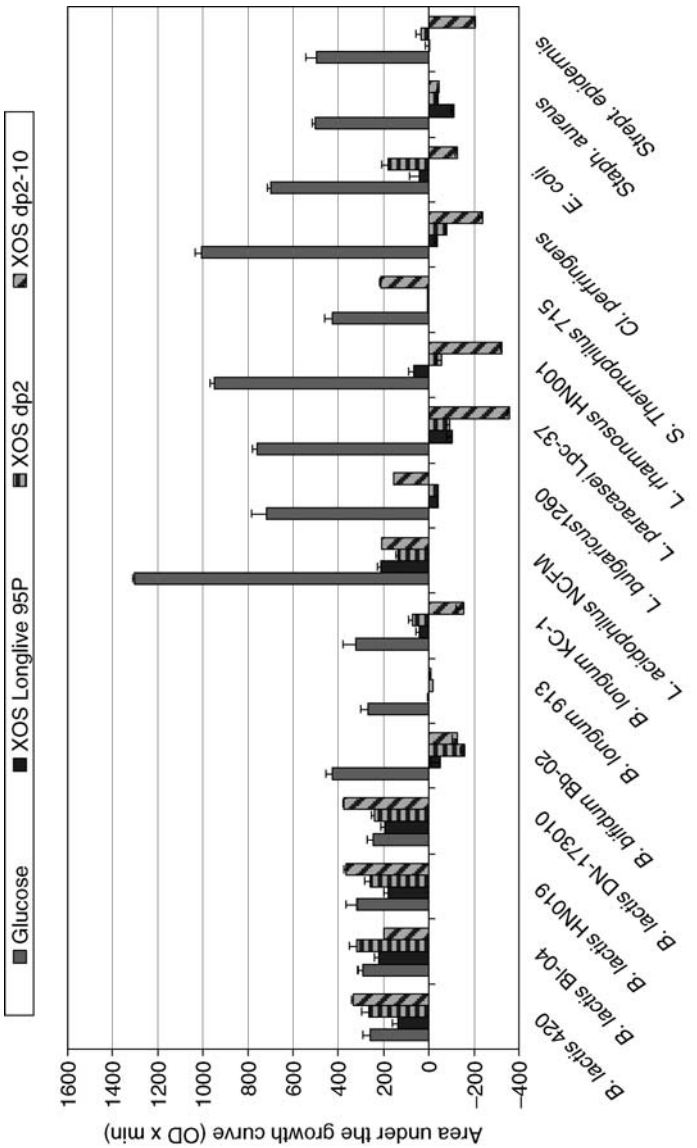

Figure 8.2
The growth of a selection of bifidobacteria, lactobacilli and other microbial strains on XOS. The growth was measured as the change in absorbance (600 nm) of liquid samples and represented the area under the growth curve (OD x minutes) obtained during the 24-h growth experiment (Jaskari et al., 1998). The area of the control medium without added carbohydrates was subtracted from results.

co., China) and hardwood-derived XOS (XOS dp2 and XOS dp2–10) when compared to growth obtained on glucose during 24-h incubation.

Interestingly, pure culture studies have also shown that for a number of *Bifidobacterium* strains the bacterial growth was higher on oligosaccharides in comparison to their monosaccharide constituents (Hopkins et al., 1998), thus, indicating that there may be a specific membrane transport mechanism for XOS but not xylose. Some bifidobacteria might import XOS before hydrolyzing it (Crittenden et al., 2002), which could offer a competitive advantage against cross-feeding by other microbes in the intestine.

8.3.2.2 Batch Culture Studies

Effects of oligosaccharide fermentation on predominant intestinal microbial groups can be monitored in batch culture fermentations and continuous or semi-continuous color simulations using colonic microbiota from fecal samples. The effects of different prebiotic oligosaccharides (FOS, inulin, lactulose, galacto-oligosaccharides (GOS), isomalto-oligosaccharides (IMO), soybean oligosaccharides (SOS), and XOS) on microbiota were compared in batch fermentations (Rycroft et al., 2001). Fermentation properties of different oligosaccharides varied and resulted in different responses in the composition and activity of microbiota. Fermentation of all oligosaccharide compounds increased the numbers of bifidobacteria and most decreased clostridia, but XOS and lactulose produced the highest increase in bifidobacteria, whereas FOS were the most effective substrate for lactobacilli. A similar significant increase in numbers of bifidobacteria, and also lactobacilli, was seen as a result of XOS fermentation in a semi-continuous simulator system inoculated with adult feces (Zampa et al., 2004). Microbial fermentation of XOS moderately increased the production of gases, and the concentrations of lactate, acetate, and propionate in both studies (Rycroft et al., 2001; Zampa et al., 2004). Zampa and co-workers also reported beneficial effects of XOS not only derived from increased populations of bifidobacteria and lactobacilli, but also from reduced concentrations of secondary bile acids, which exert negative actions on the colon and present a dose dependent toxic potential related to their co-mutagenic and tumor-promoting properties (Moure et al., 2006).

8.3.2.3 Animal Studies

The bifidogenic effects of XOS have also been observed in animal studies (*Table 8.1*). Studies in rats have demonstrated that XOS significantly stimulate

Table 8.1

Animal studies on effects of XOS

Animal studies	Major findings	Reference
Diabetic rats (n = 8), dietary sucrose and corn starch replaced with XOS for 5 weeks	Improvement in diabetic symptoms such as elevated serum glucose, cholesterol and triglycerides	Imaizumi et al. (1991)
Rats (n = 50) were fed with fiber-free diet, or diet containing 7.5% of oat fiber, gum arabic, FOS or XOS for 17 days	Oligosaccharides and gum arabic all increased the cecal wall and contents weight and decreased cecal pH with concomitant increase in SCFA. Excretion of nitrogen in feces was increased, thus, blood urea and renal nitrogen excretion decreased	Younes et al. (1995)
Rats (n = 44) and mice (n = 52) were assigned to control, FOS, XOS or gum arabic group for 14 days	XOS did not affect microbiota of animals, and only moderate effects on cecal cell proliferation were found. FOS increased significantly bifidobacteria in rats	Howard et al. (1995)
Rats (n = 10 in each group) were fed with control, cellulose, oligofructose, FOS or XOS containing diet for 14 days	Fecal pH was lowest and bifidobacteria numbers highest in the XOS group. Consumption of XOS increased most the weight of colon and cecum	Campbell et al. (1997)
Rats (n = 40) were fed with basal, XOS or FOS diet for 35 days, and treated with 1,2-dimethylhydrazine (DMH) to induce colon carcinogenesis	Oligosaccharides decreased cecal pH, increased cecal weight and bifidobacteria population, but XOS had a stronger effect than FOS. Both oligosaccharides reduced the formation of precancerous lesion	Hsu et al. (2004)
Mice (n = 16 in each group), diet supplemented (1%) with nine different oligosaccharides for a 6- month study period	XOS was most efficient prebiotic in increasing lactobacilli and bifidobacteria counts, and in reducing sulphite-reducing clostridia	Santos et al. (2007)

the growth of cecal and fecal bifidobacteria. Furthermore, XOS induced an even larger increase in bifidobacteria numbers than an equivalent dose of FOS, which had a greater effect on the *Lactobacillus* population (Campbell et al., 1997; Hsu et al., 2004). A more recent feeding-trial with mice (Santos et al., 2006) compared the long-term effects of various prebiotics on the microbial populations. In this study, 1% of FOS, inulin, lactulose, XOS, SOS, IMO, or transgalacto-oligosaccharides (TOS) were administered to the basal diet and the effects on small and large intestinal microbiota were determined after 6 months of intervention. From all the prebiotics tested, XOS was the most efficient substrate to

increase bifidobacteria, lactobacilli and total anaerobic microbial numbers in the colon. Of all the oligosaccharides, XOS supplementation also resulted in the strongest reduction of sulphite-reducing clostridial strains, thus affecting the colonic microbiota in an overall favorable manner.

8.3.2.4 Human Intervention Studies

Reports from few human interventions, mostly conducted in Japan, regarding colonic fermentation of XOS have been published to date (*Table 8.2*). Okazaki and co-workers fed 5 g of XOS daily for 3 weeks to healthy men, and found a significant increase in fecal bifidobacteria numbers. The effect of XOS

Table 8.2

Human intervention studies conducted on XOS

Human intervention study	Major findings	Reference
Healthy men (n = 9), 5 g/day XOS for 3 weeks	Significant increase in *Bifidobacterium* and *Megasphaera*, other microbes unaffected. Increased fecal acetic acid and decreased pH	Okazaki et al. (1990a)
Healthy men (n = 5), 1 and 2 g/day XOS for 3 weeks each	Significant increase in *Bifidobacterium*, other microbes unaffected, Consumption of 2 g was more effective, but even dose of 1 g of XOS increased bifidobacteria	Okazaki et al. (1990b)
Healthy men (n = 10), 2, 5 and 10 g/day XOS	Occurrence of diarrhea decreased with concomitant increase in *Bifidobacterium* with daily doses of 2 and 5 g	Kobayshi et al. (1991)
Healthy men (n = 9), 5 g/day XOS for 3 weeks	Putrefaction products in feces (*p*-cresol, indole, skatol) decreased	Fujikawa et al. (1991)
Constipated women (n = 40), 0. 4 g/day XOS for 4 weeks	Defecation frequency and stool quantity increased, self reported quality of life improved	Iino et al. (1997)
Constipated pregnant women (n = 29), 4.2 g/day XOS for 4 weeks	XOS effective in reducing severe constipation and normalizing stool consistency. Clinical symptoms scores improved	Tateyama et al. (2005)
Healthy elderly (n = 9 in control group; n = 13 in XOS group), 4 g/day XOS for 3 weeks	Significant increase in Bifidobacteria and fecal moisture content, decreased fecal pH	Chung et al. (2007)

administration on the growth of several other microorganisms was also studied, and only *Megasphera* numbers were significantly affected (increased) in addition to bifidobacteria, indicating a very selective proliferation effect (Okazaki et al., 1990a). Fecal pH reduced and acetic acid concentrations increased during this intervention. In another trial by the same researchers, *Bifidobacterium* numbers were increased in the feces of subjects consuming only 1 and 2 g of XOS per day, although the higher daily dose resulted in a more significant increase (Okazaki et al., 1990b). Even as low a daily dose as 0.4 g of XOS was shown to increase the numbers of bifidobacteria in human fecal samples, without effects on other microbial groups (Iino et al., 1997). Furthermore, decreased concentrations of putrefactive products such as *p*-cresol, indole and skatole were measured concomitantly with an increased ratio of fecal bifidobacteria (Fujikava et al., 1991). In a more recent human intervention study in elderly subjects (Chung et al., 2007), a daily XOS dose of 4 g increased significantly the *Bifidobacterium* numbers from 10^6 to 10^8 cfu/g of wet feces during a 3 week study period. *Clostridium perfringens* levels remained unchanged and the fecal pH was decreased and fecal moisture content increased during the study period. No difference in adverse gastrointestinal symptoms (flatulence, discomfort, stool consistency, defecation frequency) between the control and study group was recorded, suggesting that XOS was well tolerated by the elderly.

The limitations of the human interventions studies carried out to date are relatively small number of subjects, the (in most cases) uncontrolled study-design and the use of plating method in enumeration of microbes in feces, thus, larger controlled human intervention studies are needed to confirm the prebiotic status of XOS.

8.3.2.5 Effect of Substitution and Origin of XOS

The effect of differently substituted XOS on fermentability was first recognized by Van Laere et al., who included a linear XOS and arabinoxylo-oligosaccharides (AXOS) in a fermentation study of complex plant cell wall derived oligosaccharides (Van Laere et al., 2000). It was shown that the linear XOS were fermented by more of the tested intestinal microbial strains tested compared to the branched AXOS. The number and nature of the substitutes in XOS molecule appears to affect the fermentation speed and the metabolites produced. This was in particular demonstrated in a batch fermentation study (Kabel et al., 2002), where non-substituted XOS (nXOS) and arabino-XOS (AXOS) were reported to be fermented

more quickly than structurally more complex acetylated XOS (AcXOS) and XOS containing a 4-O-methylglucuronic acid group (GlcA(me)XOS). In this study, the fermentation of the least substituted molecules XOS and AXOS resulted in quick production of acetate and lactate, whereas the fermentation of AcXOS and GlcA (me)XOS increased the production propionate and butyrate. These results highlight the importance of detailed elucidation of the structural features of non-digestible oligosaccharides in relation to their fermentation properties.

8.3.3 Effects on Health

The formation of preneoplastic lesions (aberrant crypt foci, ACF) in the distal colon is used as a biomarker for colon carcinogenesis. In a study with rats (Hsu et al., 2004), the ACF formation was induced with 1,2-dimethylhydrazine (DMH, a tumor promoter) and the rats were fed with basal diet or diet containing XOS or FOS. Dietary supplementation with both oligosaccharides inhibited the development of precancerous colonic lesions and simultaneously lowered the cecal pH level through increased concentrations of short chain fatty acids (SCFA) and increased the *Bifidobacterium* population and cecal weight. The decreased ACF and increased cecal weight could be due to the normalization of epithelial cell proliferation via increased concentrations of SCFA from oligosaccharide fermentation by *Bifidobacterium*. In this study, XOS was more efficient in increasing the bifidobacteria numbers and relative colonic and cecal wall weight than FOS, leading the investigators to conclude that XOS could be more effective in increasing and controlling the epithelial cell proliferation through a trophic effect of produced SCFA. These findings are supported by other animal trials (Campbell et al., 1997; Howard et al., 1995; Younes et al., 1995), although Howard and co-workers found only moderate effects of XOS on cell density and cecal crypt depths in mice, and no effects on microbiota (Howard et al., 1995). Campbell et al. showed increased cecal and colonic weight in rats together with decreased pH, increased SCFA (especially lactate and acetate) and bifidobacteria as a result of XOS and FOS supplementation. In the same study, XOS supplementation resulted in more significant changes in measured parameters than FOS supplementation. Younes and co-workers found an increase in total cecal weight (wall and content weight) after rats consumed XOS and FOS. The two oligosaccharides reduced the pH in cecum significantly more than oat fiber or control diet, and increased cecal SCFA concentrations. The ratios of SCFA differed considerably between oligosaccharides with XOS producing more acetate and FOS butyrate. Also, fecal nitrogen

excretion increased and urinary nitrogen excretion decreased as a results of dietary oligosaccharide supplementations (Younes et al., 1995). Results from these animal trials indicate that fermentation of XOS could play a role in maintaining normal mucosal differentiation and, thus, the integrity of the colonic mucosa.

A study on diabetic rats found that when simple carbohydrates in the diet were replaced with XOS (10 g per day), the increased serum cholesterol and triglyceride levels seen in diabetes were reduced and liver triglycerides levels to a comparable level seen in healthy rats (Imaizumi et al., 1991). The researchers concluded that XOS could be applicable to foods as a sweetener replacing sucrose, which would benefit diabetic patients. However, these findings reported in animal trials have not been confirmed in humans yet.

In some human interventions, effects of XOS on intestinal microbiota and gastrointestinal function have been studied at the same time. Intervention in constipated pregnant women showed marked improvements in the defecation frequency and stool consistency during and after dietary supplementation with 4.2 g of XOS for 4 weeks. Before XOS administration the subjects recorded, in average, to defecate only once per week. During and after the intervention, defection frequency increased significantly to 6–7 times per week with concomitant improvement in subject's self-reported symptoms (Tateyama et al., 2005). Similar findings with adult women have been reported previously (Iino et al., 1997). Defecation frequency and abdominal symptoms improved simultaneously with increased bifidobacteria numbers, and persisted 2–4 weeks after XOS ingestion was completed. Kobayashi et al. found that administration of 2 g of XOS per day decreased the frequency of diarrhea in men (Kobayashi et al., 1991).

8.4 Safety and Regulatory Status

XOS were tested for mutagenicity, acute and subchronic toxicity. XOS were found to be non-mutagenic and showed no acute toxicity. Safety was also confirmed in a 90-day subchronic toxicity study in rats. These studies are mentioned in a Suntory product brochure and were conducted with Suntory's Xylo-oligo70 product (Biotec Suntory – Xylo-oligosaccharide brochure).

Limited data on digestive tolerability are available; however, volume of gas produced by human fecal bacteria during 24-h fermentation *in vitro* was similar for FOS and XOS (Rycroft et al., 2001) and no adverse effects have been reported in the human interventions carried out in healthy subjects (Okazaki et al., 1990a,b) including pregnant women (Tateyama et al., 2005) and elderly (Chung et al., 2007).

In Japan, XOS are approved as ingredients for Foods for Specified Health Uses (FOSHU), specifically for foods to modify gastrointestinal conditions at a recommended daily dose of 1–3 g (Japanese Ministry of Health, Labour and Welfare, www.mhlw.go.jp/english/topics/foodsafety/fhc/02.html). Use of XOS as food ingredient outside Japan and other Asian countries where XOS-containing products are currently marketed may require specific regulatory approval.

8.5 Market Information and Application

XOS were first used as food ingredient in the 1990s in Japan and since 1997, 32 product launches (including new formulations and varieties) have been recorded by Mintel's global new products database (Mintel, 2008). Use of XOS in dietary supplements accounted for 38% of all launches, use in dairy for 25%, use in sugar and gum confectionery for 16%, use in non-alcoholic beverages for 13% and use in baby food and soup for the remaining 8%. XOS is predominantly used in Asia (Japan, China, South Korea, Vietnam, Taiwan) with Japan accounting for more than half of the new product launches since 1997.

According to Suntory's product brochure (Biotec Suntory – Xylo-oligosaccharide brochure), XOS are acid- and heat-resistant. XOS remain intact after heating for 1 h at 100°C within a pH range from 2.5 to 8. Heat stability has also been confirmed at 120°C. Sweetness is claimed to be approximately 40% of sugar after comparable refinement and viscosity should allow easy use in various food applications (Biotec Suntory – Xylo-oligosaccharide brochure).

8.6 Summary

- XOS are resistant to digestion and are fermented by gastrointestinal microbiota.
- Fermentation of XOS by microbiota increases the concentrations of SCFA in colon, especially acetate, propionate and lactic acid. Gas volumes produced are moderate.
- Results from *in vitro* and *in vivo* studies are promising and suggest that XOS may selectively stimulate growth and/or activity of intestinal bacteria associated with health and well-being, particularly bifidobacteria.
- Additional controlled human intervention studies using molecular techniques to determine changes in fecal microbiota are needed to confirm the prebiotic potential of XOS and the efficacious dose, which has been suggested to be as low as 1 g/day.
- XOS can be considered an emerging prebiotic, as the scientific evidence is still not sufficient to classify XOS as an established prebiotic compound (Gibson et al., 2004).

List of Abbreviations

ACF	aberrant crypt foci
AcXOS	acetylated xylo-oligosaccharides
AXOS	arabinoxylo-oligosaccharides
DMH	1,2-dimethylhydrazine
dp	degree of polymerization
FOS	fructo-oligosaccharides
GlcA(me)XOS	xylo-oligosaccharides containing a 4-O-methylglucuronic acid group
GOS	galacto-oligosaccharides
IMO	isomalto-oligosaccharides
nXOS	non-substituted xylo-oligosaccharides
OD	optical density
SCFA	short chain fatty acids
SOS	soybean oligosaccharides
TOS	transgalacto-oligosaccharides
XOS	xylo-oligosaccharides

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9 Resistant Starch and Starch-Derived Oligosaccharides as Prebiotics

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Dietary fiber has long been recommended as part of a healthy diet based on the observations made by Burkitt and Trowell (1975). Since then, epidemiological evidence has consistently shown that populations consuming higher levels of foods containing fiber have decreased risk of a variety of chronic health disorders such as cardiovascular disease, type II diabetes, and certain cancers. Average fiber intake in the United States is approximately 13 g/day for women and 18 g/day for men (National Academy of Sciences, 2006). The FDA recommends a minimum of 20–35 g/day for a healthy adult depending on calorific intake. In many EU countries including France, Germany and the UK (see *Figure 9.1*), fiber intakes are much lower than authorities recommend for men and women (Buttriss and Stokes, 2008; Gray, 2006). Thus, there is a need to increase fiber consumption and many newly isolated or developed fibers can easily be added to beverages and processed foods. The reasons for such low compliance is somewhat complex, however the most basic rationale for not consuming fiber-rich foods is perceived bad taste and mouthfeel and the availability of conventional food items containing fiber.

Dietary fibers confer a wide range of health effects, from alleviation of constipation to reduction of cholesterol (Buttriss and Stokes, 2008). The physiological effects of dietary fibers in humans depend on the physico-chemical properties of fiber (viscosity, fermentability, bulking properties) and on the human gastro-intestinal (GI) tract (gut microbiotia, GI transit time). A specific subset of dietary fibers, so called prebiotics, convey health benefits by selectively stimulating the growth and/or activity of one or a limited number of bacteria like bifidobacteria and lactobacilli in the colon (Gibson and Roberfroid, 1995).

There is considerable industry and public interest in the capacity of foods and food components to promote health and lower risk of non-infectious

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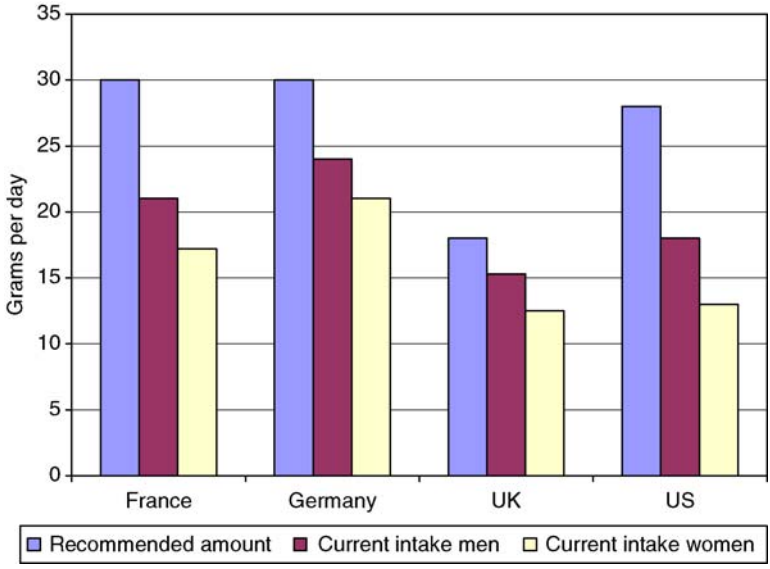


Figure 9.1
Daily intake of fibers.

diseases related to diet and lifestyle. Industry is thus challenged to develop fibers that can overcome the problems with stability under manufacturing conditions, functionality in various food systems, and taste in order to give the consumer more options when it comes to getting fiber in their diet. Newly derived starch fibers meet these requirements. In this chapter, prebiotic properties of newly derived fibers from maize and other starches will be reviewed with a specific focus on PROMITOR™ fibers, which were designed for optimal taste and texture and have prebiotic properties. The PROMITOR™ line includes a Soluble Gluco Fiber (PROMITOR™ Soluble Corn Fiber in US) and an insoluble Resistant Starch that are classified as food ingredients.

9.1 Introduction

The human gut microbiota constitutes a dynamic and ecologically diverse environment. The large intestine is by far the most heavily colonized region of the digestive tract, with up to 10^{12} bacteria for every gram of gut contents containing more than 400 different species of bacteria. The number of bacteria in the colon outnumber (10-fold) the number of human cells making it a very powerful target for nutritional interventions. Through the process of fermentation, colonic

bacteria are able to produce a wide range of compounds that have both positive and negative effects on the gut physiology as well as other systemic influences. For instance, certain microbial populations present in the gut provide an efficient barrier to invading pathogens (Macfarlane, 2008). Competition for nutrients and ecological niches, production of antimicrobial compounds, lowering of intestinal pH through production of short chain fatty acids and stimulation of the immune system play a role in limiting the ability of pathogens to colonize the gut and potentially cause disease. Many of these microbiota-associated activities have a direct impact on host health. While prebiotics are selectively interacting with the intestinal microbiota, they are being fermented by the bacteria into many different metabolites. As the composition of the microbiota is modified, the types of fermentation metabolites into which prebiotic substrates are converted are also modified. Some of these metabolites are utilized by the cells lining the intestine, while others are absorbed into the blood of the host and pass the blood barrier to enter the systemic body space, where they interact with many physiological processes in all vital organs and peripheral tissues of the host (Lenoir-Wijnkoop et al., 2007).

9.2 Resistant Starches

Resistant starches are defined as the sum of starch and products of starch degradation not absorbed in the small intestine of healthy individuals (Asp, 1997). There are four main groups of resistant starches: RS1, RS2, RS3 and RS4. RS1 is physically inaccessible starch (i.e., starch in whole grains), RS2 is granular starch i.e., starch in green bananas), RS3 is retrograded starch (i.e., starch in cooked and cooled potatoes) and RS4 is a chemically-modified starch (i.e., an esterified starch). PROMITOR™ Resistant Starch is classified as a type 3 resistant starch.

9.2.1 Introduction to a Type 3 Resistant Starch

9.2.1.1 Regulatory Status

Resistant starches occur naturally in many foods and thus have been safely consumed across the globe for years. PROMITOR™ Resistant Starch is a food ingredient in the US and EU and can be labeled as "Resistant Starch,"

"Starch," "Maize Starch" or "Corn starch." It is a non genetically-modified source of dietary fiber which enables, where relevant regulatory conditions are met; the use of fiber nutrient content claims (contains fiber, source of fiber, high fiber).

The European definition of dietary fiber published in Directive 2008/100/EC recognizes as dietary fiber, carbohydrate polymers with three or more monomeric units, which are neither digested nor absorbed in the human small intestine and belong to the following categories:

- edible carbohydrate polymers naturally occurring in the food as consumed
- edible carbohydrate polymers which have been obtained from food raw material by physical, enzymatic or chemical means and which have a beneficial physiological effect demonstrated by generally accepted scientific evidence
- edible synthetic carbohydrate polymers which have a beneficial physiological effect demonstrated by generally accepted scientific evidence

RS meets the European definition and can be incorporated into food products to meet the Regulation (EC) 1924/2006 that requires at least 3 g of fiber per 100 g or at least 1.5 g per 100 kcal for the nutrition claim "source of fiber"; and at least 6 g of fiber per 100 g or at least 3 g per 100 kcal for the nutrition claim "high in fiber." Levels necessary for nutrient content claims in the US are 2.5 g fiber/serving for a "good source" claim and 5.0 g fiber/ serving for an "excellent source" claim.

9.2.1.2 Dietary Fiber Content

PROMITOR™ Resistant Starch has an analysis of approximately 60–75% total dietary fiber per AOAC method 991.43. This AOAC method works well for fibers that are insoluble or nearly insoluble.

9.2.1.3 Calorific Value

RS has a calorific value of 1.70 kcal/g, as calculated using standard practices of subtracting the percent fiber content as analyzed by AOAC 991.43 from total carbohydrates. This is the calorific declaration used on specification sheets in the US, as specified by US regulations for insoluble fibers. EU calorific value will be 2 kcal/g as per Commission Directive 2008/100/EC.

The true metabolizable energy (TME) content was also determined in an *in vivo* avian model at the University of Illinois (Knapp et al., 2008). The model is a more precise model for calorific determination than *in vitro* models and collection of urine and feces is easier and often more precise than in humans. This method, using bomb calorimetry, determined the calorific value of RS to be in the range of 1.7–2.0 kcal. This suggests that either a small portion of the RS is digested, or that fermentation products generated in the colon adds a small fraction of calories. Fermentation is a more likely hypothesis based on human glycemic response data. The glycemic response for RS is approximately 10% that of a maltodextrin control (Kendall et al., 2008). Since maltodextrin and RS are both glucose polymers, a glycemic response of 10% suggests that the remaining 90% of RS remains undigested and enters the colon. Thus, it is likely the majority of the calories from RS may actually come from fermentation metabolites.

9.2.1.4 Digestive Tolerance

Type 3 RS was shown to be well tolerated up to doses as high as 45 g/d (Bouhnik et al., 2004). Stewart et al. (2008, 2009) conducted a study in which subjects (n = 20, 10 men, 10 women) consumed 12 g fiber/d for 14 consecutive days. Subjects were asked to report gastrointestinal symptoms (cramping, bloating, stomach noise, flatulence) on a 10 point scale (1 = minimal symptoms, 10 = severe symptoms). RS was shown to be well tolerated (Stewart et al., 2009). In a dose-response study, tolerance was assessed in 22 healthy volunteers consuming three different doses of RS (5 g, 15 g, 25 g) in acute conditions after an overnight fast (Kendall et al., 2009). Subjects were asked to report gastrointestinal symptoms (belching, bloating, flatulence, nausea and diarrhoea) on a 100 mm VAS scale at 0, 15, 30, 45, 60, 90 and 120 min after consumption. RS was shown to be well tolerated in these conditions.

9.2.1.5 Applications

RS acts as an insoluble fiber that can be added to baked products, cereal products or snacks, providing them with higher fiber levels as well as other health benefits. It is one of the most stable resistant starches on the market (unpublished data) and so can be used in extruded or sheared and baked products with less loss of fiber during processing. As a result the final product has a high fiber content while

offering a range of health benefits. With only 1.70 kcal/g, it can also be used in low calorie products reducing both calories and carbohydrates when replacing flour or other cereal-based ingredients.

Applications for RS include puffed or sheeted snacks, chips, extruded breakfast cereals, pasta, muffins, cookies and biscuits, crackers, frozen dough, breads.

It can be used as a partial replacement for flour in bakery products that exhibit characteristics comparable to those achieved using conventional wheat flour (e.g., cookie spread, golden brown color, pleasant aroma, surface cracking). Thanks to its low water holding property, it also does not affect height and spread management of biscuits, cookies or other baked goods.

RS enhances crispiness of cookies and crackers as well as the surface of baked sheeted crackers and extruded products. Furthermore, the induced reduction of water activity and moisture content, enhance sensory characteristics as well as the shelf life of goods. Notably, it tends to decrease bulk density, improving expansion in extruded cereals and snacks. In fried snacks, fat uptake may be reduced by up to 25% when RS is used, helping to meet "high/rich in fiber" claims.

Moreover, with a thermal stability as high as 150°C, it will retain more fiber content and structure than other resistant starches, which start to break down below 120°C.

9.2.2 Prebiotic Properties of Various Resistant Starch Products (RS2 and RS3)

9.2.2.1 Effect on Microbiota Modulation

The ability of RS to favor growth of bifidobacteria and lactobacilli within the gut flora has been assessed *in vitro*, in animal models and in humans.

In a study Le Blay et al. (2003) showed the prebiotic properties of RS2. Eighteen rats were fed a low-fiber diet (Basal) or the same diet containing raw potato starch (RS2) (9%) or short-chain FOS (9%) for 14 days. Changes in wet-content weights, bacterial populations and metabolites were investigated in the caecum, proximal, distal colon and feces. Both substrates exerted a prebiotic effect compared with the Basal diet. All bacteriological analysis were performed within 2 h after sampling. Samples were diluted and dilution were applied on plates using both unselective and selective media. After incubation, single colonies were counted. FOS increased lactic acid-producing bacteria throughout the caecocolon and in feces, whereas the effect of RS2 was limited to the caecum and proximal

colon. As compared with RS2, FOS doubled the pool of caecal fermentation products, while the situation was just the opposite distally. This difference was mainly because of the anatomical distribution of lactate, which accumulated in the caecum with FOS and in the distal colon with RS2. Feces reflected these impacts only partly, showing the prebiotic effect of FOS and the metabolite increase induced by RS2. In conclusion, this study demonstrates that FOS and RS2 exert complementary effects and combined ingestion could be beneficial by providing health-promoting effects throughout the colon.

Brown et al. (1997) also observed prebiotic effects in 12 young male pigs fed with high amylose cornstarch diet (RS2). Starch provided 50% of total daily energy either as a low amylose cornstarch or as a high amylose (amylomaize) cornstarch. Fecal output, fecal concentrations and excretion of total SCFA (notably propionate and butyrate), fecal culture-based bifidobacteria counts (expressed per gram of wet feces) and their daily fecal excretion were higher when pigs were fed the high amylose cornstarch.

Similar prebiotic effects have been reported for retrograded RS (RS3) in several animal models. Dongowski et al. (2005) and Jacobasch et al. (2006) have shown in rats fed with RS3 that the growth of bifidobacteria and the production of SCFA were increased throughout the digestive tract, favoring thus a decrease of the pH in the caecum, colon and feces.

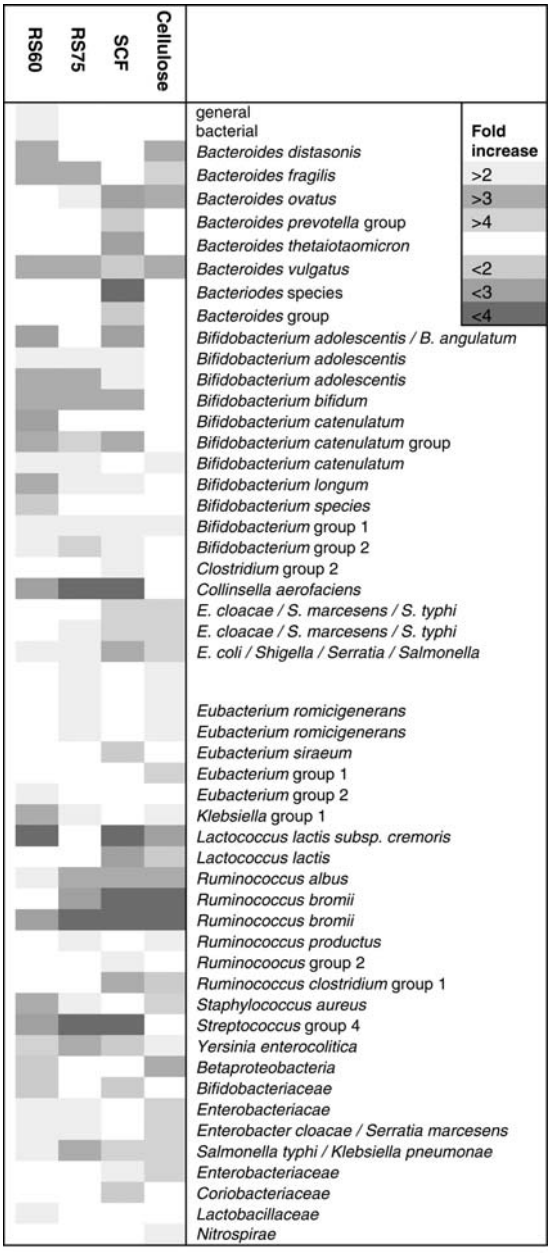
Using another model of human flora-inoculated gnotobiotic rats (HFA), colonized with microbiotas from UK or Italian donors, Silvi et al. (1999) looked at the prebiotic effects of a RS3. Consumption of this RS3 (15 g/100 g diet) resulted in significant changes in both the UK and Italian flora-associated rats: numbers of lactobacilli and bifidobacteria were increased 10–100-fold, and there was a concomitant decrease in enterobacteria when compared with sucrose-fed rats (control). The induced changes in caecal microbiota of both HFA rat groups were reflected in changes in bacterial enzyme activities and caecal ammonia concentration. This RS3 markedly increased the proportion of n-butyric acid in both rat groups, lowered caecal ammonia concentration, caecal pH and beta-glucuronidase activity.

The prebiotic properties of RS3 have also been demonstrated in humans (Bouhnik et al., 2004). First, this study determined the bifidogenic properties of a RS3 at 10 g/d; Second, the dose-response relationship of the bifidogenic effects of this RS3 at doses ranging from 2.5 to 10 g/d in comparison with a placebo were assessed. Faecal samples were diluted and dilutions were applied on plate using different selective media. RS3 was shown to be bifidogenic at a dose of 10 g/d. However, bacteria counts increased at doses of 5 and 7.5 g/d, but

decreased at doses of 2.5 and 10 g/d. Thus, no firm conclusions could be drawn from this study on bifidogenic properties of RS3 in humans, however the authors observed that only a low baseline bifidobacteria count was significantly associated with a bifidogenic response to treatment. This observation was recently corroborated by Roberfroid who highlighted that the baseline level of bifidobacteria together with the time of exposure to a prebiotic are more determinant factors than the amount of prebiotics consumed to assess the potential prebiotic properties of a fiber (Roberfroid, 2007). The lack of bifidogenic effect of RS3 in this study at 2.5 and 10 g/d in phase 2 might be primarily linked to an elevated baseline bifidobacteria count in the groups of volunteers.

In this respect, a recent *in vitro* study also showed that the RS3 crystalline polymorphism can impact the RS fermentability by human gut microbiota as well as the short chain fatty acids production. Human fecal pH-controlled batch cultures showed that RS induces an ecological shift in the colonic microbiota, with polymorph B being much more efficient in inducing *Bifidobacterium* spp. growth than polymorphism A. Interestingly, polymorph B also induced higher butyrate production to levels of 0.79 mM (Lesmes et al., 2008). Type-A crystalline polymorph is found in typical cereal starch granules while the type-B polymorph is found in potato and high amylose cereal starch granules. The A polymorph has a much lower water content in the crystal lattice compared to the B polymorph.

PROMITOR™ Resistant Starch has shown to be a potential prebiotic in *in vitro* studies at TNO (Maathuis et al., 2008). The aim of the study was to investigate the effect of newly developed maize-based fibers on the activity and composition of the microbiota in the colon. The tested fibers were glucose-based and have variable structures including two resistant starch preparations. The fibers were pre-digested, mono- and di-saccharides were removed, and the residual polymer was used to assess the production of microbial metabolites and changes in composition of the microbiota using a dynamic, validated, *in vitro* model of the large intestine (TIM-2). Microbial metabolite analysis showed an increase in health-promoting metabolites (short-chain fatty acids) and a reduction in toxic metabolites from protein fermentation compared to the poorly-fermentable control (cellulose). The lactate production was also relatively low, indicating that it is slowly fermented. This may contribute to its excellent tolerance and extend its health benefits throughout the entire large intestine. Using microarray technology to compare multiple species and groups of colonic microbiota, RS was found to promote the growth of beneficial bacteria such as bifidobacteria and lactobacilli (*Figure 9.2*). Further studies are underway to determine if these *in vitro* effects are also seen *in vivo*.



SGF: Soluble Gluco Fibre/Soluble Corn Fibre; RS 60: Resistant Starch 60%; RS 75: Resistant starch 75%

Figure 9.2

Use of the microarray technology to evaluate the effects of PROMITOR™ fibers on multiple species and groups of colonic microbito.

Based on the above-mentioned studies, it can be reasonably concluded that RS2 and RS3 have prebiotic activity. The mechanisms involved in promotion of bifidobacteria/lactobacilli growth may be different than those observed for the other prebiotics (e.g., FOS or inulin). A study with FOS and high amylose starch (RS2) in pigs fed a diet based on human foods showed that both raised fecal bifidobacteria numbers by approximately equal amounts when fed separately. When fed together there was an increase that exceeded the individual increases, suggesting that they operate by different mechanisms (Brown et al., 1997). If FOS acts as a metabolic substrate for bifidobacteria and lactobacilli, RS2 seems to function differently. Indeed, *in vitro* studies showed on one hand that pure bifidobacteria strains have limited capacity to use RS2 as a substrate (Topping et al., 2003); on the other hand, they also showed physical adhesion of several bifidobacteria species to RS2 (Topping et al., 2003), suggesting thus that the prebiotic properties of RS2 may be linked to its ability to confer physical protection on the bifidobacteria/lactobacilli throughout the upper digestive tract. The same reasoning may apply to RS3, as Brouns et al. (2007) have shown that breast-fed babies' microbiota, mainly composed of bifidobacteria was unable to use RS3 as a substrate, though several animal and human studies have shown prebiotic effects of RS3.

Nevertheless, it seems that a limited number of bifidobacteria strains has the ability to use RS2 as a substrate as shown in the study of Crittenden et al. (2001). In this study, 40 *Bifidobacterium* strains were examined to complement RS in a synbiotic yogurt. Only *B. lactis* Lafti B94 possessed all of the required characteristics. This isolate was the only one able to hydrolyze Hi-maize (RS2), to survive well in conditions simulating passage through the gastrointestinal tract and to possess technological properties suitable for yogurt manufacture. *Bifidobacterium lactis* Lafti B94 survived without substantial loss of viability in synbiotic yogurt containing Hi-maize during storage at 4°C for 6 weeks. In this study, RS2 was seen as a good complementary prebiotic ingredient for new synbiotic functional food products.

9.2.2.2 Health Benefits Associated with Prebiotic Properties of Resistant Starch

Several health benefits have been linked to prebiotics, notably to FOS, GOS and inulin. One of these benefits is enhancement of the body's natural immune defenses (Schley and Field, 2002; Vos et al., 2007). This effect is primarily

localized to immune defenses in the gut, such as the gut-associated lymphoid tissue, however some studies have shown systemic effects. The precise mechanisms by which prebiotics exert their immune effects are unclear; whether it is through changes in the microbial population, through fermentation metabolites (i.e., SCFA) or through direct interaction of the prebiotics with mucosal membrane receptors. There have been very few investigations into the immune-modulating effects of resistant starch. Preliminary data from a recent study including PROMITOR™ Resistant Starch demonstrates a potential immune-modulating response in an animal model of inflammatory bowel disease. Animals supplemented with Resistant Starch had fewer macroscopic lesions in the gut and a reduction in the size of mesenteric lymph nodes (unpublished data) as compared to animals without Resistant Starch in the diet.

Another benefit seen with fermentable and prebiotic dietary fibers is enhancement of mineral absorption and/or increase in bone mineralization and bone density. There are numerous hypothesized mechanisms by which fermentable fibers may alter mineral absorption and impact bone density, a few of which include increased solubility of minerals, increased gastrointestinal surface area for absorption (by means of SCFA production), and alteration of the microbial population (Scholz-Ahrens et al., 2007). Many of the studies surrounding mineral absorption and bone formation have included prebiotics, such as inulin and FOS. However, RS has also been reported to enhance the ileal absorption of a number of minerals in rats and humans. Lopez et al. (2001) and Younes et al. (2001) reported an increased absorption of calcium, magnesium, zinc, iron, and copper in rats fed RS2-rich diets. Similar preliminary results have been seen in an animal study investigating PROMITOR™ Resistant Starch where it was shown to increase femur calcium concentration after 12 weeks of supplementation (Martin et al., 2009).

9.2.2.3 Gut Health Biomarkers

It has been shown that RS has health-promoting actions on the colonic microbiota beyond the prebiotic effect. For instance, studies in children with cholera-induced diarrhoea having consumed RS (high-amylose starch) plus the rehydration therapy have shown major reduction in fluid loss and a halving of time to recovery (Ramakrishna et al., 2000, 2008). This study has been replicated in babies with other forms of infectious diarrhoea where it was shown that both RS (as green bananas) and non-starch polysaccharides (NSP) facilitated recovery and improved intestinal permeability (Rabbani et al., 2001). Accelerated recovery

from infectious diarrhoea has also been shown in animals. A specific microorganism, *Brachyspira hyodysenteriae*, causes substantial economic losses in the commercial pig breeding industry through morbidity and mortality in the weaning period. The effect is expressed as diarrhoeal disease on the introduction of solid food. Feeding with cooked rice, an established source of RS (Marsono et al., 1993), lowers the incidence and severity of disease with a consequent reduction in mortality (Hampson et al., 2000). Part of the benefit seems to be due to increased fluid absorption through greater SCFA production, as these acids stimulate the uptake of water and cations (Na^+, K^+, and Ca^{2+}), particularly in the proximal colon. Several studies in humans (*Table 9.1*) as well as in animals (*Table 9.2*) have shown the ability of RS to enhance production of SCFA in the ceacum and throughout the colon. This outcome is an obvious mechanism for reversing diarrhoea-induced fluid loss. SCFA also appear to modulate the muscular activity of the large bowel and to promote the flow of blood through the viscera; both these actions could assist in lowering the severity of diarrhoea. In addition to these well-documented effects, it is possible that RS could limit the viability of the cholera organism in the gut. It may be hypothesized that the bacteria could adhere to the starch granules, very much in the same way as bifidobacteria, and thus be removed from the site of action (Topping et al., 2003). The production of SCFA (Cummings et al., 1996; Heijnen et al., 1998; Jenkins et al., 1998; Muir et al., 2004), the reduction of colonic pH (Birkett et al., 1996; Heijnen et al., 1998; Phillips et al., 1995) together with a beneficial change in microbiota metabolism pattern induced by RS intake (*Tables 9.1* and *9.2*) are further means of biocontrol for pathogens and potential pathogens.

Diet and its interaction with the gut microbiota, and reduced protection from the microbiota with age are likely contributory factors to colorectal cancer (CRC). Genotoxic or carcinogenic metabolites produced or activated by the gut microbiota provide a diversity of environmental insults, which play a role in the initial stages of cancer (Tuohy et al., 2005). There is considerable interest in using microbiota-modulating tools such as prebiotics (Burns and Rowland, 2000) or RS (Cassidy et al., 1994) to protect against colonic tumor development or to maintain a good colonic physiology.

To date, several human studies have examined the effect of RS on human colonic function (*Table 9.1*). These intervention studies have evaluated different types of RS, in combinations or alone, at different amounts. Some studies have mimicked human diets in that they have used a range of food-based sources, whereas others have used single manufactured forms.

Table 9.1

Human studies investigating the effects of RS intake on gut health biomarkers (*Cont'd* p. 272)

Sample size and study length	Intervention	Parameters measured / outcome	Authors
14 healthy subjects - 4 weeks	- 45g Hylon VII (62% RS) to a standard diet - standard diet low in RS	Cytotoxicity of fecal water on colon cell line: – Cell proliferation in rectal biopsies: – Fecal output: ++ Fecal SCFA: ++ Fecal bile acids: ++ Fecal secondary bile acids: –	Van Munster et al, 1994
11 healthy subjects in a randomized controlled cross-over study - 3 weeks	- High RS diet (raw banana flour + unprocessed wheat seeds + high-amylose maize cornbread, 26 to 50g/d) - Low RS diet (cooked banana flour + processed wheat seeds + low-amylose maize cornbread, 3 to 8g/d)	Butyrate: ++ Faecal pH: – Faecal excretion of NSP: ++ Feacal ammonia: – Fecal output: ++	Phillips et al, 1995
11 subjects in a randomized controlled cross-over study - 3 weeks	- High RS diet (≈40g/d, RS1, RS2, RS3 mix) - Low RS diet (5g/d)	Faecal nitrogen excretion: ++ Feacal ammonia: – Faecal phenols: – Faecal pH: –	Birkett et al, 1996
12 healthy subjects in a controlled cross-over study - 15d periods	- 17-30g RS2 diets - potato and banana starch - 17-30g RS3 diets - retrograded maize and wheat starch - RS-free diet	Faecal output: ++ Faecal SCFA: ++ Faecal excretion of NSP: ++ Fecal nitrogen excretion: ++ Transit time: –	Cummings et al , 1996

Table 9.1

Sample size and study length	Intervention	Parameters measured / outcome	Authors
23 hypertriglyceridemic subjects in a randomized controlled cross-over study - 4 weeks	- High amylose maize starch diet (RS:17-25g/d) - Oat bran: low-RS diet	Faecal pH: – Faecal SCFA: ++, butyrate Secondary faecal bile acids excretion: –	Heijnen et al, 1998
12 subjects in a controlled, cross-over study - 4 weeks	- Controlled basal diet enriched in RS (amylomaize starch, 55g/d RS) - Controlled basal RS-free diet	Faecal output: ++ Faecal SCFA: no effect β-glucuronidase: – Faecal bile acids excretion: – Faecal secondary bile acid: – Feacal concentration of neutral sterols: –	Hylla et al, 1998
24 healthy subjects in a controlled, randomized, cross-over study - 2 weeks	- RS2 (≈20g/d) - RS3 (≈25g/d) - Low fibre diet	Feacal output : ++ Butyrate: ++	Jenkins et al, 1998
57 babies (5-12 months) with persistent diarrhoea	- Rice-based diet containing green banana (250g/L diet) - Rice-based diet	Intestinal permeability: ++ Diarrhoea: –	Rabbani et al, 2001
23 patients with recently removed colonic adenomas in a randomized, controlled, parallel study - 4 weeks	- 45g amylomaize (28g/d RS2) in capsules - 45g maltodextrin in capsules	Cell proliferation: no effect Feacal output: no effect Faecal pH: no effect Feacal SCFA: no effect Faecal bile acids excretion: – Faecal secondary bile acids: –	Grubben et al, 2001

12 healthy volunteers in a controlled, cross-over study - 4 weeks	- High RS diet: Hylon VII in foods (RS: ≈ 55g/d) - Low RS (cornstarch, RS:2.8-3.7g/d)	Cell proliferation: no effect DNA adducts: ++	Wacker et al, 2002
20 volunteers with a family history of colorectal cancer in a randomized, cross-over study - 3 weeks	- Basal diet + RS (22g/d) + wheat bran (12g/d) - Basal diet + Wheat bran (12g/d) - Control diet	Feacal output: ++ Transit rate: ++ Faecal pH: –, only for RS Faecal SCFA: ++, only for RS Feacal phenols and ammonia: ++	Muir et al, 2004
8 healthy volunteers per fibre tested	- Phase 1: RS3 (10g/d) - Phase 2: RS3 (2.5, 5, 7.5 and 10g/d)	Prebiotic effects: ++ in phase 1 Prebiotic effects: ++ at 5 and 7.5g/d only in phase 2	Bouhnik et al, 2006
12 healthy volunteers with 3-14 bowel movements/ week, in a cross-over, randomized, double-blind, controlled study	- 25g/d Promitor™ Resistant Starch - Low fiber diet (<2g/d)	Fecal output: ++ Tolerance: good	Maki et al, 2009 (submitted)

++: increased or improved, –: decreased

Table 9.2

Animal studies investigating the effects of RS intake on luminal and epithelial colorectal cancer biomarkers (*Cont'd* p. 276)

Animal model	Intervention	Parameters measured	Author
Wistar rats (azoxymethane)	Dietary carbohydrate replaced with sucrose, cornstarch or RS (RS_2, 67g/100g)	ACF: –	Thorup et al, 1995
Sprague Dawley rats (Dimethylhydrazine)	3 or 10g/100g cellulose RS_3 (hydrolyzed high amylose maize starch)	Faecal output: ++ SCFA and butyrate production: ++ Tumor incidence: no effect Tumor size and multiplicity: no effect	Sakamoto et al, 1996
Sprague Dawley rats (dimethylhydrazine)	- Low RS, low fibre diet - 14.4g/100g diet RS (RS_2)	ACF: ++ Cell proliferation: ++ Faecal output: ++ Tumor size and multiplicity: ++	Young et al, 1996
C57BL/6 min mice	- RS-free diet (2% cellulose, no RS) - RS_3 (high amylose cornstarch, 18g/100g)	Gut-associated lymphoid tissue: no effect Tumor incidence: no effect	Pierre et al, 1997
Cannulated piglets	- RS-free diet - RS_2 (uncooked high amylose cornstarch) diet, 17% - RS_3 (retrograded high amylose cornstrarch) diet, 17%	Fecal nitrogen excretion: –, for RS_3 only Urine nitrogen excretion: –, for RS_3 only	Heijnen et al, 1997

Sprague Dawley rats (dimethylhydrazine)	- Retrograded high-amylose cornstarch (RS_3), 25% - RS_3, 25% + vitamin A diet Control diet	ACF: – Faecal output: ++ Faecal pH: – Butyrate: ++	Cassand et al, 1997
Sprague Dawley rats (dimethylhydrazine)	- RS-free diet (2% cellulose) - 25g/100g RS_3 (retrograded amylose maize starch)	ACF: no effect Caecal pH: – Faecal weight and output: ++ β-glucuronidase: –	Mazière et al, 1998
Human-flora associated Fisher rats	- Retrograded amylose (15g/100g diet) - RS free diet	Ammonia: – β-glucuronidase: – Caecal SCFA: ++ Cell proliferation: ++ Prebiotic effect: ++	Silvi et al, 1999
Min mice	RS free diet 1:1 potato RS and high-amylose maize diet	Tumor incidence: ++	Williamson et al, 1999
BDIX rats	- Low-fiber control diet - RS_3 (30%)	ACF: – Caecal butyrate: ++ Mucosal cell proliferation: no effect	Perrin et al, 2001
Wistar rats (IQ)	- RS free diet - Potato starch and high amylose maize starch (35%)	Excretion of the food carcinogen, Faecal output: ++ IQ: ++ Transit : ++	Ferguson et al, 2003

Table 9.2

Animal model	Intervention	Parameters measured	Author
Sprague Dawley rats	15g/100g casein with or without ≈50% Hi-Maize or 25g/100g casein with or without ≈50% Hi-Maize	Caecal SCFA: ++ DNA damage: – p-cresol: – Thinning of mucosal layer: –	Toden et al, 2005
Sprague Dawley rats (azoxymethane)	Low RS diet + 1% lyophilized *Lactobacillus acidophilus* and *Bifidobacterium lactis* - 10% RS diet (Hi-Maize) +1% lyophilized probiotics	Acute apoptotic response: ++ Cecal pH: – Cell proliferation: ++ Crypt column height: ++ Fecal and caecal SCFA: ++ Prebiotic effect : ++	Le Leu et al, 2005
Rats	- RS-free diet - Retrograded potato debranched maltodextrin (RSA(RS3), 5.7g/100g - RSB(RS3), 5.7g/100g)	Bile acids fecal excretion: ++ Caecal pH: – Caecal SCFA: ++ Fecal output: ++, for RSA	Dongowski et al, 2005
Sprague Dawley rats (DMH)	- RS-free diet - Heat-treated Novelose 330 (RS3, 10%)	Apoptosis: ++ Epithelial proliferation: – Tumor incidence: – Mucin properties: ++ for acidic mucin	Bauer-Marinovic et al, 2006
Wistar rats	- Heat-treated Novelose 330 - Normal Novelose 330	Caecal and colonic pH: – Crypt length: ++ Formation of secondary bile acids: – Fecal bile acids: ++ SCFA, butyrate: ++ Prebiotic effect: ++	Jacobash et al, 2006

++: increased or improved, –: decreased

In most of the studies, RS intake (17 g/d up to 50 g/d) was shown to increase fecal output, to increase fecal SCFA and to decrease fecal pH (Birkett et al., 1996; Cummings et al., 1996; Heijnen et al., 1998; Hylla et al., 1998; Jenkins et al., 1998; Maki et al., 2009; Muir et al., 2004; Phillips et al., 1995; van Munster et al., 1994).

The effect of RS consumption on fecal output is dose-dependent and increasing doses are associated with increasing recoverable starch in stools (Phillips et al., 1995). An increased fecal output is generally seen as being beneficial to gut health as it helps dilute the carcinogenic compounds and reduce thus their time contact with the epithelium.

The reduction of the fecal pH reflects an acidification of the colonic lumen due to the production of SCFA, notably butyrate linked to the fermentation of RS by the microbiota. The fact that RS favours butyrate production has been largely demonstrated *in vitro* (Brouns et al., 2007; Fassler et al., 2006, 2007) and in animal studies (Cassand et al., 1997; Dongowski et al., 2005; Jacobasch et al., 2006; Le Leu et al., 2005; Perrin et al., 2001; Sakamoto et al., 1996; Silvi et al., 1999; Toden et al., 2005). Butyrate plays a critical role in mucosal physiology and metabolism, by providing 50% of the daily energy requirements of the colonic mucosa, while also being implicated in cellular differentiation and proliferation. It has been seen *in vitro* that butyrate could be involved in cancer prevention through hyperacetylation of histone proteins in the cell nucleus (Tran et al., 1998) and selectively induce apoptosis in colon cancer cells but not in healthy colonocytes (Ruemmele et al., 2003).

The fermentation of RS induces changes in the microbiota metabolism pattern reducing either the numbers and/or activities of putrefactive bacteria in the colon, leading to a reduction in enzyme activities involved in carcinogenic pathways (e.g., β-glucuronidase) as well as toxic metabolites such as amines, indoles, p-cresol (Birkett et al., 1996; Heijnen and Beynen, 1997; Hylla et al., 1998). The use of protein fermentation products as a source of nitrogen for the microbiota to grow favours a reduction of fecal ammonia and phenols (Birkett et al., 1996; Muir et al., 2004). As protein fermentation products seem likely to be carcinogenic, this might be an additional mechanism by which RS might be protective. A low colonic pH is also associated with a decreased conversion rate of primary to secondary bile acids, which are thought together with other toxic metabolites like ammonia, phenol, amines, etc, to impact fecal water cytoxicity. In this respect, some human studies have shown that RS intake, due to its fermentation in the colon, can help reduce the production of secondary

bile acids (Grubben et al., 2001; Hylla et al., 1998; van Munster et al., 1994). Seeing the fact that RS intake helps in reducing the amount of several toxic compounds in the colon lumen via several mechanisms, a reduction of the fecal water cytoxicity is expected and this has been observed in a human study (van Munster et al., 1994) as well as in *in vitro* studies (Fassler et al., 2007).

One study in humans did consistently not show any effect (Grubben et al., 2001). That study was conducted in subjects with recently removed colonic adenomas, and the RS was administered in capsule form. They also failed to observe any changes in fecal fermentation, which suggests that the capsules did not effectively release the RS.

Generally speaking, similar effects as in humans have been observed in animals for luminal biomarkers of gut health. That is to say that in all animal studies, RS intake has favored butyrate production, increased fecal output, decreased pH, enzyme activity, ammonia, p-cresol and other toxic metabolites (Dongowski et al., 2005; Ferguson et al., 2003; Jacobasch et al., 2006; Maziere et al., 1998; Perrin et al., 2001; Sakamoto et al., 1996; Silvi et al., 1999; Toden et al., 2005; Young et al., 1996).

In rats, challenged with 1,2-dimethylhydrazine or azoxymethane or again in genetically modified mice models (min mice), the effects of RS intake on colorectal neoplasia and on epithelial markers of colorectal cancer have also been investigated (*Table 9.2*). However, depending on the type of animal model, on the nature and the amount of carcinogenic compounds used to induce colorectal cancer in rats, on the feeding time period, the effects of RS intake on aberrant crypt foci (ACF), cell proliferation, DNA damages, tumor incidence are highly variable. It is therefore very difficult to draw any conclusions on RS intake on markers of colorectal-cancers in animal studies. However, whether these particular animal models are relevant to investigate nutritional benefits of RS on gut health is a question mark as they induce drastic effects that do not reflect what occurs in physiological conditions and may wipe out any potential bioactive-associated preventive effects.

Toden et al. (2005) in this respect used a more appropriate rat model, which consisted in feeding rats with a western type diet enriched in proteins. They demonstrated that RS fermentation in the colon is beneficial to health in the sense that it helps counteract the deleterious effect induced by a high protein intake. The high protein diet resulted in a twofold increase in damage to colonocyte DNA compared to a low-protein diet. This was associated with thinning of the colonic mucosa barrier and increased level of p-cresol. The addition of

RS (high amylose starch) to the diet increased SCFA and attenuated DNA damages, suggesting protection against genotoxic agent and lesser genotoxocity of the fecal water.

These observations have been corroborated by a recent study that investigated the effects of *in vitro* fermentation products of *in-vitro*-digested or *in-vivo*-digested RS2 and RS3 on Caco-2 cells (Fassler et al., 2007). Compared to control, the cytotoxicity, anti-genotoxicity against hydrogen peroxide (comet assay) and the effect on barrier function measured by trans-epithelial electrical resistance of fermented samples of RS were determined. Batch fermentation of RS resulted in an anti-genotoxic activity ranging from 9–30% decrease in DNA damage for all the samples. Additionally, *in vitro* batch fermentation of RS caused an improvement in integrity across the intestinal barrier by approximately 22% for all the samples.

9.3 Other Starch-Derived Fibers with Potential Prebiotic Effects

Many new starch-derived prebiotic candidates are now available (e.g., Nutriose®, Fibersol-2® and PROMITOR™ Soluble Gluco Fiber).

Made from starch, Nutriose® can be described as a resistant dextrin. During the process of dextrinisation, the starch undergoes a degree of hydrolysis followed by repolymerization. It is this repolymerization step that makes starch become indigestible, due to many α 1,6, α 1,2 and α 1,3 linkages. According to the AOAC method 2001–2003, Nutriose® contains 85% fiber (Lefranc-Millot, 2008). The calorific value of Nutriose® has been reported to be 1.7 kcal/g (Lefranc-Millot, 2008) and is claimed to be consistent with clinical determination in healthy young men (Vermorel et al., 2004) and to be in agreement with the consensual calorific value of soluble dietary fibers (Livesey, 1992). Only 15% is enzymatically digested in the small intestine, while 75% reaches the colon where it is slowly fermented and 10% is excreted in fecal matters (van den Heuvel et al., 2005).

Fibersol-2® is a spray-dried powder produced by a pyrolysis and a controlled enzymatic hydrolysis of cornstarch. It was estimated that most of Fibersol-2 escapes digestion in the upper gastrointestinal tract and that 90% reaches the colon, where half of this fraction is fermented by the microbiota (Flickinger et al., 2000).

9.3.1 Introduction to PROMITOR™ Soluble Gluco Fiber (SGF)

9.3.1.1 Regulatory Status

PROMITOR™ Soluble Gluco Fiber is a regular food ingredient in the EU and can be labeled as "Soluble Gluco Fiber," "Glucose Syrup" or "Dried Glucose Syrup." The name "Soluble Gluco Fiber" is consistent with EU Directive 2000/13/EU as amended on labeling, presentation and advertising of foodstuffs as it indicates both the precise nature of the food in that it is a glucose type of food and distinguishes the fiber content of the glucose syrup. SGF is a non-genetically modified source of dietary fiber, which enables the use of fiber nutrient content claims (contains fiber, source of fiber, high fiber) where relevant regulatory conditions are met.

In the US, PROMITOR™ Soluble Corn Fiber (SGF in EU) is GRAS and can be labeled as "Soluble Corn Fiber," "Corn Syrup," or "Corn Syrup Solids."

9.3.1.2 Dietary Fiber Content

SGF is obtained from a partially hydrolyzed starch-made glucose syrup, using an existing production process that yields approximately 70–85% fiber with exceptional color, clarity and flavor (*Figure 9.3*).

SGF has a typical analysis of approximately 72% total dietary fiber per AOAC method 2001.03. Highly water soluble fibers such as SGF and some resistant maltodextrins contain digestion-resistant material that is not precipitated by the addition of ethanol as prescribed in the 991.43 method.

In three different human trials SGF has demonstrated a consistent glycemic response, approximately 30% that of rapidly digestible carbohydrates (i.e., glucose, maltodextrin) (Kendall et al., 2009). This correlates well to the amount of digestible carbohydrate (~70% fiber, ~30% digestible carbohydrate) based on the AOAC 2001.03 method.

9.3.1.3 Calorific Value

SGF, has a calorific content of 2 kcal/g (Fastinger et al., 2007). True metabolizable energy content, was determined in an *in vivo* avian model which utilizes bomb calorimetry of the food prior to consumption by the animal and

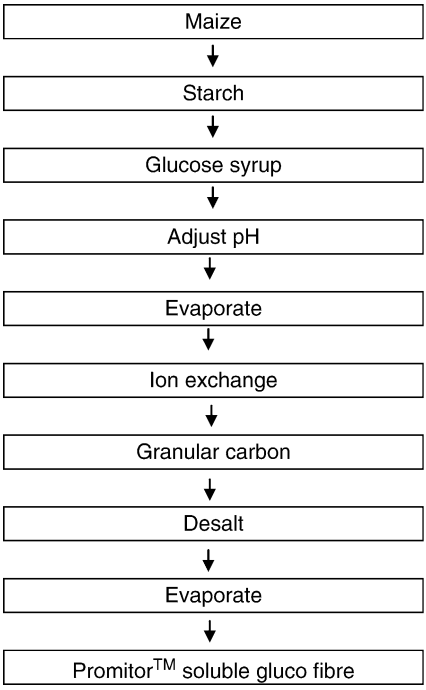


Figure 9.3
Production process of PROMITOR™ Soluble Gluco Fiber.

subsequent bomb calorimetry of the collected waste (urine and feces) after consumption. Additionally, glycemic response studies in humans support this calorific value (Kendall et al., 2007, 2008). Using the calculation (4 kcal/g × 30% digestible), SGF would have 1.2 kcal/g. However, energy yield from fermentation cannot be estimated by the blood glucose response and likely yields a small amount of additional kcals (<1 kcal). *In vitro* analysis has also shown it to be approximately 25% digestible, 50% fermented and 25% unchanged. Using a fermentation value of 2 kcal/g, as suggested by (Oku and Nakamura, 2002), the following calculations yield a calorific value for Soluble Gluco Fiber of 2 kcal/g.

$$\begin{aligned}\text{Soluble Gluco Fiber kcal/g} &= (4\ \text{kcal/g}) \times (25\%\ \text{digestible}) \\ &\quad + (2\ \text{kcal/g}) \times (50\%\ \text{fermentable}) \\ &\quad + (0\ \text{kcal/g}) \times (25\%\ \text{unchanged}) \\ &= 2\ \text{kcal/g}\end{aligned}$$

9.3.1.4 Digestive Tolerance

The gastrointestinal tolerance has been assessed in human trials and was shown to be well tolerated up to doses as high as 25 g/d. Stewart et al conducted a study in which subjects (n = 20, 10 men, 10 women) consumed 12 g fiber/d SGF for 14 consecutive days (Stewart et al., 2008, 2009). Subjects were asked to report gastrointestinal symptoms (cramping, bloating, stomach noise, flatulence) on a 10 point scale (1 = minimal symptoms, 10 = severe symptoms). SGF was shown to be well tolerated (Kendall et al., 2009; Sanders et al., 2008). In an acute dose-response study (Kendall et al., 2009), tolerance was assessed in 22 healthy volunteers consuming three different doses (5 g, 15 g, 25 g) in acute conditions after an overnight fast. Subjects were asked to report gastrointestinal symptoms (belching, bloating, flatulence, nausea and diarrhoea) on a 100 mm VAS scale at 0, 15, 30, 45, 60, 90 and 120 min after consumption. It was also shown to be well tolerated in these conditions up to 25 g.

9.3.1.5 Applications

SGF is an easy-to-formulate fiber which functions like glucose syrup in most systems. It can be used as a partial or total replacement for regular glucose syrup, other sweeteners, or low calorie bulking agents such as polyols, whilst maintaining texture and mouthfeel.

With 2 kcal/g, it also reduces calories when replacing sugar/glucose syrups (4 kcal/g). It has little to no sweetness and so can be used in combination with high intensity sweeteners to balance sweetness to the level of the standard product (in accordance with the EU legislation on sweeteners use in foodstuffs – Directive EU 94/35). As a result of its high solubility and stability in acidic conditions it is suitable for high solids low pH food matrices such as fruit filling. Its low impact on flavor and viscosity – similar to glucose syrup (*Figure 9.4*) – allows to reduce sugar and calories significantly, while adding fiber which does not affect the food product's organoleptic quality.

Main applications include cereals bars and breakfast cereals, cookies and biscuits, snacks, beverages, yogurts, ice creams, desserts, fruit fillings, sauces, confections and processed meats.

Beverages

SGF is a 100% water soluble, fiber source which does not cause any sedimentation or dramatically increase viscosity as some hydrocolloids do. These negative effects

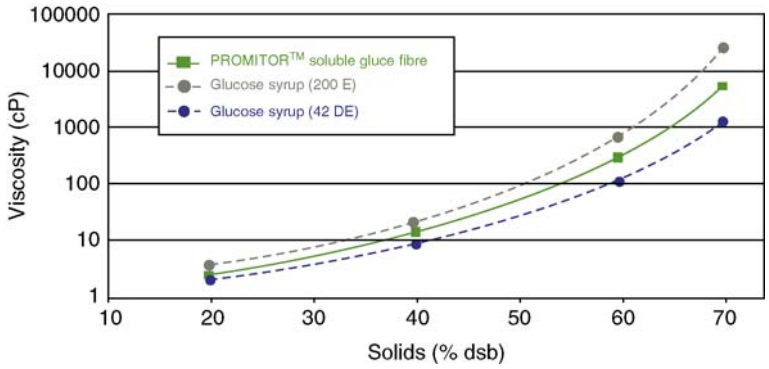


Figure 9.4
Viscosity of PROMITOR™ Soluble Gluco Fiber at 20°C.

can occur with other ingredients before or after heat treatment, dependent on the force and the type of shear applied. It also does not create cloudiness or turbidity. As a result, replacing sugar, either partially or totally, has no significant impact on the total "dry matter" or viscosity of the beverage, maintaining the original body and mouthfeel and avoiding the creation of a long or slimy structure.

As the energy is 2 kcal/g, it can help to deliver a significant sugar reduction. The sweetness is about 20% of the sweetness of sucrose, so adding a high intensity sweetener will create the desired taste. As a practical and simple example, replacing 4.5 g of sucrose per 100 mL in flavored and sweetened water with SGF will reduce energy load from 17.2 kcal/100 ml to less than 10 kcal/100 mL whilst adding 3 g fiber/100 mL. It can also be added to an existing beverage formulation and will then enhance its mouthfeel whilst simultaneously improving taste.

SGF is a maize starch derivative with a very bland flavor and does not contain any compounds responsible for off flavors sometimes caused during extraction and other processing stages. Its impact on the flavor of energy reduced drinks is not normally noticeable, with a smooth and non-grainy or powdery perception.

During beverage processing, pasteurization or flash pasteurization is often used to obtain microbiological stability. The combination of a heat treatment together with a low pH (as most beverages are) can damage some ingredients leading to breakdown before and during storage. SGF, however is stable throughout all normal processes in the beverage industry, which means there is no need to "overdose" it in formulations.

Bakery

Cereals bars are mainly composed of glucose syrups which work as a binder to maintain the cohesive structure of the particles of cereal and fruit. It is therefore particularly effective to replace glucose syrups with SGF, which can work as the sole binder thanks to its cohesive properties. Any reduction in sweetness can be compensated by ingredients such as fructose, although in many cases the dried part of the bar provides enough sweetness already. In addition SGF permits to achieve a fiber content of up to 35 g/100 g, corresponding to approximately 10 g/serving.

In moist matrices such as muffins, it decreases the water activity of the system and helps to maintain the stability of the product. In some cases a humectant (e.g., glycerol, sorbitol) can help to maintain the same water activity, ensuring that the product is preserved.

In biscuit-like and soft dough products, the low sweetness and the textural role of sugar, mean that the percentage of SGF incorporated does not generally exceed 50% of the total sugar.

Combining SGF and RS is a simple way to increase fiber content to reach the fiber amount necessary to make a "high fiber" claim, without affecting sensory characteristics of biscuits or muffin-like cakes whose regular flour based equivalents in the market have less than 1.5 g fiber/100 g.

Dairy

Because SGF behaves in a similar way to glucose syrup, it can effectively replace sugar while adding fiber in dairy products. At the same time it can contribute to a richer texture and a similar mouthfeel in low fat or non fat dairy products compared to full fat references.

Dairy processing typically includes heat treatment and homogenization steps which are synonymous to high shear, high temperature conditions. The stability in these harsh conditions makes it ideal for such dairy products.

Completely soluble at acidic and neutral pH, SGF adds texture and fibers with a smooth non sandy or powdery mouthfeel. For instance, it will compensate for the lack of body of a low fat and/or low sugar dairy dessert mousse, while increasing fiber and enhancing sensory characteristics.

It is also suitable for formulating fermented products due to its survivability during fermentation and can be added at the beginning of the process with no loss of fibers. (*Figure 9.5*).

In fruit preparations, it can replace the sweetener (either partly or totally) without impacting texture, while maintaining the pleasant mouthfeel stemming from its glucose syrup-like viscosity.

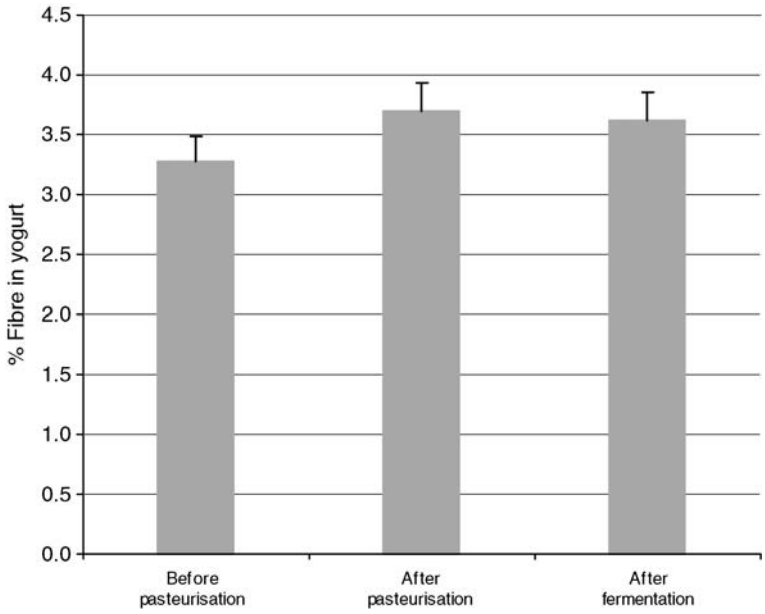


Figure 9.5
Stability of PROMITOR™ Soluble Gluco Fiber under Yogurt processing.

As SGF demonstrates high stability at low pH (pH < 4), it is particularly suitable for fruit preparations – where typical pH is around 3.8 to ensure good microbial stability during shelf-life – offering shelf-life stability with no loss of fiber content. Any sweetness loss is usually compensated by a high intensity sweetener such as sucralose.

In desserts and ice creams, SGF will add texture while replacing sweeteners (sugar or glucose syrup) and/or some fat, and will improve the nutritional profile of the end product. For instance, it is possible to achieve 30% fat and 60% sugar reductions in ice creams. In such formulations, it will help to keep a creamy taste and a mouthfeel similar to the full fat and sugar alternative.

9.3.2 Growing and Preliminary Evidence on Prebiotic Properties of New Starch-Derived Fibers

Fifteen grams per day Nutriose® intake over 2 weeks has been reported to decrease fecal pH and to reduce significantly *Clostridium perfringens* in humans (Lefranc-Millot, 2008).

Fibersol-2® has been shown to increase both bowel regularity, fecal volume and to favor growth of bifidobacteria in 20 healthy volunteers with a fecal frequency lower than three times a week (Satouchi et al., 1993). In this study, it has been observed that consumption of 3.75 g/d of Fibersol-2® for 5 days increased weekly fecal frequency from 2.6 times to 4.0 times and doubled fecal output. Bifidobacteria counts were also significantly increased. The prebiotic properties of Fibersol-2® have also been demonstrated in dogs (Flickinger et al., 2000). Though, a recent study, during which 38 healthy volunteers consumed 7.5 and 15 g Fibersol-2® over 3 weeks showed that resistant maltodextrin supplementation altered ($p < 0.05$) bacterial populations from baseline to treatment and increased butyrate production, but failed showing a significant effect ($p = 0.12$) on fecal *Bifidobacterium* populations during the treatment period (Fastinger et al., 2008).

SGF has been shown to be a potential prebiotic in *in vitro* studies at TNO (Maathuis et al., 2008). As described previously, the aim of the study was to investigate the effect of five newly developed maize-based fibers on the activity and composition of the microbiota in the colon. The fibers were pre-digested, mono- and di-saccharides were removed, and the residual polymer was used to assess the production of microbial metabolites and changes in composition of the microbiota using a dynamic, validated, *in vitro* model of the large intestine (TIM-2). Microbial metabolites analysis showed an increase in health-promoting metabolites (short-chain fatty acids) and a reduction in toxic metabolites from protein fermentation compared to the poorly-fermentable control (cellulose). Using microarray technology to compare multiple species and groups of colonic microbiota, it was found to promote the growth of beneficial bacteria such as bifidobacteria (*Figure 9.2*).

Human studies are ongoing to confirm the prebiotic properties of SGF.

9.4 Conclusion

The prebiotic effects of RS are promising. Existing data suggest that RS would be able to positively impact immune response, modulate inflammation, improve mineral absorption and help maintain a good colic function. Preliminary data on PROMITOR™ Resistant Starch suggest similar properties as those that have been reported so far for RS2 and RS3.

New soluble non-viscous fibers like PROMITOR™ Soluble Gluco Fiber are easy to integrate into new or existing formulations without compromising flavor or texture. Very well tolerated and clean tasting, the new generation of

fibers can help develop new health-plus versions of products in a wide range of food categories. In particular their low calorific value (0–2 kcal/g), makes them preferable alternatives to high calorific ingredient (sugar 4 kcal/g, fat 9 kcal/g). The fact that they can compensate for a lack of body and texture in many low calorie – sugar or fat reduced – products means that they are frequently used in the dairy and bakery sectors in particular. They have also shown high process and acid stability allowing manufacturers to formulate and guarantee fiber content (and other associated benefits) throughout the entire shelf life of products.

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10 Oligosaccharides Derived from Sucrose

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10.1 Introduction

Sucrose is a non-reducing disaccharide, consisting of an α-D-glucopyranosyl residue and a β-D-fructofuranosyl residue linked covalently by their respective anomeric carbons (α-D-glucopyranosyl-1,2-β-D-fructofuranoside). It is not just a simple disaccharide, among others: in fact, the energy of its glycosidic bond is higher than that of a usual glycosidic bond. It is equal to 27.6 kJ/mol, which is similar to the energy of a nucleotide-sugar bond as in UDP-glucose or ADP-glucose. This means that sucrose is a protected and activated form of D-glucose (as well as of D-fructose), which plays a key role in the metabolism of plants, for a wide variety of synthesis reactions.

Sucrose is essentially produced by extraction from cane (75% of sugar production is form cane which contains 20% sucrose by weight) and beet (25% production; 15% sucrose by weight). The total production is higher than 120 million metric tons per year. The crystallization-purification process yields a highly pure compound (>99.9%), at a very reasonable price.

Such characteristics make sucrose a very interesting renewable raw material for synthesis reactions, and particularly for the synthesis of prebiotic oligosaccharides, using either fructosyl transferases or glucosyl transferases.

10.2 Fructo-Oligosaccharides or Fructans

Like starch and sucrose, fructans are naturally present in many plants as reserve carbohydrates. Fructans may be involved in the protection of plants from water-related stresses: drought, salt and cold stress.

They can also be produced by a wide range of microorganisms, bacteria, yeast or fungi.

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In this chapter, we give an overview of fructan synthesis from sucrose by plants or microorganisms, their use and their beneficial effects on human and animal health.

10.2.1 Structural Diversity of Fructans

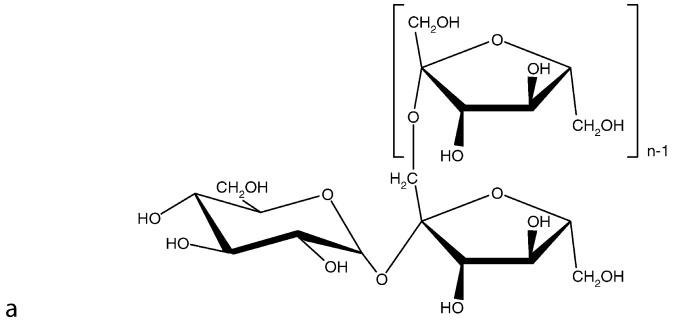
Fructans, also named fructooligosaccharides (FOS, Sc-FOS), oligofructoses oligofructans or inulin, are complex carbohydrates found in several plants.

Four major classes of structurally different fructans can be distinguished: inulin, levan, mixed levan, and neoseries:

- *Inulin-type fructans* consist of linear (2-1)-linked β-D-fructofuranosyl units in which the fructofuranosyl units (F) are bound to the β-2,1 position of sucrose or GFn (1^F(1-β-D-fructofuranosyl)$_{n-1}$ sucrose) (*Figure 10.1a*). They are usually found in plant species belonging to the order Asterales, such as chicory and Jerusalem artichoke. The chain length can vary from 2 to a few hundred fructose units depending on the plant (Ritsema and Smeekens, 2003).

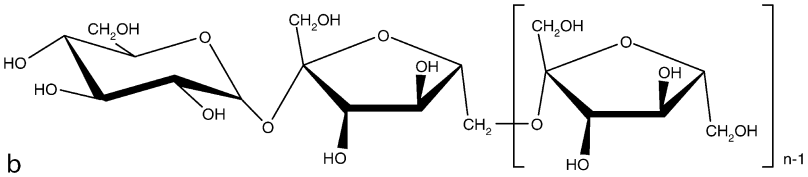
  Fructosyl transferases derived from fungi such as *Aureobasidium pullulans* and *Aspergillus niger* also produce 1F type FOS that are mainly composed of 1-kestose (GF2), nystose (GF3) and fructosyl-nystose (GF4). The terms fructooligosaccharides (FOS) or short chain fructooligosaccharides (Sc-FOS) have been used but only for (1^F(1-β-D-fructofuranosyl)$_{n-1}$ sucrose, GFn, $2 < n < 10$) excluding long chain fructans such as inulin.
- *Levans* consist of linear (2–6)-linked β-D-fructofuranosyl units in which fructofuranosyl units (F) are β linked to the 6 position of sucrose (GFn or 6^F(1-β-D-fructofuranosyl)$_{n-1}$ sucrose) with n = 1–3,000 possessing minor amounts of β-2,1 branching (*Figure 10.1b*). In plants, they are usually found in grasses but can also be produced by several bacteria, notably *Bacillus polymyxa, Bacillus subtilis,* and *Streptococcus mutans.*
- *Mixed levans* are composed of both (2–6)- and (2-1)-linked β-D-fructofuranosyl units. This type of fructan is found in many plant species such as wheat and barley. An example of this type of fructan is the molecule bifurcose (*Figure 10.1c*).
- *Neoseries* consist of linear (2-1)-linked β-D-fructofuranosyl units in which fructofuranosyl units (F) are bound to C6 of the glucose moiety of sucrose (6^G(1-β-D-fructofuranosyl)$_{n-1}$ sucrose, *Figure 10.1d*). In plants, they can be found in onions.

  In some cases, for example in *Asparagus officinalis*, Shiomi et al. (1979) described inulin neoseries consisting in linear (2-1)-linked β-D-fructofuranosyl units in which the fructofuranosyl units (F) are bound to both C1 of the fructose moiety and C6 of the glucose moiety of sucrose. This results in a fructan polymer with two



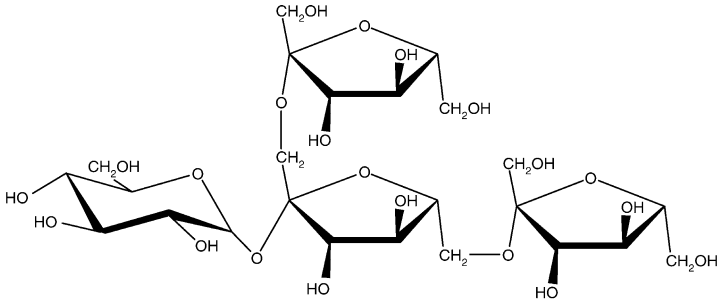
a

Inulin type fructans → 1^F (1-β-D-frutofuranosyl)$_{n-1}$ sucrose



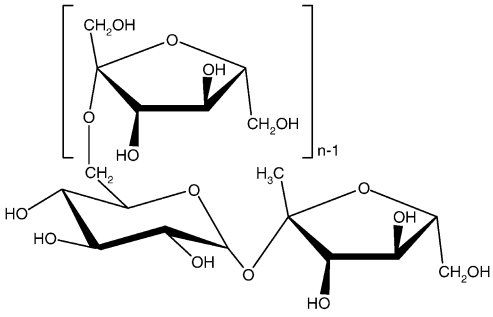
b

Levans → 6^F (1-β-D-frutofuranosyl)$_{n-1}$ sucrose



c

Mixed levan → 6^F(1-β-D-frutofuranosyl)1^F(1-β-D-frutofuranosyl) sucrose or bifurcose



d

Neoseries → 6^G(1-β-D-frutofuranosyl)$_{n-1}$ sucrose

Figure 10.1

Structural representation of different types of fructans (a) inulin type, (b) levan type, (c) Mixed levan type, (d) Neoseries type.

fructan chains on both ends of the sucrose molecule (1^F(1-β-D-fructofuranosyl)$_m$ 6^G(1-β-D-fructofuranosyl)$_{n-1}$ sucrose).

If we consider the definition of dietary fibers: prebiotics or dietary fibers are mainly non-digestible oligosaccharides of natural or synthetic origin. They are based on the monosaccharides glucose, fructose, galactose and mannose with a polymerization degree from 2 to 20 monosaccharide units. Therefore, all the types of fructans described here can be considered as dietary fibres.

The fructans can also be classified as prebiotics. They are characterized by the fact that they are neither hydrolyzed by mammalian digestive enzymes nor absorbed from the gastrointestinal tract by the host animal. Consequently, they can only be fermented in the gastrointestinal tract, which "*beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves host health*" (Gibson and Roberfroid, 1995). This definition was updated by Roberfroid (2007): "A prebiotic is a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microbiota that confers benefits upon host well-being and health."

The benefits of inulin-type fructans and short chain fruto-oligosaccharides to humans have now been well studied and described for more than two decades. These molecules have been recognized as prebiotics.

Other fructans have been less studied for their prebiotic effects. It has, for instance, been demonstrated that neokestose is also a good promoter for the growth of beneficial bacteria (Kilian et al., 2002).

Levans have also been recognized as non-digestible food fibers. *In vitro* investigation showed that bifidobacteria can use levans as a source of carbon. However, this depends on the degree of polymerization – various researchers have suggested that the maximal molecular weight for a prebiotic effect from levans is 4,500 Da (Marx et al., 2000).

The production yield of fructans using enzymes from plants is low, and mass production of enzyme is quite limited by seasonal conditions. Therefore, industrial production chiefly depends on fungal enzymes, principally from *Aspergillus niger* or *Aureobasidium pullulans.*

10.2.2 Fructans From Plants

Fructans of various types can be found as natural components in honey, fruits and vegetables such as Jerusalem artichoke, banana, chicory, onion, leek, garlic,

rye, barley, yacon and salsify. For most of these sources, concentration ranges are between 0.3 and 6% (w/w). For chicory and salsify, they are between 5 and 10% (w/w), while in Jerusalem artichoke and yacon they can reach up to 20% (w/w) (Mussato, 2007).

In plants, fructans are synthesized by the action of two or more fructosyl transferases.

Fructan synthesis starts with the conversion of sucrose to 1-kestose. The enzyme that performs this reaction, sucrose-sucrose 1-fructosyl transferase (1-SST, EC 2.4.1.99) is found in all fructooligosaccharide producing plants. The resulting 1-kestose is, in general, used as a substrate by more species specific fructosyl transferases to synthesize longer and/or more complex fructans.

One of the simplest fructans in plants is linear inulin. This fructan is present in plants belonging to Asterales. In members of the *Liliaceae*, inulin neoseries have been found.

Fructans of the levan type have been found in grasses, where mixed fructan types, also called, Gramminans, can also be present. Gramminans sometimes consist of even more complex structures in which neosugars are combined with branched fructan chains (Ritsema and Smeekens, 2003).

The reason for the variety of structures in plants is unknown; it might be based on different physiological needs or could be the consequence of different evolutionary origins of fructan biosynthesis in different families.

10.2.3 Fructans From Microorganisms

The nomenclature of fructan-producing enzymes remains polemical. Some workers still use the term of β-D-fructofuranosidase (hydrolase numbered EC.3.2.1.26), whereas others designate it as fructosyl transferase (EC.2.4.1.9), concentrating on the nature of transfructosylation of the reaction catalyzed by the enzyme. The reason why many authors have used the name β-D-fructofuranosidase is probably because the transfructosylating activity was first found in an invertase preparation used at high sucrose concentrations. Transfer and hydrolysis reactions are very similar; as hydrolysis is a transfer reaction onto water as acceptor. In each case fructose is transferred from sucrose donor to (i) water acceptor for hydrolysis reaction or (ii) fructan / sucrose for transfer reaction.

Recently different enzymes designated as β-D-fructofuranosidases were compared for their transferase activity (Arrojo et al, 2005). The transferase activity of these enzymes was analysed in detail and the maximum Sc-FOS synthesis yield, expressed as weight percentage of the total amount of carbohydrates

in the final mixture, was determined. While the β-fructofuranosidase from *Saccharomyces cerevisiae* gives a maximum yield of 8% fructooligosaccharides, the enzyme from *Aspergillus aculeatus* produces 61% of fructooligosaccharides, close to the value obtained with the enzyme from *Aspergillus niger* ATCC 20611. Some other strains such as *Xanthophyllomyces dendrorhous, Schwanniomyces occidentalis and Rhodotorula gracilis* produced 17–19% yield fructooligosaccharide syrups. These results clearly demonstrate that there is no obvious relationship between enzyme name in literature and the corresponding enzyme activity.

These results greatly underline the necessity to characterize an enzyme preparation not only by its name, but also experimentally, and to determine the ratio transferase/hydrolase activity at 50 Brix sucrose (concentration above which this transferase/hydrolase ratio generally does not change).

10.2.3.1 Fructans From Fungi

Many articles on fructan synthesis have been published and a lot of fructooligosaccharide producing fungi studied for their potential application to short-chain fructooligosaccharide (Sc-FOS) production.

Generally, fructosyl transferases from fungi produce 1F-type FOS with a high regiospecificity. Using sucrose as substrate, these enzymes are able to transfer fructofuranosyl groups from sucrose as donor to yield the corresponding series of Sc-FOS: 1-kestose (GF2), nystose (GF3) and fructosyl-nystose (GF4).

The first fungus reported to achieve a high yield of Sc-FOS production was described in by Hidaka et al. (1987). Using this enzyme, from *Aspergillus niger* ATCC 20611, the maximum Sc-FOS conversion from sucrose reached 55–60% (w/w) with respect to total sugar. Rapidly Hidaka et al. (1988) and Hirayama et al. (1988) fully characterized this enzyme and developed it in Japan for an industrial production of Sc-FOS syrup Neosugar®, Meiji Seika Co.).

At the same time, Jung et al. (1987) found another fungal enzyme for Sc-FOS production using *Aureobasidium pullulans*. This enzyme gave rise to another industrial process (Cheil Foods and Chemicals Co. in Korea), well described by Yun et al. (1990, 1992).

Rapidly (Su et al. 1991) discovered another fungus producing a transfructosylating enzyme for fructooligosaccharide synthesis: *Aspergillus japonicus* isolated from a natural habitat was found to produce a significant amount of fructosyl

transferase with a good potential for industrial production of fructooligosaccharides. The fructooligosaccharide yield from initial sucrose was about 60%.

Fructosyl transferase activity was also found in a commercial fruit juice processing enzyme preparation (Pectinex Ultra SPL, Novo Nordisk) from *Aspergillus foetidus* (Hang and Woodmans, 1996). The conditions affecting the enzymatic production of high fructooligosaccharide content syrup from sucrose were investigated. As for the enzymes previously described, Pectinex Ultra SPL showed a high transferase/hydrolase activity ratio. The enzyme can convert a 50 Brix sucrose syrup to a 55–60% (on total sugar) fructooligosaccharide syrup conferring a great potential for industrial production.

The synthesis of fructooligosaccharides using whole *Penicillium citrinum* at high sucrose concentration has also been investigated (Lee and Shinohara, 2001). In this case, both 1-kestose and neokestose were produced. However, no reaction product was obtained from neofructooligosaccharides. The oligosaccharides produced were 1-kestose, neokestose, nystose and fructosyl-nystose. Based on these experimental results, a hypothetical reaction pathway was proposed to illustrate how neofructooligosaccharides are formed from 1-kestose.

Penicillium citrimum KCTC 10225BP of soil origin was also described to produce both 1-kestose and neokestose oligosaccharide series, in which the degree of polymerization of each type was reported to be 3–6 (Han et al., 2003).

In Katapodis et al. (2003), production of β-fructofuranosidase from the thermophilic fungus *Sporotrichum thermophile* was studied. This enzyme was optimally active at 60°C. The optimal pH described for transfructosylation was 6.0. The major sugar produced by the transfructosylating activity of the enzyme was 1-kestose. The optimum initial sucrose concentration of 250 g/l allowed the production of only 12.5 g FOS/l.

More recently, Sangeetha et al. (2004), described the production of 1-F type fructooligosaccharides from *Aspergillus oryzae* CFR 202 and *Aureobasidium pullulans* CFR 77. The conversion yield was limited to 55–60% as the glucose released during the enzymatic reaction acts as a competitive inhibitor. These studies gave rise to a US patent application (Ramesh et al., 2005) for the preparation of prebiotics.

The use of recombinant *Aspergillus niger* enzyme was also described (Zuccaro et al., 2008). The combination of sucrose analogs as novel substrates with highly active recombinant *Aspergillus niger* enzyme provides a new and powerful tool for the efficient preparative synthesis of tailor-made saccharides. Molecules of the important 1-kestose and nystose types, headed with different modified monosaccharides of interest were prepared.

10.2.3.2 Fructans From Yeast

The recent increase in the use of prebiotic oligosaccharides in the food industry has lead to the search for new microorganisms and enzymes for their production.

Maugeri and Hernalsteens et al. (2007) screened yeast obtained from fruits and flowers from the Brazilian tropical forest, capable of secreting extracellular enzymes with high fructosyl transferase activity. The screening and isolation procedure resulted in the isolation of one potentially interesting yeast strain, *Rhodotorula spp.* (LEB-V10). This enzyme showed no hydrolytic activity with respect to the Sc-FOS produced and a conversion yield from sucrose of 50% (w/w).

Inulinase from *Kluyveromyces marxianus* was also studied for fructo-oligosaccharide production (Santos and Maugeri, 2007). This enzyme is able to specifically produce 1-F fructooligosaccharides. In this work, experimental factorial design was applied to optimize the fructooligosaccharide synthesis. The variables studied were temperature, pH, sucrose concentration and enzyme concentration. Only temperature and sucrose concentration were shown to be significant parameters. In this case, the maximum conversion yield from sucrose was only 10%.

Other yeast such as *Schanniomyces occidentalis* has been described for fructosyl transferase activity yielding the prebiotic 6-kestose (Álvaro-Benito et al., 2007). However, the main reaction catalyzed by this enzyme is sucrose hydrolysis.

10.2.3.3 Fructans From Bacteria

Bacterial Levansucrase

Fructan-producing bacteria can be found in a wide range of taxa, including plant pathogens and bacteria present in oral and gut flora of animals and humans. In general, bacteria produce levan-type fructan molecules consisting mainly of β, 2–6 linked fructosyl residues, occasionally containing β, 2–1 linked branches.

These polymers are found in many plants and microbial products and are useful as emulsifying and thickening agents in the food industry. Plant levans have a shorter chain length (about 100 fructofuranosyl residues) than microbial levans that contain up to three million residues.

Bacterial levans are synthesized extracellularly by a single enzyme, levansucrase, which produces levan directly from sucrose. Examples of bacterial genera in which fructan-producing strains can be found are *Bacillus, Aerobacter, Streptococcus and Zymomonas.*

Levansucrase (sucrose:2,6-β-D-fructan 6-β-D-fructosyl transferase; E.C. 2.4.1.10) secreted by *Bacillus subtilis* after induction by sucrose was purified by Dedonder (1966). Since, it has been extensively investigated. Detailed enzymological studies by Chambert and Gonzy-Treboul (1976) showed that the enzyme obeys a ping-pong mechanism, which involves a covalent fructofuranosyl-enzyme intermediate that has been isolated.

Production of short-chain levans (fructooligosaccharides) by levansucrase from *Bacillus subtilis* C4 was also investigated (Euzenat et al., 1991).

More recently, according to the potential of short-chain levans to be used by bifidobacteria (Marx et al., 2000), several studies on the use of levansucrase for fructooligosaccharide production have been reported.

As in previous work by Euzenat, Ahmed et al. (2005) described the production and use of extracellular levansucrase from *Bacillus subtillis*. With partially purified enzyme, in optimal conditions, conversion of sucrose to levan reached 84%. Sucrose concentration is the most effective factor controlling the molecular weight of the synthesized levan. The molecular weight of levan decreased from 60 to 0.5 KDa with increasing sucrose concentration from 2.5 to 40%.

Byun et al. (2007) optimized the conditions for the formulation of fructooligosaccharides from sucrose by a transfructosylation reaction using levansucrase from *Pseudomonas aurantiaca* and *Zymomonas mobilis*. As with the other bacteria, formation of fructooligosaccharides by levansucrases of both origins was sucrose concentration dependent. The optimum initial sucrose concentration for the formation of short-chain fructooligosaccharides (DP 3–6) was 70% (w/w). Under these conditions, yields of levan-type fructooligosaccharides were 24–26%. These effects of sucrose concentration on molecular weight are in agreement with those reported by numerous authors.

Modification of ionic strength can also modify chain length. Under conditions of high ionic strength, only levan with an average DP of 120 was synthesized with a reasonable yield by Tanaka et al. (1979). It was suggested in this work that the DP of the levan is generally regulated by ionic strength.

Another method for producing short-chain levan from long-chain levan, consists of hydrolyzing the molecule by levanase from fungus to give levan octaose (DP8, Lizuka et al., 1995).

Bacterial Fructosyl Transferase

Some bacteria such as *Bacillus macerans* produce fructosyl transferase. The crude enzyme unexpectedly selectively synthesized GF5 and GF6 fructooligosaccharides

whereas purified enzyme produced mainly 1-kestose and nystose as do other transfructosylating enzymes (Park et al., 2001).

A process for fructooligosaccharide production from sucrose using extracellular enzyme from *Zymomonas mobilis* has also been developed (Bekers et al., 2004). The fructan syrup obtained contained 45–48% (w/w) of fructans with respect to total sugar (1-kestose, 6 kestose, neokestose and nystose).

10.2.4 Kinetic Modeling of Fructooligosaccharide Synthesis

Fructosyl transferases catalyze the transfer of fructofuranosyl residues from sucrose to various acceptor substrates. The synthesis involves group transfer without intervention of a cofactor. Two different reactions area catalyzed:

- Transglycosylation using the growing fructan chain (oligosaccharide synthesis) as the acceptor substrate.
- Hydrolysis of sucrose and/or oligosaccharides, when water is used as an acceptor.

Fructosyl transferases belong to Glycoside Hydrolase family 68. They are β-retaining enzymes, employing a double-displacement mechanism that involves formation and subsequent hydrolysis of a covalent glycosyl-enzyme intermediate (a ping-pong type mechanism, Chambert et al., 1974).

For short chain fructooligosaccharides (Sc-FOS), different kinetic models of fructooligosaccharide synthesis have been studied for *Aureobasidium pullulans* (Jung et al., 1989), *Aspergillus niger* (Ouarne and Guibert, 1992) and more recently, *Rhodotorula spp.* (Alvarado and Maugeri, 2007). For these enzymes, during the time course of the reaction, the medium contains unreacted sucrose, glucose from the sucrose used as fructosyl donor and synthesis products, GF2, GF3 and GF4. The presence of fructose in very limited concentrations can also be noted.

In these studies, the initial reaction rate was determined independently by varying the concentrations of sucrose, GF2, GF3 and GF4. The initial inhibitory effect was also studied by adding glucose to pure substrate solutions (sucrose, GF2, GF3 and GF4) at different concentrations.

Different mathematical models have been proposed and found to fit well with experimental data. All authors agree to describe the chain of reactions below:

$$GF + GF \rightarrow GF_2 + G$$

$$GF_2 + GF_2 \rightarrow GF_3 + GF$$

$$GF_3 + GF_3 \rightarrow GF_4 + GF2$$

$$GF + G \rightarrow G + GF$$

The formation of fructooligosaccharides resulted from a consecutive set of transfer reactions. For sucrose, the enzyme kinetics was characteristic of a Michaelis-Menten type with inhibition at high substrate concentrations.

In addition to these basic equations, the fructosyl transfer (equation below) from GFn to G should also be considered. For all oligosaccharides including sucrose, transfructosylation was found to be inhibited competitively by glucose:

$$GF_{n(1<n<4)} + G \rightarrow GF_{n-1} + GF$$

GFn hydrolysis was also described:

$$GF_{n(1<n<4)} + H_2O \rightarrow GF_{n-1} + G$$

In particular, the enzymes from *Aureobasidium pullulans* and *Aspergillus niger* have a high regiospecificity. They selectively transfer the fructosyl moiety of sucrose to the 1-OH fructofuranoside of the other sucrose molecules resulting in the formation of very predominantly 1-kestose based Sc-FOS.

10.2.5 Process

Recent developments in industrial enzymology have made possible the large-scale production of Sc-FOS by enzymatic synthesis. It appears that industrial production of Sc-FOS by enzymatic synthesis can be divided into two types of processes. The first one is the batch system using soluble or immobilized enzyme and the second one the continuous process using immobilized enzyme or whole cells.

Industrial processes for the synthesis of fructooligosaccharides were studied using enzymes with high transfructosylating activity. The best enzymes described were from fungi such as *Aspergillus niger, Aspergillus japonicus* and *Aureobasidium pullulans.* All produced Sc-FOS.

The industrial processes for fructooligosaccharide synthesis are schematized in *Figure 10.2*. The steps are described in detail below.

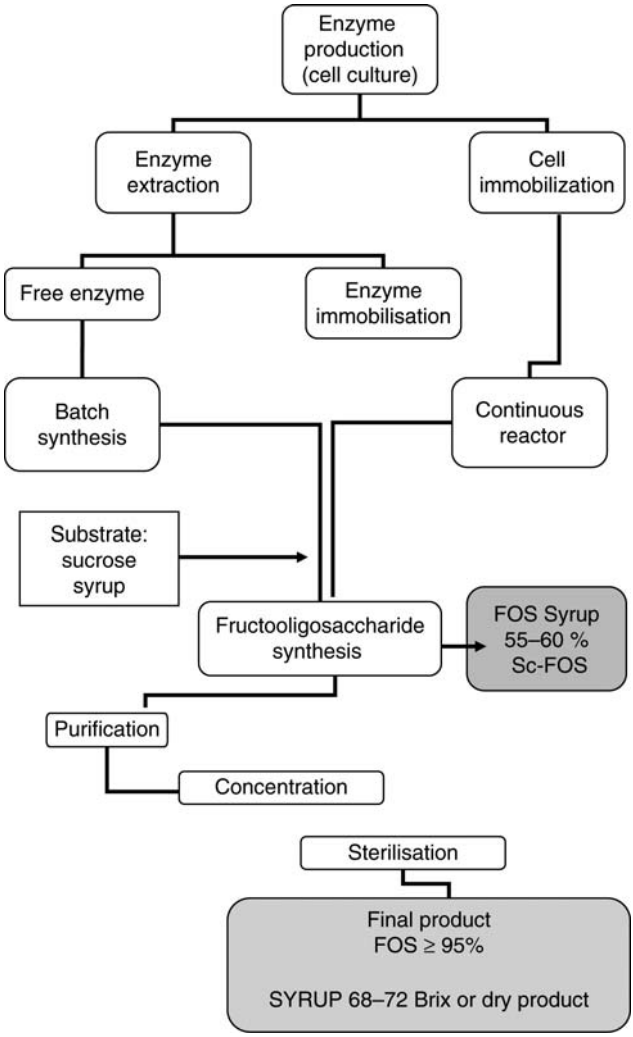


Figure 10.2
Flow chart of typical process of Sc-FOS production, by free enzyme, immobilized enzyme or immobilized cells.

10.2.5.1 Enzyme Production

For some fungal strains, production of fructosyl transferase can be by aerobic submerged culture, by fluid-bed culture, culture broth or semi-solid culture medium. Fermentation parameters (temperature, pH, aeration, agitation) should be established for each microorganism. Generally, sucrose is the best carbon

source for both cell growth and enzyme activity production. The optimum temperature for growth is often around 30°C and the pH above 5.5.

The enzymes thus obtained are cell bound and require complex operations for separating the enzyme from the mycelium. At the end of the culture period, cells are easily harvested by a basket or continuous centrifuge, and the enzyme extracted.

Cells can also be directly immobilized for Sc-FOS production.

Non cell-bound enzyme can be obtained using semi-solid *Aspergillus niger* 489 culture medium, with the addition of cereal bran. The enzyme thus obtained was easily separated from the cells without any extraction step (Park and Pastore, 1998).

The example of *Aspergillus oryzae* grown in liquid medium with sucrose and yeast extract was also described (Rao et al., 2005) for soluble extracellular enzyme production – a process that is easy to carry out and that is of low cost.

Using an optimized medium, *Aspergillus oryzae* produces round pellets which were stable during the fermentation period (Sangeetha et al., 2005a).

For industrial scale fermentation, these pellets consisting of compact masses of hyphae are advantageous, since the filamentous form of fungi may wrap around the impeller and damage the agitator blade. Also pellet formation makes downstream processing easier in industrial fermentation.

10.2.5.2 Enzyme Extraction

At the end of the log-phase period, cells are collected by centrifugation and washed twice with deionized water, physiological saline solution or buffer. For mycelium-bound enzyme, washed cells are resuspended in water or buffer before enzyme extraction.

Different methods can be used for enzyme extraction:

- Ultrasonication
- Lysozyme
- Cell grinding

An additional centrifugation or filtration step is then needed to produce clear supernatant containing free enzyme. In some cases (Yun and Song, 1999), the enzyme solution thus obtained required an additional concentration step by ultrafiltration or dialysis (molecular weight cutoff 10 KDa). The resulting enzyme is used crude without further purification.

10.2.5.3 Substrates

Sucrose is the natural substrate of fructosyl transferase.

The effect of the sucrose concentration was investigated to minimize the hydrolysis reaction. The maximum yield of Sc-FOS is currently obtained with an initial sucrose concentration of 55 Brix.

Moreover, there is a consensus that a high concentration of commercial food grade sucrose syrup (60–70 Brix) should be used for both the batch and continuous process of fructooligosaccharide production. At such high concentrations, sucrose syrups have the benefit of a low water activity reducing the risk of contamination during the enzymatic synthesis step and reducing the final evaporation costs.

Commercial food grade Sc-FOS is being produced from pure food grade sucrose.

In order to use Sc-FOS as animal feed additive, it would be necessary to reduce production costs. With batch production based on free enzyme or cells, the use of molasses was described to produce 166 g/l fructooligosaccharides from 360 g/l molasses sugar (Shin et al., 2004). Raw sugar can also be used.

10.2.5.4 Cell Immobilization

When enzymes are cell-bound, a method in which microorganisms are directly immobilized on a carrier can be used for Sc-FOS synthesis. This method does not require separation of enzyme from cells and can prevent the reduction of enzyme activity during the extraction step.

Meiji Seika Co., which was the first to produce Sc-FOSs, used initially a continuous process involving immobilized *Aspergillus niger* cells entrapped in calcium alginate beads.

Yun and Song (1999) described *Aureobasidium pullulans* cell immobilization with calcium alginate. Cells were suspended with 3% sodium alginate solution and extruded through syringe needles to form small beads dropping into calcium chloride solution.

Cheil Foods and Chemicals Co. also developed a continuous process using immobilized cells of *Aureobasidium pullulans* entrapped in calcium alginate beads. Two one-cubic meter packed bed reactors have been in commercial operation since 1990. With concentrated sucrose as substrate, the stability of the immobilized cells is about 3 months at 50°C (Yun, 1996).

β-fructofuranosidase from *Aspergillus japonicus* mycelium was also immobilized by entrapment in calcium alginate gel (Cheng et al., 1996). A packed bed reactor was employed for the production of fructooligosaccharides at 42°C. The reaction was continued for 35 days and only 17% of enzyme activity was lost during this period.

10.2.5.5 Enzyme Immobilization

A column reactor filled with immobilized cells, is hard to operate at high flow rate due to a diffusional limitation of substrate and products within the calcium alginate gel matrix. The immobilized enzyme column is essentially superior to the immobilized cell column from the practical point of view:

- The immobilization process is quite simple.
- The unit volumetric immobilized activity is higher.
- There are fewer diffusional limitations.

For these reasons, a lot of studies on fructosyl transferase immobilization have been done. Several of adsorbents, ion exchangers and carriers for covalent enzyme linkage have been studied for the immobilization of fructosyl transferase.

The following examples are illustrative of some of the relevant studies for industrial immobilization of fungal fructosyl transferase.

Yun and Song (1999) described *Aureobasidium pullulans* fructosyl transferase immobilization process on a highly porous anion exchange resin (Diaion HPA25, Samyang Co., Korea). The support consisted of styrene-divinylbenzene polymer with quaternary alkylamine as functional group. Immobilization was very simple: after intracellular enzyme extraction with lysozyme, soluble enzyme was passed through a column containing the porous ion exchange resin (10 h at room temperature). After immobilization, only washing was done. In order to simplify the immobilization procedure, no activation of the support was carried out before or after immobilization.

Kono et al. (1994) patented for Meiji Seika Co. a fructose transferring enzyme immobilization on a granular carrier with or without crosslinking agent. Fourteen carriers were tested and in accordance with the results obtained, anion exchange resin which carrying primary to quaternary amines as functional groups were defined to be essential for immobilization. Immobilization resulted in 63–100% relative activity.

More recently, another industrial immobilization process was described for *Aureobasidium pullulans* fructosyl-transferase (Vankova et al., 2008).

As in the previous case, an anion-exchanger suitable for food applications (Dowex Marathon MSA resin, Dow Chemical, USA) which is a styrene-divinyl-benzene matrix with quaternary amine as a functional group was used. For enzyme extraction, the cells were disrupted in a bead mill and the debris removed in a centrifuge. In order to increase the efficiency of the immobilization process, the low molecular weight solutes present in the enzyme solution were removed by ultra-filtration. For immobilization, resin particles were simply mixed gently with free purified enzyme, for 12 h at 12°C and washed to eliminate unbound compounds. Immobilization resulted in 65% relative activity.

Pectinex ultra SP-L (a commercial preparation from Novozymes) containing fructosyl transferase activity, was covalently immobilized onto Eupergit C (Tanriseven and Asla, 2005). Immobilization resulted in 96% relative activity and immobilized enzyme retained its activity for 20 days without any decrease. This enzyme was also covalently immobilized on an industrial polymer polymethacrylate-based (Sepabeads EC) activated with epoxy groups. The influence of pore volume and average pore size on biocatalyst performance was studied. Several parameters that affect immobilization, such as buffer concentration, pH and amount (mg) of protein added per gram of support were analyzed. Authors found that Pectinex Ultra SP-L can be efficiently immobilized on these supports without adding any external salt or buffer (Ghazi et al., 2005).

10.2.5.6 Fructooligosaccharide Synthesis

Two enzymatic reaction systems were described for industrial fructooligosaccharide (Sc-FOS) production:

- A batch system with soluble fructosyl transferase or cells containing fructosyl transferase activity.
- A continuous system using immobilized cells entrapped in calcium alginate gel or immobilized enzyme on insoluble carrier.

Batch Production

For batch production of fructooligosaccharides, enzymes are suspended in high sucrose concentration syrup (55–70 Brix) and incubated at 55–60°C for

20–25 h with gentle agitation. From a practical standpoint, the batch production with free enzyme needs an additional process to destroy and remove the residual enzymes or cells after conversion. This process results in high costs for the production of a unit amount of Sc-FOS, therefore, a continuous process employing immobilized enzyme or cells is a better alternative.

The use of *Aspergillus oryzae* mycelium pellets was also described (Sangeetha et al., 2005a) for batch production of fructooligosaccharides. The pellets, incubated with 60% sucrose solution at 55°C, gave a Sc-FOS yield of 53%. This Sc-FOS yield was maintained up to the sixth cycle. However, it was not possible to recycle the pellets beyond the sixth cycle due to their disintegration. Compared to conventional batch production, the system was described as advantageous and economical because it does not require supplementation of any additional nutrients or the use of fresh inocula.

In the process of Sc-FOS production with fructosyl transferase, the main problem is the generation of by-products, principally glucose and residual sucrose. At the end of the production step, the maximum Sc-FOS content is known to be 55–60% per unit dry substance. Some authors have focused on searching for an economical method for the production of higher Sc-FOS contents.

- Jung et al. (1993) examined a mixed-enzyme system of fructosyl transferase and glucose oxidase. The mixed enzyme reaction was carried out in a stirred tank reactor containing, sucrose, fructosyl transferase and glucose oxidase. The net Sc-FOS content in the commercial syrup was limited to only 55–60% due to the accumulation of glucose which acts as an inhibitor of the fructosyl transferase. By supplementing glucose oxidase to the conventional Sc-FOS reaction system, it was possible to convert the glucose into gluconic acid readily separable from neutral sugars by simple ion exchange operation. By reducing or eliminating the glucose produced during fructooligosaccharide synthesis, the conversion of fructooligosaccharides was enhanced to reach up to 90%. This process was only described for batch reactions.
- Another process with simultaneous removal of glucose was described with free purified enzyme (Nizikawa et al., 2001). A membrane reactor system was developed using a nanofiltration membrane through which glucose permeated but sucrose and fructooligosaccharides did not. Percentage fructooligosaccharide reaction product was thus increased to above 90%, a yield comparable to that of the product obtained following chromatographic separation.

Continuous Production

From the standpoint of production cost, a continuous process using an immobilized enzyme or an immobilized microorganism is preferred to a batch process with native enzyme.

For continuous production of fructooligosaccharides, particles of immobilized biocatalyst were packed into fixed bed reactors. Industrial reactor volumes are conventionally between 1 and 2 m^3. To avoid channeling problems in the reactor, column operation is facilitated by the upward flow of substrate, in this way, compression of the immobilized enzyme is minimized (Yun and Song, 1999). The reactor temperature is kept constant by circulating hot water through a jacket.

In case of fructosyl transferase from *Aureobasidium pullulans*, for cell immobilization fixed bed reactors, the initial flow rate at 50°C is about 0.15–0.3 BVH and 1.5 to 2 BVH for immobilized enzyme fixed bed reactors (Yun and Song, 1999). Authors concluded that immobilized enzyme column is essentially superior to the immobilized cell column

For immobilized fructosyl transferase from *Aureobasidium pullulans* CCY 27-1–94, the initial flow rate described (Vankova et al., 2008) was about 0.8 BVH for a constant temperature of 50°C.

10.2.5.7 Fructooligosaccharide Purification

Considering a maximum Sc-FOS content for fructosyl transferase, enzyme processes are known to yield 55–60% on a total sugar basis. Numerous studies have focused on searching for an economical method for the production of high Sc-FOS levels.

In 1984, the separation of Sc-FOS from residual sucrose and monosaccharides was performed by Meiji Seika Co by simulated moving bed chromatography (SMB). But as would be expected, the recovery yield of this process was far too low to be applicable to the production scale (Yun, 1996).

Over the last two decades there has been remarkable progress toward the understanding of the phenomenon of chromatographic separation. This has expanded the field of application of this technology and led to major improvements in process control and column design. More recently, Vankova et al. (2008) described an optimized simulated moving bed separation for fructooligosaccharide purification. A cation exchange adsorbent tailored for saccharide separation (Amberlite™ 1320 Ca, Rhom and Hass Company, France) which is based on a poly(styrene-codivinylbenzene) matrix with a functional group – $(SO_3)_2Ca^{2+}$,

was used. The Sc-FOS solution obtained contains less than 5% monosaccharides and disaccharides.

The separation yield of Sc-FOSs in recent SMB processes is 95%. This type of separation allows high yield at low capital investment and running costs.

The potential of cross flow nanofiltration has been assessed in the purification of oligosaccharide from mixtures containing contaminant mono and disaccharides (Nobre et al., 2006). The work showed the concentration of the high molecular weight sugar fraction from a Sc-FOS mixture obtained by fermentation. Fractionation of sugar was studied using 0.5 KDa membranes. The retention yields obtained for the oligosaccharides were 27 and 8% for mono and disaccharides with a volume concentration ratio of 3.6 as compared to the initial conditions. The fraction of Sc-FOS was concentrated during the separation process from 52 to 72%. To be applied for fractionation of oligosaccharides on a large scale, this process requires further optimization.

10.2.5.8 Concentration

For concentration, evaporation of the reaction products is performed by a traditional process for sucrose concentration.

For the final dry product, spray drying of purified syrup can also be carried out.

10.2.5.9 Sterilization

In the sterilization procedure, heat, ultraviolet radiation or sterilizing filtration can be used. To avoid coloring the reaction products, high-temperature sterilization is not recommended.

10.2.6 Physicochemical Properties

Short chain fructooligosaccharides are water soluble, non-viscous and easy-to-use ingredients. Indeed, their technological properties are similar to those of sucrose. They also enhance aromas and mask the off-flavors coming from intense sweeteners.

Fructooligosaccharides have a nice, clean sweet taste typically 0.3–0.6 times as sweet as sucrose depending on the chain length – sweetness decreases with increasing chain length.

The relatively low sweetness makes oligosaccharides useful in food production when a bulking agent with reduced sweetness is desirable to enhance other food flavors.

They are stable in the pH range of 5.0–10.0 and thermally stable at food processing temperatures. Nevertheless, as a whole, when the pH falls below 4.0 and treatment temperature is high, they can become hydrolyzed.

Oligosaccharides can also be used to alter the freezing temperature of frozen food and to control the intensity of browning due to the Maillard reaction in heat-processed food. They also provide high moisture retaining activity preventing excessive drying (Mussato and Mancilha, 2007).

The caloric value of value of purified fructooligosaccharides (%Sc-FOS > 95%) has been estimated to be 1.5–2.0 kCal/g. This is approximately 40–50% the caloric value of digestible carbohydrates such as sucrose.

10.2.7 Functional Properties of Fructooligosaccharides

Fructooligosaccharides are prebiotic agents. The dietary modulation of the gut microbiota by prebiotics is designed to improve health by stimulating the numbers and/or activities of bifidobacteria and lactobacilli.

For a dietary substance to be classified as a prebiotic, at least three criteria are required:

- It must not be hydrolyzed or absorbed in the stomach or the small intestine.
- It must be selective for beneficial commensal bacteria in the colon such as bifidobacteria.
- Fermentation of the substrate should give beneficial luminal/systemic effects within the host.

The human digestive system plays a crucial role in overall body health. Far from being a purely energy absorbing system, the gut is sometimes compared to a second brain for is high number of nerve cells, whose operating system is very similar to the brain. The colonic ecosystem with billions of bacteria is also recognized to have major, immunological, hormonal, and physiological roles influencing many aspects of general health.

In the past decades, numerous *in vitro*, clinical works have done and reviews have detailed the beneficial effects of non-digestible oligosaccharides on animal and human health (Gibson, 2004; Grizard and Bartomeuf, 1999; Hirayama, 2002;

Mussato and Mancilha, 2007; Sangeetha et al., 2005b; Swennen et al., 2006). The interactions between dietary carbohydrates reaching the colon and microbiota have proved to be of a great importance.

After few days, the use of fructooligosaccharides in human or animal diets leads to changes in the balance of colonic bacteria in a favorable manner. Interestingly, one of the main potential benefits of increasing fecal bifidobacteria is that the bifidobacteria could maintain potentially pathogenic bacteria such as *Escherichia coli* and clostridia at low level.

For the metabolism of non-digestible oligosaccharides, bacteria produce glycolytic enzymes which hydrolyze them into mono and disaccharides. The sugars can then be transported into the cell where they can be further metabolized to short chain fatty acids (SCFAs), in particular, acetate, butyrate and propionate with a resulting acidification of the colon environment.

This pH decrease is beneficial for the development of bacteria able to metabolize Sc-FOS such as bifidobacteria or lactobacilli and detrimental to the growth of the species potentially involved in bowel diseases.

The SCFAs produced are rapidly absorbed by the hindgut and provide energy to colon cells, but are also important regulators of many physiological processes influencing gut health.

As hypothesized by various authors:

- Butyrate particularly plays a key role in the large intestine mucosa maintenance and the regulation of the epithelial cell renewal.
- Propionate is metabolized in the liver influencing hepatic gluconeogenesis, inhibiting ureagenesis and influencing cholesterol production.
- Acetate, the major SCFA in the gut, is directly used by muscle and influences cholesterol production.

The relative proportions of the different SCFAs depends on the type of carbohydrate entering into the colon. It was described, from *in vitro* cultures, animal experiments or gut models that Sc-FOS will lead to the production of high levels of butyrate. This influences the mucosa depth by increasing cell differentiation at the bottom of the villi and reducing apoptosis at their apex. As mentioned above, this mucosa is the first barrier against pathogens arriving from the gut content, and thus its integrity and thickness are of great importance.

This colonic ecosystem and gut component modifications lead to several benefits for the host. Physiological functions are classified into three types:

- The primary function, approved by FOSHU in Japan (Food of Specified Health Use) in 1993, encourages good gastrointestinal conditions including normal stool frequency, less constipation, healthy intestinal microbiota.
- The second function, approved by FOSHU in 2000, is related to better mineral absorption, including an increase in bone density and relief of anemia.
- The third concern involves immunomodulation, such as allergy, cancer prevention, effect on lipid metabolism. Studies on these functions are in progress, and FOSHU should approve some of them in the near future.

Unfortunately, some undesirable effects have also been described but the purpose of this section is to summarize the role of non-digestible oligosaccharides in promoting health and treating diseases so these negative aspects will not be dealt with here.

10.2.7.1 Improved Gastrointestinal Condition

As described previously, the metabolic end products of non-digestible oligosaccharides (NDOs) in the gut are SCFAs. SCFAs are efficiently absorbed by colonic epithelial cells, stimulating their growth as well as water and salt absorption, increasing the humidity of the fecal mass through osmotic pressure. This consequently improves the intestinal mobility allowing reduction in constipation.

Inhibition of diarrhea was also observed with fructooligosaccharide diets especially when diarrhea is associated with intestinal infection. *In vitro* studies confirm the potential indirect protective effect of fructans via bifidobacteria. It has been reported that bifidobacteria may secrete a bacteriocin-type substance against *E. coli, Salmonella, Listeria, Camylobacter, Shigella* and *Vibrio cholerae.* These results are in accordance with the fact that Sc-FOS reduces susceptibility to *Salmonella* colonization in chickens.

A decrease in putrefactive substances with an increase of bifidobacterial ratio and SCFA amount was also measured. As a practical application, Sc-FOS has been added to the feed of livestock and pet animals to decrease fecal odor.

10.2.7.2 Effect on Mineral Absorption

Several mechanisms have been postulated for increased mineral absorption induced by Sc-FOS.

SCFAs produced by bifidobacteria lead to a reduction of lumen colonic pH, increasing calcium and magnesium solubility, mucosal surface production and absorptive capacity of the epithelium.

It is also likely that NDOs increase the water content in the colon and thus increase the solubility of some minerals.

This has been confirmed by a long-term beneficial effect of bone mineral content in growing rats or prevention of bone loss in ovarectomized rats. The addition of 5% fructooligosaccharides prevented bone loss significantly in femur and lumbar vertebra in the presence of 1% calcium. At 0.5% calcium in diet, 10% Sc-FOS was necessary to significantly increase bone mineralization.

Improved calcium and magnesium absorption in adolescents and/or menopausal women has been demonstrated for Sc-FOS.

A better absorption of iron was also demonstrated in rats, this supports the claim that Sc-FOS diet can relieve anemia caused by malabsorption of iron.

10.2.7.3 Modulation of the Immune System

The mucosal barrier is a complex structure that physically separates the internal milieu from the environment in the digestive tract. It includes an epithelium covered in a vascular system and also mucus inhabited by intestinal bacteria, which all play a role in host defense. To protect against infection, the human intestine can benefit from three lines of defence, namely the gut mucosa itself, the gut lymphoid-associated tissue and the gut microbiota.

Feeding mice with diets supplemented with Sc-FOS increased the activities of natural killer cells and phagocytes and enhanced lymphocyte functions compared to mice fed a diet without Sc-FOS (Sangeetha et al., 2005b).

Decrease in the Risk of Colon Cancer

Diets are well known to be a major factor influencing colon cancer prevalence. Diets containing high concentrations of animal protein have been associated with greater risk.

In agreement with the beneficial effect of fruits and vegetables and the positive effect on colon cancer preservation, research suggested that NDOs such as fructooligosaccharides can decrease the entire process of colon cancer development (Swennen, 2006).

This study showed that Sc-FOSs induced a decrease or even a suppression of colon tumor in Min mice. This effect may have been related to stimulation of gut-associated lymphoid tissue, owing to the development of Peyer's patches.

This anticarcinogenic effect appears to be relative to cellular immunity

More generally FOSs were described as fermentable fibers providing protection against earlier stages of colon carcinogenesis.

- Butyrate produced in the colon is known to stimulate apoptosis in colonic cancer cell lines.
- Health promoting bacteria inhibit the growth of pathogenic bacteria and thus decrease the production of carcinogenic substances and bacterial enzymes that play a role in colon carcinogenesis (e.g., bile acid, p-cresol).
- At the same time, bacterial growth accelerates the colonic transit time. As a result, the duration of exposure to potential carcinogens decreases.

These are still results on animal studies, but now several clinical studies on preventing cancer by dietary fibers are under throughout the world.

10.2.7.4 Effect on Lipid Metabolism

The first data on the effect of NDOs on serum cholesterol and lipid level suggested that they have hypocholesterolemic and hypotriglyceridemic effects (Grizard and Bartomeuf, 1999). Besides its effect on the intestinal tract, FOS was described to exert a systemic effect, namely by modifying the metabolism of lipids in several animal models.

FOS were described to reduce postprandial triglyceridemia by 50% and avoid the increase in serum free cholesterol levels occurring in rats fed with a western-type high-fat diet. Changes in the concentration of serum cholesterol have been related with changes in the intestinal microbiota. Some strains of *Lactobacillus* spp. have been described to assimilate cholesterol while others appear to inhibit the absorption of cholesterol through the intestinal wall (Mussato and Mancilha, 2007).

The change in lipid metabolism was also suggested to be a consequence of the action of propionate on the liver.

However, these results were modulated by Giacco et al. (2004). In individuals with mild hypercholesterolemia, they showed that moderate intake of Sc-FOS (10.8 g/kg) had no major effects on lipid metabolism (either in the fasting or in the postprandial period). However, a small but significant increase in lipoprotein

A concentration was observed with Sc-FOS consumption together with a reduction of the postprandial insulin response. The clinical relevance of these small effects is unclear.

Human studies on the lipid-lowering properties of prebiotics when consumed at a moderate dose are sometimes contradictory, and the role in lipid reduction remains to be confirmed.

In order to quantify the effects in humans of dietary 1F-type fructans on serum triacylglycerols, Brighenti (2007) conducted a meta-analysis of the available literature. Fifteen eligible randomized, controlled trials published from 1995 to 2005, for a total of 16 comparisons, were identified from databases. Standardized mean effect sizes were calculated for net changes in serum triacylglycerol concentrations using a random-effect model.

The intake of fructans was associated with significant decreases in serum triacylglycerols by 0.17 mmol/L or 7.5%. Given the limited number of studies, no specific effects for gender, amount fed, duration of the study, background diet, overweight, hyperlipidemia, or diabetes were further formally investigated. But, from the test for heterogeneity it appears that the effect of inulin-type fructans on circulating triacylglycerols is consistent across conditions. In conclusion, dietary 1F-type fructans significantly reduced serum triacylglycerols. The mechanisms, possibly related to colonic fermentation and/or incretin release from the distal gut, warrant further studies.

Role of FOS in Blood Glucose Control

It was found (Luo et al., 2000) that the daily consumption of 20 g FOS decreased basal hepatic glucose production in healthy subjects without any effect on insulin-stimulated glucose metabolism.

This study allowed authors to evaluate the effects of the chronic ingestion of FOS on, plasma, lipid and glucose concentrations but also hepatic glucose production and insulin resistance in type 2 diabetics. Contrary to other studies, the results showed that 4 weeks of 20 g/d of FOS had no effect on glucose or lipid metabolism in type 2 diabetics. The subjects had had type-2 diabetes for a long time, so their metabolism might not be modified as easily as that of healthy subjects.

10.2.7.5 Undesirable Effects

In spite of the above, Sc-FOS have some undesirable effects. As other non-digestible carbohydrates, they have osmotic properties and are extensively

fermented in the large bowel. As consequence, at high daily doses (25–30 g/day), they may cause flatulence and or function as a laxative. These effects are clearly dose related and may vary significantly from one individual to another.

10.2.8 Applications of Fructooligosaccharides

Considering all the claims detailed above combined and the physiochemical properties of fructooligosaccharides, their use as food ingredients has increased rapidly.

Development of synbiotics combining FOS with targeted probiotic strains or other NDOs, are increasingly described. These synbiotics promote the growth and/or activate the metabolism of various health promoting bacteria.

Fructooligosaccharides are used in dairy products for sugar reduction and prebiotic effects. They are also associated with probiotic bacteria in yoghurts and fermented drinks. In dairy products, FOS improves the mouth feel by increasing unctuousness, which is appreciated in low-fat reduction creams, sorbets, chocolate, mousse, milk, etc.

Other applications in food industries include desserts such as, fruits preparations, confectionary, cereals, bakery products, biscuits, sweet, gums, soup, salad dressing.

Fructooligosaccharides also find uses in processed meat, canned fish, sauces and pet food with health and nutrition claims.

In addition, fructooligosaccharides are used for health benefits in feed for many animals including: broilers, layers, rabbits, calves, lambs, kids, piglets, sows, fattening pigs, pets (dog, cat), horses and fish.

10.2.9 Market

Due to differing definitions, there are specific difficulties to analyze the development of the Functional Foods market, resulting in strongly varying estimations concerning the market volume of such products.

The global market for Functional Foods was estimated in 2000 at around US $ 33 billion. The largest and moat dynamic market is the USA with an estimated market share of around 50%.

Japan is another important market for functional foods, with a specific health related food category called FOSHU. An estimated market value of FOSHU was US$ three billion in 2000 with an estimated turnover of 14 billion US$.

Sales of functional food and drink in Europe have experienced considerable growth, with a doubling of sales between 2000 and 2005. In 2005 the value of European sales of functional foods was US$ eight billion and likely to reach in excess of ten billion euros by 2010.

Prebiotics concern a very small part of the functional food market but this is growing rapidly. Prebiotics are associated with breakfast cereals, baked goods, cereal bars, baby food as well as some dairy products. The main commercial food oligosaccharides (DP 2–10), used as prebiotic agents, are essentially obtained by enzymatic technology. They include non-digestible oligosaccharides such as inulin, fructooligosaccharides, galacto-oligosaccharides, lactulose, isomalto-oligosaccharides, soybean oligosaccharides, xylo-oligosaccharides.

In 2007 an analysis by Frost and Sullivan found that the US prebiotics markets, earned revenues of US$ 69 million, estimated to reach 198 million in 2014.

In Japan the demand for prebiotic oligosaccharides was 69,000 tons/year. Among these oligosaccharides the demand for starch oligosaccharides was the largest. The fructooligosaccharide market was only 6.5% of the global demand of prebiotics (Nakakuki, 2002). FOS products were first approved as food for specified health uses in 1993 and are sold as functional ingredients in Japan.

In Europe, total sales of functional food was estimated in 2005 at around eight billion euros while the prebiotics sector was valued at 87 million euros, with expectations to reach 180 million euros in 2010 (Source: Frost and Sullivan). Currently, the European market is believed to be dominated by fructans and galacto-oligosaccharides. Resistant starch products are at the development stage. The production of prebiotic fibers in Europe is estimated of about 30,000 tons, including inulin, oligofructose, FOS, GOS and lactulose.

FOS are available worldwide, mainly in East Asia, North America and Europe as prebiotics and/or dietary fiber like oligosaccharides. Market growth of oligosaccharides is about 15% per year.

In 1984, Meiji Seika Co. first succeeded in the commercial production of FOS using *Aspergillus niger*. The product was marketed in Japan under the name "Meioligo." This company later established a joint venture with Beghin-Say from France called Beghin-Meiji-Industry. They produced FOS under the trademark "Actilight."

Some other companies produced fructooligosaccharides from sucrose, Golden Technology (USA) under the trade mark NutaFlora and Cheil Food and Chemical Co (Korea) with Oligo-Sugar.

The global market for fructooligosaccharides from sucrose was estimated in 1995 to be 20,000 tons.

10.3 Lactosucrose From Sucrose

Lactosucrose is a functional trisaccharide consisting of D-galactose, D-glucose and D-fructose (O-β-D-galactopyranosyl-(1–4)-O-α-Dglucopyranosyl-(1–2)-O-β-D-frucofuranoside) used as an artificial sweetener. It is 30% as sweet as sucrose. It is produced enzymatically by the enzymatic transfer of the fructofuranosylsyl residue of sucrose to lactose. This fructofuranosyl transfer was principally described with bacterial enzymes from *Arthobacter sp, Bacillus sp, Klebsiella pneumonia* and *Rahnella aquatilis*. Lactosucrose can be obtained either by trans-fructosylation reaction catalyzed by β-D-fructofuranosidase or levansucrase in which lactose is used as a fructofuranosyl acceptor and sucrose as fructofuranosyl donor.

10.3.1 Lactosucrose Production

10.3.1.1 Lactosucrose Synthesis Using Bacterial β-D-Fructofuranosidase

Production of lactosucrose (4G-β-D-galactosylsucrose) using β-fructofuranosidase from *Arthrobacter* sp K-1 was first described by Fujita et al. (1990) who investigated the transfructosylation reaction with sucrose and various acceptors such as lactose or xylose. When incubated with sucrose alone, this enzyme catalyzed both transfructosylation and hydrolysis. But in the presence of a suitable acceptor such as lactose, the enzyme catalyzed mostly transfructosylation and transferred the fructose residue preferentially to the C1-position of the glucose moiety of lactose, producing a non-reducing oligosaccharide.

Lactosucrose transfer ratio with 10% (w/v) sucrose and 20% (w/v) lactose concentrations was around 50% with lactosucrose yield close to 27% (w/w) of total sugar.

More recently, Pilgrim et al. (2001) once more studied lactosucrose synthesis from sucrose and lactose by using β-fructofuranosidase from *Arthrobacter* spp. K-1. The transfructosylation mechanism was studied and reaction rate constants, equilibrium constants, dissociation Michaelis constants, glucose and sucrose inhibition were determined at 35°C and 50°C. The transfer reaction was found to be of an ordered bi-bi type in which sucrose first bound to the enzyme and lactosucrose was released last. The maximum yield attainable in a batch process with equimolar concentrations of substrates was reported to be around 50%.

This group also investigated (Kawase et al., 2001) lactosucrose production using a simulated moving bed. Simulation of the batch process showed that product removal could increase the lactosucrose yield from 50 to over 80%. Unfortunately, the expected optimum was not attained due to strong product hydrolysis around the raffinate outlet port.

10.3.1.2 Lactosucrose Synthesis Using From Bacterial Levansucrase

Production of lactosucrose by levansucrase from *Aerobacter levanicum* was first described by Avigad (1957) who reported the synthesis of a new trisaccharide, lactosucrose (α-lactosyl-β-fructofuranoside), by levansucrase acting on a system comprised of sucrose as fructosyl donor and lactose as acceptor.

Later, production of lactosucrose, by various microorganisms containing levansucrase activity was also investigated (Park et al., 2005). Among the tested bacteria, *Bacillus subtilis* was the most effective producer using lactose as an acceptor and sucrose as a fructosyl donor. Lactosucrose production by this strain was optimal at pH 6.0 and 55°C where from 225 g/l lactose and 225 g/l sucrose in 10 h lactosucrose yield was 32.9% (w/w).

Another way to produce lactosucrose was described by Choi et al. (2004). In this case, whole cells harboring transfructosylation activity of levansucrase was used. Levansucrase-induced cells of *Paenibacillus polymyxa* were obtained in the medium containing sucrose, and the transfructosylation activity in the whole cell was optimized for lactosucrose production. The optimal cell concentration was 2.0% (w/v) with substrate ratio of lactose and sucrose 22.5% (w/v) each, 55°C, and pH 6.0. Under these conditions, lactosucrose yield was 30.9% of total sugar with a productivity of 2.8% (w/v)/h.

10.3.2 Functional Properties of Lactosucrose

Lactosucrose was described to be resistant to digestion in the stomach and small intestine.

It was reported to be selectively utilized by intestinal *Bifidobacterium* species resulting in significant induction in the growth of these bacteria in the colon. It is widely used in Japan as a dietary supplement and in functional foods, including yoghurt. Lactosucrose is being developed in the United States for similar uses (Park et al., 2005).

However, Park et al. (2008) concluded, with regard to *in vitro* and clinical works, "the evidence for prebiotic status of lactosucrose is still not sufficient and lactosucrose cannot, at present, be classified as a prebiotic."

10.4 Glucooligosaccharides

A wide range of non-digestible glucooligosaccharides can be synthesized from sucrose using a variety of glucosyltransferases with different transglucosylating specificities. Such enzymes, named glucansucrases (E.C. 2.4.1.X), catalyze the synthesis of high molecular weight glucan polysaccharides (Monsan et al., 2000) following the reaction:

$$n\ G - F \xrightarrow{\text{Glucansucrase}} (G)_n + n\ F$$

The specificity of the main glucansucrases is described in *Table 10.1*. The structure of corresponding glucan polymers is given in *Figure 10.3*.

This reaction involves the formation of an intermediary D-glucopyranosyl-enzyme covalent intermediate (*Figure 10.4*). In the presence of an efficient

Table 10.1

Glucansucrases and their regiospecificity

Glucansucrase	Glucan	Osidic linkages
Dextransucrase (E.C. 2.4.1.5)	Dextran	α-1,6 (>50%)
Mutansucrase (E.C. 2.4.1.5)	Mutan	α-1,3 (>50%)
Alternansucrase (E.C. 2.4.1.140)	Alternan	Alternating α-1,3/ α-1,6
Amylosucrase (E.C. 2.4.1.4)	Amylose	α-1,4 (100%)

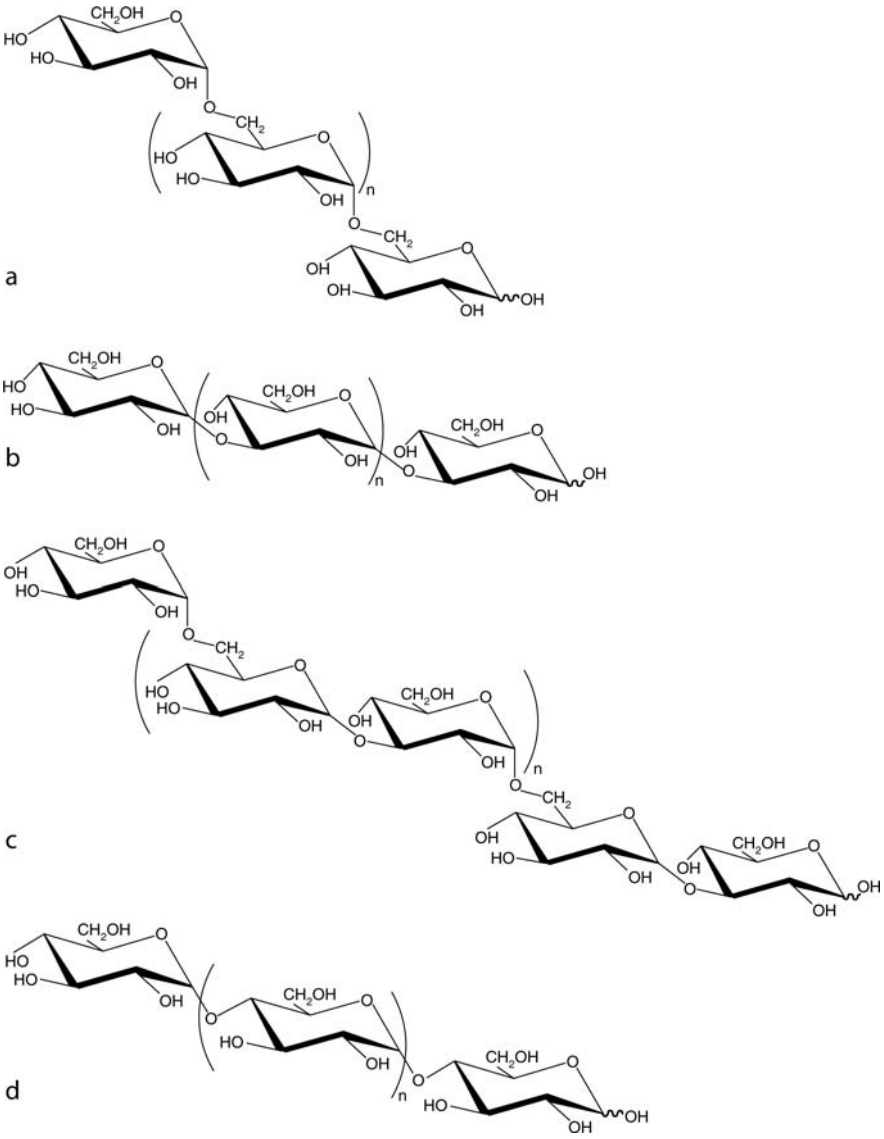


Figure 10.3
Structural representation of different types of glucans synthesized by glucansucrases (a) dextran, (b) mutan, (c) alternan, (d) amylose.

carbohydrate acceptor, like maltose, the polymerization reaction is limited, and low molecular weight glucooligosaccharides (*Figure 10.4*) are obtained (Koepsell et al., 1952). Very interestingly, in such acceptor reactions, glucansucrases present the same regiospecificity as for glucan polymer synthesis.

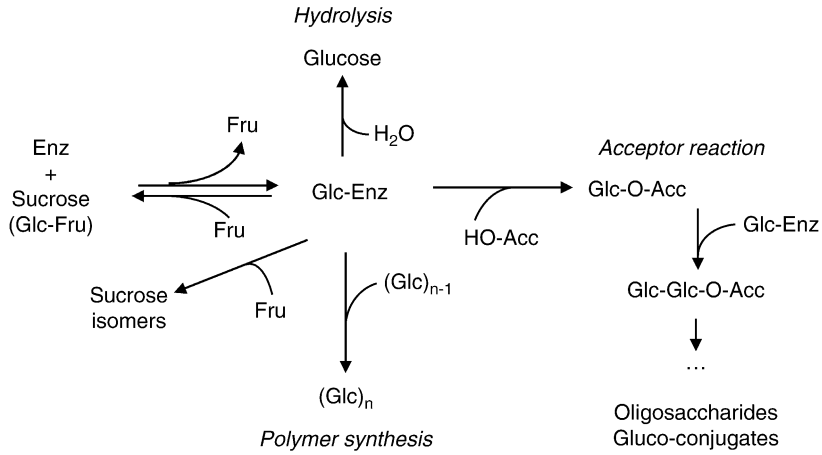


Figure 10.4
Different reactions catalyzed by glucansucrases.

This results in the possibility to use sucrose to directly synthesize several types of glucooligosaccharides of interest for prebiotic applications.

In addition, the D-glucopyranosyl residue can be transferred onto water (sucrose hydrolysis) or onto a non-carbohydrate acceptor to yield a gluco-conjugate (*Figure 10.4*).

10.4.1 Dextransucrases

Dextransucrases are extracellular enzymes produced by lactic acid bacteria of the genera *Leuconostoc*, *Streptococcus*, *Lactococcus* and *Lactobacillus* (Monsan et al., 2001). They belong to Family 70 of glycoside-hydrolases (Moulis et al., 2006). They share a general common structure consisting of (*Figure 10.5*):

- An N-terminal signal peptide involved in their excretion
- A variable region of unknown role
- A highly conserved N-terminal catalytic domain which contains the amino acids involved in the catalytic mechanism and presents a permutated $(\beta/\alpha)_8$ barrel structure (Moulis et al., 2006)
- A C-terminal glucan binding domain, involved both in polysaccharide and oligosaccharide synthesis, and containing a series of repeating amino acid sequences (Moulis et al., 2008)

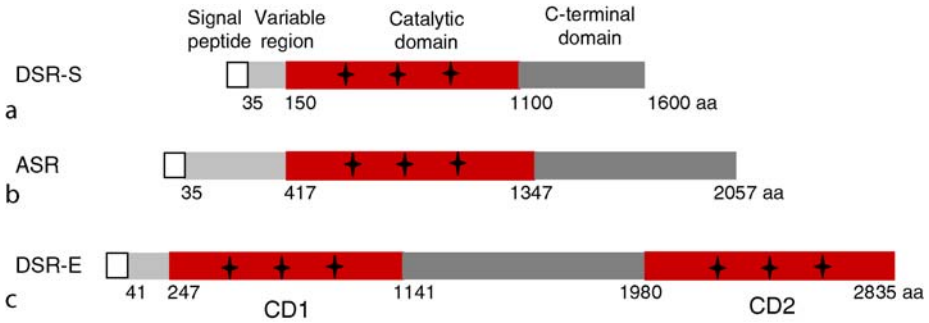


Figure 10.5

Structural organization of glucansucrases (a) dextransucrase from *Leuconostoc mesenteroides* NRRL B-512F, (b) alternansucrase from *Leuconostoc mesenteroides* NRRL B-1355, (c) dextransucrase from *Leuconostoc mesenteroides* NRRL B-1299. The position of catalytic amino-acids is indicated.

The dextransucrase from *Leuconostoc mesenteroides* NRRL B-512F is widely used for producing industrial dextran. It is a large protein of 1,527 amino acids. After acidic hydrolysis, dextran is mainly used for chromatograpy support (Sephadex®) production, as well as blood plasma substitutes and iron carriers (dextran-sulphate).

The *in vitro* utilization by human gut microbiota of the following carbohydrates:

- Industrial-grade dextran
- Oligodextran fractions produced by controlled enzymatic hydrolysis of dextran (Mountzouris et al., 1999)
- Maltodextrin

was studied using anaerobic batch culture fermenters (Olano-Martin et al., 2000). Glucose and fructooligosaccharides were used as reference carbohydrates. Fructooligosaccharides selectively increased numbers of bifidobacteria in the early stages of fermentation. Dextran and oligodextran resulted in an enrichment of bacteria with high levels of persistence up to 48 h, with production of elevated levels of butyrate ranging from 5 to 14.85 mmol/l. A three-stage continuous culture cascade system was used for a more effective simulation of the conditions that prevail in different regions of the large intestine. A low-molecular-mass oligodextran fraction was then shown to be the best substrate for bifidobacteria and lactobacilli, when compared to dextran and maltodextrin. In addition,

dextran and oligodextran stimulate butyrate production more efficiently, which has been shown to present potentially very interesting anti-neoplastic properties (Olano-Martin et al., 2000). These results underline the interest of oligodextrans as modulators of the gut microbiota.

In the presence of glucose or isomaltose as acceptors, dextransucrase synthesizes a series of glucooligosaccharides of the isomaltosaccharide type (IMOs). Such partially non-digestible oligosaccharides are presently the main prebiotics produced for the Japanese market (Monsan and Auriol, 2004). They are obtained from starch hydrolysates (maltooligosacharides) by the action of an α-transglucosidase from *Aspergillus* spp. They only present short chain oligosaccharides: commercial preparations contain (Mountzouris et al., 1999) a mixture of isomaltose (DP 2: 23%), isomaltotriose (DP 3: 17%), oligosaccharides (DP 4–6: 26%), non-isomalto-oligosaccharides (panose, maltose, maltotriose, nigerose, kojibiose: 30%).

Recently, the possibility to engineer dextransucrase at the molecular level by truncating its C-terminal domain has resulted in variants able to directly synthesize isomaltooligosaccharides of controlled molecular weight. Synthesis yields of up to 69 and 75% have been obtained for 40 and 10 kDa dextran, respectively (Moulis et al., 2008). This opens the way to the direct production of prebiotic IMOs from sucrose, and particularly with increased chain lengths to limit their digestibility.

The dextransucrase from *Leuconostoc mesenteroides* NRRL B-1299 produces a dextran containing about 30% α-1,2 linkages (Monsan et al., 2000). This type of glycosidic bond is still synthesized by the enzyme when maltose is used as an acceptor (Remaud-Siméon et al., 1994). A mixture of three families of oligosaccharides is then obtained, which contain a maltose residue at the reducing end and either (1) only α-1,6 linkages (series OD), (2) α-1,6 linkages and one α-1,2 linkage at the non-reducing end (series R), or (3) α-1,6 linkages and one α-1,2 linkage on the penultimate D-glucosyl residue (series R′, *Figure 10.6*). The corresponding glucooligosaccharides (GOS) are highly resistant to attack by digestive enzymes from humans and many other animals (Valette et al., 1993), and are not metabolized by germ-free rats (Djouzi et al., 1995). They induce a broad range of glycolytic enzymes without increased production of gases (Djouzi and Andrieux, 1997). Bifidobacteria, lactobacilli and particularly bacteroides specifically utilize such GOS as carbon source (Monsan and Auriol, 2004).

Dextransucrase is obtained by fed-batch culture of *L. mesenteroides* NRRL B-1299, using sucrose as carbon source and specific enzyme inducer (Dols et al., 1997a). As sucrose is also the substrate of the extracellularly

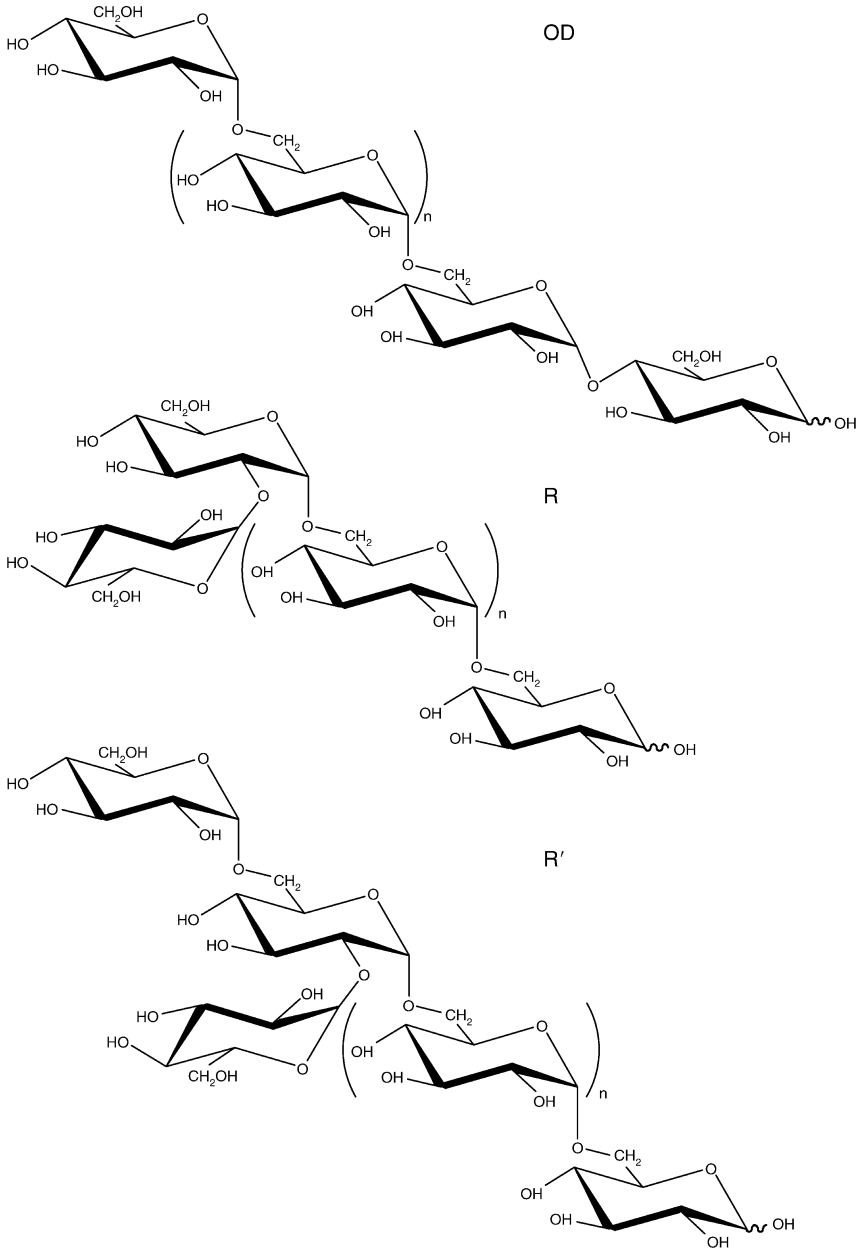


Figure 10.6

Structure of the glucooligosaccharides synthesized by the dextransucrase from *Leuconostoc mesenteroides* NRRL B-1299 from sucrose when using maltose as acceptor: series OD, series R and series R′.

obtained dextransucrase, dextran is synthesized during microbial growth and D-fructose is produced. The presence of the D-fructose results in the repression of dextransucrase production. This repression effect can be suppressed in the presence of D-glucose. The simultaneous fed-batch addition of sucrose and D-glucose thus increased the dextransucrase activity obtained in the culture medium by 100% to reach a final activity of 9.7 U/ml (Dols et al., 1997b). Very fortunately, more than 90% of this dextransucrase activity is associated to the cells and the surrounding dextran slime. It can thus be very easily recovered from the culture medium by centrifugation.

To develop a continuous process for glucooligosaccharide synthesis, the dextransucrase must be immobilized. This can be easily done, thanks to the strong association of dextransucrase with dextran slime and cells, by simply entrapping the catalytic activity within calcium alginate beads. The immobilization yield is 93%, and a specific activity of 4.1 U/ml of gel can be obtained (Dols et al., 1997b). The present industrial production is of 60 tons per year, and is obtained by operating the immobilized dextransucrase in a 1-m^3 continuous packed-bed reactor. The key parameter for efficient operation is the sucrose-to-maltose ratio, which controls both the glucooligosaccharide yield and the size distribution (Dols et al., 1997b). Sucrose conversion at the outlet of the reactor is 100%.

Roberfroid (2008a,b) stressed the fact that this type of oligosaccharide cannot be classified as a prebiotic, due to the lack of evaluation in humans. In fact, these GOS are produced on the industrial scale essentially for cosmetic application, to stimulate the growth of beneficial skin microorganisms (Lamothe et al., 1994).

It is increasingly accepted that the concept of prebiotic need not only apply to the gastrointestinal microbiota. It could also be applied to any other microbiota, like the skin microbiota or the vaginal microbiota, for example. Different oligosaccharides have been evaluated for their prebiotic effect using selected human vaginal lactobacilli presenting a high level of hydrogen peroxide production, and pathogenic microorganisms (Rousseau et al, 2005).

In any case, the evidence of a prebiotic effect is clear in animal trials, e.g., the administration of 0.15% (w/w) GOS to young calves (two populations of 1,300 animals) resulted in 20% decrease in veterinary costs (Monsan and Auriol., 2004), without any side effects.

In addition, it was demonstrated that GOS prevent the installation of type II diabetes in over-fed mice. Female C57/Bl6/J mice were fed with a high-fat diet (45% fat, 35% carbohydrate, 20% protein) supplemented or not with 1.5 g/kg/day of GOS. The GOS supplementation did not change body weight nor fat pad mass,

nor any of the blood parameters measured (glucose, insulin, leptin, triglycerides, and free fatty acids). Mice which received the GOS supplemented diet showed increased glucose utilization after a 1 g/kg load of glucose compared with the mice fed the high-fat diet alone (Boucher et al., 2003).

It must be underlined that the effect of the glycosidic linkage involved in a wide range of disaccharides upon selectivity of fermentation by intestinal bacteria has been recently determined, showing that α-1,2 linkages are particularly selective (Sanz et al., 2005).

The originality of the dextransucrase from *Leuconostoc mesenteroides* NRRL B-1299 is to present not only one, but two catalytic sites (*Figure 10.3*): one is specific for α-1,6 linkage synthesis and is located at the N-terminal end, while the second one is specific for α-1,2 linkage synthesis and is located at the C-terminal end (Bozonnet et al., 2002). The N-terminal truncation of the native corresponding gene results in the design of a new enzyme able to catalyze the controlled synthesis of α-1,2 branched oligodextrans, with different sizes and branching degrees (Fabre et al., 2005). These products are presently under evaluation for their prebiotic properties.

10.4.2 Alternansucrase

When grown on sucrose, *Leuconostoc mesenteroides* NRRL B-1355 produces a glucosyltransferase, alternansucrase, which catalyses the synthesis from sucrose of a glucan containing alternating α-1,3 and α-1,6 linkages, alternan (Côté and Robyt, 1982a; Jeanes et al., 1954; Lopez-Munguia et al., 1993; Seymour et al., 1979).

The corresponding encoding gene was independently isolated by two groups (Argüello-Morales et al., 2000; Kosmann et al., 1999). Alternansucrase is a 2,057 amino acid enzyme, with a molecular weight of 245 kDa. Its structural organization (*Figure 10.5*), with an N-terminal catalytic domain and a C-terminal glucan binding domain containing amino-acid repeats, is similar to that of most of the glucansucrases of Family GH-70 of the glycoside-hydrolases (Argüello-Morales et al., 2000).

In the presence of acceptor carbohydrates, such as maltose, alternansucrase presents the same specificity as for alternan synthesis and catalyses the production of oligoalternans (Argüello-Morales et al., 2001; Côté and Dunlap, 2003; Côté and Robyt, 1982b; Pelenc et al., 1991). Such oligosaccharides have been demonstrated to efficiently control enteric bacterial pathogens (Côté and Holt, 2007; Holt et al., 2005).

Gentiobiose was also used as an acceptor for the synthesis of oligosaccharides with alternansucrase. These products were compared to gentio-oligosaccharides (containing D-glucopyranosyl units linked with β-1,6 linkages) to study their effect on the growth of predominant gut bacteria. A prebiotic index (PI) was calculated to obtain a general quantitative comparative measurement of the selectivity of fermentation and to compare the influence of size and structure in the selective fermentation. This index sets a relationship between changes in the "beneficial" and "undesirable" elements within the microbiota (Sanz et al., 2006a). DP4–5 alternansucrase gentiobiose acceptor products generated the highest PI values (PI of 5.87). Regarding the production of short-chain fatty acids, the mixture of DP6–10 gave the highest levels of butyric acid but the lowest levels of lactic acid. From a general point of view, for similar molecular weights, alternansucrase gentiobiose acceptor products gave higher PI values than gentio-oligosaccharides (Sanz et al., 2006a).

The influence of glucosidic linkages and molecular weight on the fermentation by gut bacteria of maltose-based oligosaccharides synthesized by alternansucrase and dextransucrase has been determined (Sanz et al., 2006b). When using an anaerobic *in vitro* fermentation method, DP3 oligosaccharides showed the highest selectivity. Oligosaccharides with higher molecular weight (DP6–7) also resulted in selective fermentation. Oligosaccharides with DP above seven did not promote the growth of beneficial bacteria.

The C-terminal domain of alternansucrase is composed of two different series of homologous repeating units, the CW-like repeats and the APY-repeats (Joucla et al., 2006). The CW-like repeats are 20-amino-acid-long motifs with a high representation of conserved glycine and aromatic residues. APY-repeats are 79-amino-acid-long motifs, with a high number of conserved glycine and aromatic residues, specifically characterized by the presence of the three consecutive residues alanine, proline and tyrosine. Seven APY repeats are present within the last 550 C-terminal amino acids. Fully active variants of alternansucrase, truncated of its C-terminal glucan-binding domain have been successfully designed (Joucla et al., 2006): the truncation of the APY repeats, keeping four CW-like repeats, results in a variant with a molecular weight of 175 kDa. This variant presents a high specific activity and the same specificity of product synthesis as the native enzyme. In addition, it is more soluble and suffers less degradation when the corresponding gene is expressed in *E. coli* than full length alternansucrase (Joucla et al., 2006).

Alternansucrase is used by Cargill (Carlsson et al., 2006) to produce from sucrose and maltose a sweetening prebiotic mixture, Xtend™ Sucromalt,

containing residual fructose (37%), leucrose (13%) and alternan oligosaccharides (48%).

10.4.3 Amylosucrase

Amylosucrase catalyses the transfer of D-glucopyranosyl units from sucrose to synthesize only α-1,4 linkages. This reaction yields highly pure amylose chains. The gene coding for the amylosucrase from *N. polysaccharea* has been isolated, cloned and sequenced (Potocki de Montalk et al., 1999). Amylosucrase has been purified to homogeneity as a fusion protein with glutathion-S-transferase, using glutathion-Sepharose-4-B affinity chromatography. Its molecular mass is 70 kDa.

In the presence of sucrose alone, amylosucrase simultaneously catalyses sucrose hydrolysis, and maltose and maltotriose synthesis (using D-glucose as acceptor), and high molecular weight glucan synthesis (containing only α-1,4 linkages). Very surprisingly, amylosucrase is activated by high sucrose concentrations: while Vmax and Km values are equal to 510 U/g and 2 mM respectively when sucrose concentrations are lower than 20 mM, they are equal to 906 U/g and 26 mM respectively when sucrose concentrations are higher than 20 mM (Potocki de Montalk et al., 1999).

In the presence of sucrose and glycogen, amylosucrase is highly activated: at 105 mM sucrose, the addition of 30 g/l glycogen results in a 100-fold increase in amylosucrase activity (Potocki de Montalk et al., 1999). This activator effect decreases when increasing sucrose concentration, suggesting a competition between sucrose and glycogen. This reaction results in the obtention of modified glycogen, which contains extended linear chains with an average DP of 75 D-glucosyl units according to iodine-complex characterization.

This enzyme is the only glucansucrase for which the 3D structure has been solved (Skov et al., 2001). It belongs to Family 13 of glycoside-hydrolases, which is the family of α-amylases and CGTases. The amino-acid residues involved in the catalytic mechanism of D-glucopyranosyl unit transfer have been identified (Albenne et al., 2003). The structure of the α-glucan (Rolland-Sabaté et al., 2004) and amylose elongation products has been determined. Such amylose products present a nanoparticle size and are highly resistant to the attack of digestive enzymes, which corresponds to a new range of resistant nano-starches (Potocki-Véronèse et al., 2005).

The German company Südzucker is presently developing the production of such resistant amyloses, obtained by amylosucrase action, under the brand name NEO-amylose® (Südzucker 2005/2006).

10.5 Summary

- Sucrose is not a simple carbohydrate, but a protected and activated form of D-glucose and D-fructose
- Fructosyl transferases catalyze the synthesis of a variety of fructans and fructooligosaccharides using sucrose as a D-fructofuranosyl unit donor
- When using a fungal fructosyl transferase as catalyst, non-digestible prebiotic short-chain fructooligosaccharides (Sc-FOS) containing β-2,1 linkages are mainly obtained: kestose (GF2), nystose (GF3) and fructosyl-nystose (GF4)
- Sc-FOS are very efficient prebiotics which are produced on the industrial scale in continuous plug-flow reactors containing immobilized fructosyl transferase from *Aspergillus oryzae*
- Lactosucrose is a functional trisaccharide obtained by transfer of a β-D-fructofuranosyl residue from sucrose onto the reducing group of lactose, catalysed by a fructofuranosidase or a levansucrase
- Glucosyltransferases catalyze the synthesis of a variety of glucans and glucooligosaccharides using sucrose as D-glucopyranosyl unit donor
- In the presence of efficient carbohydrate acceptors, like maltose, glucosyltransferases catalyze the synthesis of low-molecular-weight oligosaccharides instead of high molecular weight polymers (acceptor reaction)
- Dextransucrase, alternansucrase and amylosucrase are microbial glucosyltransferases (transglucosidases) which can be efficiently used to synthesize non-digestible prebiotic carbohydrates, glucooligosaccharides (GOS), oligoalternans, and amylose respectively.

List of Abbreviations

Brix	degrees brix, sucrose concentration in % (w/w)
BVH	bed volume per hour
Da	daltons
DP	degree of polymerization
F	fructose
FOS	fructooligosaccharides
FOSHU	food of specified health use in Japan
G	glucose
GF	sucrose

GF2	1-kestose
GF3	nystose
GF4	fructosyl-nystose
G(n)	dextrans
Kda	kilo daltons in g/mole
NDOs	non-digestible oligosaccharides
SCFAs	short chain fatty acids
Sc-FOS	short chain fructooligosaccharides
w/w	weight per weight
w/v	weight per volume

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11 Prebiotic Potential of Polydextrose

Julian D. Stowell

11.1 Introduction

Polydextrose is a randomly bonded polymer of glucose with some sorbitol end-groups. It was originally developed by scientists at Pfizer seeking a low calorie bulking agent to be used in conjunction with intense sweeteners. Polydextrose has been used for more than 25 years in human food and beverage products around the world. It is currently marketed by Danisco A/S as Litesse® Two and Litesse® Ultra™ and by A E Staley as Sta-Lite III.

11.2 Characteristics of Polydextrose

11.2.1 Manufacture and Structure

Polydextrose is produced by the bulk melt polycondensation of glucose and sorbitol in conjunction with small amounts of food grade acid in vacuo. Typically, corn glucose is used. Further purification steps are then involved to generate a range of products suited to different applications. A representative structure of polydextrose is given in *Figure 11.1*. Recent studies have shown that polydextrose has a highly branched structure with a wide spectrum of glycosidic linkages represented, 1–6 linkages predominate. The average degree of polymerization (DP) is 12 glucose units. However, a complete spectrum of DPs up to 30 and above have been detected in the polydextrose mix (Stowell 2009).

Polydextrose is manufactured and marketed in accordance with the Food Chemical Codex Specification (Anon 2004). AOAC Method 2000.11 is used to measure polydextrose in foods (Anon 2002).

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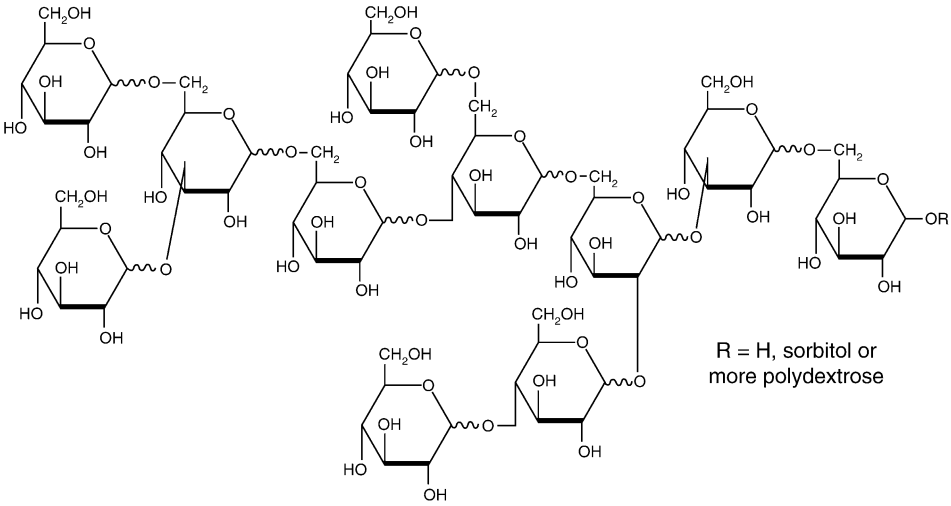

Figure 11.1
Representative structure for polydextrose. R = H, sorbitol, sorbitol bridge, or more polydextrose.

11.2.2 Safety

The safety of polydextrose in the human diet has been comprehensively demonstrated (Burdock and Flamm 1999). The data have been examined by many expert national and supra-national panels. Both the Joint Food and Agriculture Office of the United Nations (FAO) and World Health Organization (WHO) Expert Committee on Food Additives (JECFA) and the European Commission, Scientific Committee on Food (EC/SCF) have assigned an acceptable daily intake (ADI) "not specified", meaning that polydextrose can be added to foods at the level required to achieve the desired functionality (JECFA 1987; EC/SCF 1990).

11.2.3 Tolerance

Nine clinical studies have been conducted with polydextrose to evaluate the extent of gastrointestinal symptoms in the lower gastrointestinal tract. These studies showed that polydextrose is better tolerated than most other low digestible carbohydrates. This is to be expected as polydextrose has a higher molecular weight, leading to a lower risk of osmotic diarrhea and also it is only partially

fermented (see below). Having evaluated the data JECFA and EC/SCF concluded that polydextrose has a mean laxative threshold of about 90 g/day (1.3 g/Kg bodyweight) or 50 g as a single dose (Flood et al. 2004).

11.2.4 Polydextrose as a Bulking Agent

Polydextrose is resistant to digestion in the upper gastrointestinal tract and is partially fermented in the large intestine producing volatile fatty acids. Approximately 50% of the glucose equivalents of polydextrose are excreted. A number of energy balance and isotope-label disposition studies have been conducted in animals and man to evaluate the energy contribution of polydextrose. A review of the data from 14 studies concluded that polydextrose contributes an energy value of approximately 1 Kc/g (Auerbach et al. 2007).

Polydextrose can be incorporated into a wide variety of foods and beverages in place of fully caloric carbohydrates and, to some extent, dietary fats. Polydextrose itself is only slightly sweet and intense sweeteners can be used to adjust the overall sweetness of the product. In this way polydextrose facilitates the production of reduced energy foods and beverages. Indeed, this application as a low calorie bulking agent was the main *raison d'être* for polydextrose during the initial commercialization phase in the 1980's. Since then a diverse programme of investigations has been undertaken to evaluate the physiological implications of polydextrose and today the emphasis has shifted towards the use of polydextrose for its physiological benefits. These include:

- Oral health – polydextrose has been shown to be non-cariogenic
- Dietary fiber properties
- Reducing glycaemic impact – polydextrose can be used to replace glycaemic carbohydrates to reduce the overall glycaemic response to foods and diets, and
- Prebiotic properties

Only the latter will be considered in detail here. However, it is appropriate to summarize the fiber attributes of polydextrose as these overlap with its consideration as a prebiotic. Danisco currently markets its Litesse® polydextrose as the "*Single ingredient with multiple benefits*" (www.litesse.com). In this way polydextrose differs from most other commercially available fibers in that it is typically added to foods to facilitate a number of claims and not just for its potential prebiotic properties.

11.2.5 Polydextrose as Dietary Fiber

The lack of a globally accepted definition of dietary fiber has led to confusion. The WHO/FAO Codex Alimentarius Committee on Nutrition and Foods for Special Dietary Uses (CCNFSDU) has been debating the subject since 1992. In 2005 CCNFSDU proposed the following definition of dietary fiber:

"Dietary fiber means carbohydrate polymers with a degree of polymerizations (DP) not lower than three, which are neither digested nor absorbed in the small intestine. A degree of polymerization not lower than three is intended to exclude mono- and disaccharides. It is not intended to reflect the average DP of a mixture. Dietary fiber consists of one or more of:

- *Edible carbohydrate polymers naturally occurring in the food as consumed*
- *Carbohydrate polymers, which have been obtained from food raw material by physical, enzymatic or chemical means*
- *Synthetic carbohydrate polymers"*

With the exception of non-digestible edible carbohydrate polymers naturally occurring in foods a physiological effect would need to be scientifically demonstrated (*http://www.ccnfsdu.de/fileadmin/user_upload/PDF/ReportCCNFSDU2005.pdf*).

There is widespread international support for this definition of dietary fiber. It takes into account the latest science and ingredient developments and relates best to potential consumer benefits. It is hoped that a definition along these lines will soon be finalized. On this basis polydextrose would be considered as dietary fiber.

Human feeding studies have consistently reported improved bowel function in diets supplemented with polydextrose. Animal and human data indicate that polydextrose has a moderate beneficial effect on cholesterol metabolism, similar to other soluble fermentable dietary fibers. Polydextrose elicits a negligible glycaemic and insulinaemic response (*Figure 11.2*) and, in addition, has been shown to attenuate the blood glucose raising potential of glucose itself (Stowell 2009).

Even in the absence of a harmonized definition, the fiber status of polydextrose is widely accepted in most countries around the world. The Fibe Mini produced and marketed by Otsuka Pharmaceutical Company since the 1980s (http://www.fibemini.hk/) is an excellent example of a functional food targeted at improving bowel function. This product is based on polydextrose as the active ingredient.

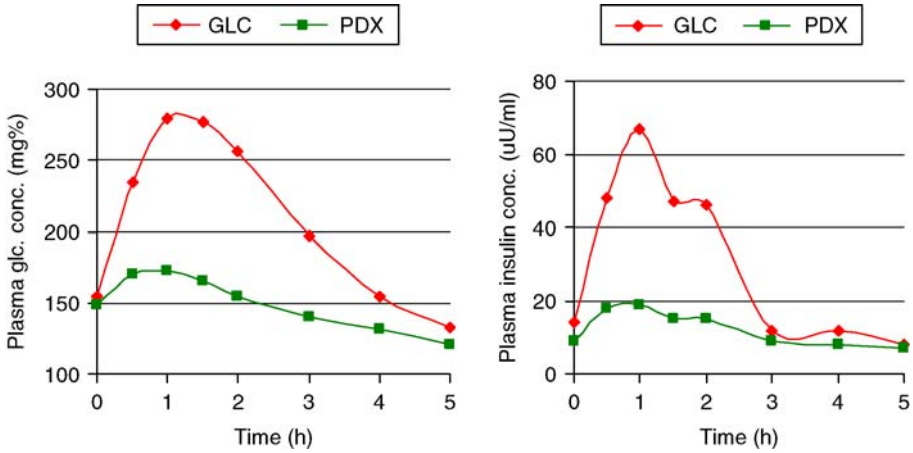

Figure 11.2

A comparison of postprandial plasma glucose and insulin response to either glucose or polydextrose ingestion. The mean response has been calculated from ten subjects (McMahon 1978).

11.3 Application of Polydextrose as a Food Ingredient

Polydextrose is a highly versatile food ingredient with excellent stability (both heat and acid) and compatibility with most food and beverage matrices. In some systems it can act as a humectant, facilitating moisture management, and it may also form a stable glass structure which can be used to positive effect in a variety of applications. It is used on a global basis in a bewildering array of commercial food and beverage products, to facilitate the production of foods with:

- A reduced or low energy content and/or energy density
- A reduced or low glycaemic impact
- An enhanced fiber content, and
- A prebiotic effect where accepted

Applications include:

- Confectionery – both chocolate and sugar confectionery
- Baked goods
- Frozen dairy desserts
- Cultured dairy products
- Beverages and dairy drinks

- Fruit spreads and fruit fillings
- Meat applications
- Pasta and noodles, and
- Pharmaceuticals
- Among others

The technological aspects of polydextrose and its food applications have been well described elsewhere (Auerbach et al. 2006; Mitchell et al. 2001; Stowell 2009).

11.4 Evaluation of Polydextrose as a Prebiotic

11.4.1 Introduction

In considering the potential prebiotic properties of polydextrose it is important to refer to the accepted definition of a "prebiotic." Until recently it appeared that consensus existed with regard to this definition. Gibson and Roberfroid (1995) first defined a prebiotic as "*a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth/and or activity of one or a limited number of bacteria in the colon, and thus improves host health*". The concept was then updated and it was proposed that the ability of a food component to function as a prebiotic should be assessed against the following criteria (Gibson et al. 2004):

- Resistance to gastric acidity, hydrolysis by mammalian enzymes and gastrointestinal absorption
- Fermentation by the intestinal microbiota, and
- Selective stimulation and/or activity intestinal bacteria associated with health and wellbeing (currently agreed at the present time to include bacteria of the genera *Bifidobacterium* and *Lactobacillus*)

Recently the definition of a "prebiotic" has been revisited both by the Food and Agriculture Organization of the United Nations (FAO – www.fao.org/ag/agn/agns/files/Prebiotics_Tech_Meeting_Report.pdf) by the International Life Sciences Institute (ILSI – http://europe.ilsi.org/activities/taskforces/diet/PrebioticsTF.htm). And by the International Scientific Association for Probiotics and Prebiotics (ISAPP) who state that:

"Prebiotics are selectively fermented, dietary ingredients that result in specific changes in the composition and/or activity of the gastrointestinal microbiota, thus conferring benefit(s) upon host health." (http://www.isapp.net/docs/Consumer Guidelines-prebiotic.pdf)

A range of studies have been undertaken on polydextrose to evaluate its prebiotic properties. These include:

- *In vitro* studies
- Metabolism studies in animals, and
- Human intervention studies.

11.4.2 Resistance to Gastric Acidity, Hydrolysis by Mammalian Enzymes and Gastrointestinal Absorption

Because of its range of glycosidic linkages, polydextrose has a compact structure that resists hydrolysis by digestive enzymes found in the upper gastrointestinal tract of mammals.

Oku et al. (1991) studied the hydrolysis of polydextrose *in vitro* using a homogenate of rat intestinal mucosa and found it to be very weakly hydrolyzed. It was concluded that polydextrose contains only a small proportion of α-1,4 and/or α-1,6 glycosidic linkages that are accessible to hydrolysis by mammalian GI enzymes. The low frequency of linkages susceptible to enzymatic hydrolysis was confirmed by Kobayashi and Yoshino (1989) and Stumm and Baltes (1997). In metabolic studies in rats Figdor and Rennhard (1981) concluded that approximately 60% of the orally administered polydextrose was eliminated in the feces. In a study by Fava et al. (2007) in which pigs were fed diets supplemented with polydextrose, residual polydextrose was found to be present in the distal colon.

In a study in humans by Figdor and Bianchine (1983) in which polydextrose was consumed daily for 10 days, 50% of a radiolabel incorporated in the polydextrose consumed on day 8 was recovered in the feces over the subsequent 2 days. In a study by Achour et al. (1994) in which humans consumed polydextrose over a 30-day period, 33% and 32% of a radiolabel incorporated in the polydextrose consumed on days 5 and 27 respectively was recovered in the feces and considered to represent intact polydextrose.

The glycaemic response to polydextrose is given in the International table of glycaemic index and glycaemic load values as 7 ± 2 compared to glucose at 100. Litesse® UltraTM, Danisco's premium branded polydextrose, mediates a glycaemic response of 4 ± 2 (Foster-Powell et al. 2002).

These studies confirm that polydextrose is poorly digested and poorly absorbed from the GI tract in humans.

11.4.3 Fermentation by the Intestinal Microbiota

Animal data (Figdor and Rennhard, 1981; Juhr and Franke, 1992) and human data (Figdor and Bianchine, 1983; Achour et al., 1994; Endo et al. 1991) indicate that the proportion of polydextrose fermented is of the order of 30 to 50%, which is similar to that for other types of dietary fibre such as cellulose. In an *in vitro* study in which the fermentation of polydextrose by human stool homogenates was assessed by hydrogen evolution, polydextrose was found to have a fermentability of 24.8% compared to a glucose reference (Solomons and Rosenthal 1985). When the fermentability of polydextrose was studied in an *in vitro* four-stage model using human fecal inocula and simulating digestion from the proximal to the distal colon, progressive degradation of polydextrose throughout the four stages was observed, accompanied by a progressive increase in short chain fatty acids (SCFAs) (Mäkivuokko et al. 2005). Fava et al. (2007) studied the effect of polydextrose on intestinal microbes and immune function in pigs. They demonstrated that polydextrose is fermented gradually throughout the colon and total concentrations of SCFAs increased in the colon.

Another *in vitro* study using human fecal homogenates to compare the fermentation profiles of a range of carbohydrates found that the quantities of SCFAs produced by polydextrose were similar to those produced by other non-digestible carbohydrate substrates while the quantity of gas produced was lower (Arrigoni et al. 1999). Within the SCFAs, the molecular ratio of acetate/propionate/butyrate produced by the fermentation of polydextrose was found to be similar to that produced by fermentations of fructo-oligosaccharides (FOS) and xylo-oligosaccharides. A further *in vitro* study using human fecal homogenates compared the compounds resulting from fermentation of polydextrose with those produced by fermentation of other carbohydrate substrates (oligofructose, inulin, glucose, arabinose, galactose, fructose, lactose, sucrose, lactulose, cellobiose, sorbitol, lactitol, Litner starch, pectins, maltitol and arabinogalactan) and concluded that

the quantities of SCFAs produced in 48 h were comparable to those seen with the other carbohydrates (Wang and Gibson 1993). These studies confirm that polydextrose is partially fermentable by human colonic bacteria. The extent of this fermentation varies but is typically in the region of 50% as measured by glucose equivalents.

One positive impact of selectively enhanced saccharolytic (carbohydrate) versus putrefactive (protein) fermentation is a reduction in colonic pH which discourages the growth of pathogens which favor a higher pH.

Recent colon simulator studies, summarized in *Figure 11.3*, have shown that polydextrose enhances the production of butyrate at each stage in the colon. Acetate and propionate were also enhanced (data not shown). It is well established that butyrate acts as a substrate for the colonic mucosa and enhanced butyrate would be expected to contribute positively to mucosal integrity. The enhancement of butyrate was achieved without any accumulation of lactic acid. Hence, a balance was established between lactic acid generation and its subsequent fermentation to short chain fatty acids.

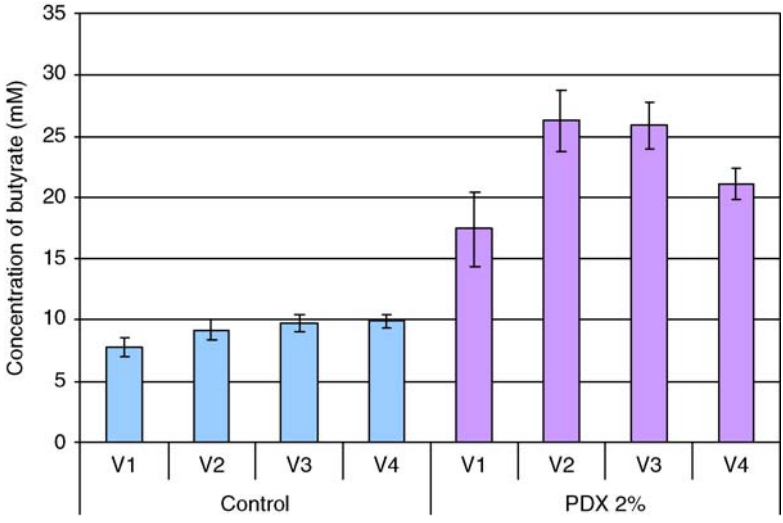

Figure 11.3

Butyrate concentrations in colon simulator vessels after 48 h simulations (average of 7 control and 8 polydextrose [PDX] simulations). V1 represents the ascending part of the colonic model, V2 represents the transverse colon, V3 the descending colon, and V4 the sigmoid and rectum area. Different parts of the model (V1–V4) differ from each other in pH levels and volume of the media and hence in microbial numbers (Mäkivuokko et al., 2005; Mäkeläinen et al., 2007).

11.4.4 Selective Stimulation and/or Activity of Intestinal Bacteria Associated with Health and Wellbeing

In a randomized double-blind, placebo-controlled study by Jie et al. (2000), in which 120 subjects consumed polydextrose over a 28-day period at different dose levels, increases in the numbers of viable bacteria of the *Lactobacillus* and *Bifidobacterium* species were detected in the subjects' stools. The increases in numbers of these bacteria were dose-dependent and were statistically significant from a dose of 4g/day (the lowest dose tested) upwards. At the same time, the number of *Bacteroides spp.* organisms in subjects' stools was reduced. In a study in human subjects by Endo et al. (1991), cited above, in which eight healthy volunteers were fed a diet rich in cholesterol and had a daily intake of 15 g of polydextrose for 6 weeks, changes in colonic flora were accompanied by a decrease in fecal concentrations of *Clostridium spp.* In a study in humans by Tiihonen et al. (2008), supplementation with polydextrose at 5 g/day and a probiotic mixture together was found to increase culturable fecal bifidobacteria over supplementation with the probiotic mixture alone when compared over a 2 week period in twenty subjects (summarized in *Table 11.1*). Consumption of the probiotic mixture increased the faecal levels of culturable lactobacilli and propionibacteria. These levels were not further increased by the addition of polydextrose to the probiotic mixture.

In an *in vitro* model simulating bacterial activity in different segments of the human colon, Probert et al. (2004) investigated the effect on bacterial populations of a range of fermentable substrates (polydextrose, lactitol and FOS).

Table 11.1

A probiotic mixture supplemented with polydextrose increased cultured bifidobacteria (Tiihonen et al. 2007)

Period	Bifidobacteria (Log_{10}cfu/g wet weight feces)		
	Mean	S.D.	p
Run-in	7.0	2.2	NS
Probiotic	7.0	2.0	NS
PDX + Probiotic	8.9	2.5	<.001
Follow-up	8.5	1.5	<0.05

Study involved 22 young adults (15 female/5 male). Polydextrose was fed at 5 g/day. The probiotic mixture included: *Lactobacillus* GG, *L. rhamnosus* LC705, *Propionibacterium shermannii* JS and *Bifidobacterium breve* Bbi. Fecal samples were analyzed after each 2 week period. Total bifidobacteria counts were measured by plating

Polydextrose was found to stimulate the number of bifidobacteria and to support the growth of a wide variety of bifidobacteria species in all three of the colon segments modelled. In an *in vitro* study using human faecal homogenates Wang and Gibson, 1993, previously referenced above, found that polydextrose stimulated bacterial growth but less selectively so than other substrates studied.

Taken together, these studies suggest that administration of polydextrose can favorably influence the balance of the microbial genera present in the colonic microbiota.

11.4.5 Other Studies

Several additional studies have assessed the prebiotic potential of polydextrose. Some of the findings are in line with those for other prebiotics whilst some point to interesting and possibly unique effects of polydextrose.

Hara et al. (2000) showed that dietary polydextrose increased calcium absorption and bone mineralization in rats. The effect may partly have been due to colonic acidification which is known to increase calcium solubility. However, the main positive effect was, unexpectedly, seen in the small intestine.

Ishizuka et al. (2003), using a rat model, showed that polydextrose considerably reduced the formation of aberrant crypt foci (ACF) in the presence of a carcinogen. The effect was most pronounced in the rectum where the reduction was up to 65%. The authors concluded that ingestion of polydextrose may prevent colorectal carcinogenesis. This study highlights the fact that polydextrose mediates its effect into the distal colon and colorectal area (at least in the rat model).

As seen above, fermentation of polydextrose has beneficial effects for mucosal functions. Enhanced butyrate production serves as an important energy source, not only for epithelial cells, but also for mucosal immune cells. Polydextrose has been shown to increase production of immunoglobulin A (IgA) in the large intestine of rats and a synergistic effect was seen with a polydextrose: lactitol combination (Peuranen et al. 2004).

Balancing immune responses in the large intestine is especially important for reducing the risk of colon cancer development. A possible mechanism for reduction in cancer development involves the regulation of mucosal gene expression. Over-expression of the cyclo-oxygenase 2 (cox-2) gene is related to early stages of colon cancer development and chronic inflammatory diseases in the intestine. Mäkivuokko et al. (2005) combined two different *in vitro* systems, namely a four-stage simulator of colonic fermentation and a cell-culture based

model of human intestinal epithelial function, in order to study the effects of polydextrose on colon cancer development. A dose-dependent decreasing effect on cox-2 expression was observed in Caco-2 cells (a human colon cancer cell line). This reduction of cox-2 expression associated with the colonic fermentation of polydextrose further suggests a protective role of polydextrose against colon cancer. Fava et al. (2007) showed a reduction in the cox-2 inflammatory biomarker in association with polydextrose consumption in a pig model. They did, however, not observe a change in the microbiota composition as determined by fluorescent *in situ* hybridisation, this may relate to the relatively high fibre content of the test feed, and the young age of the animals. The reduction in cox-2 is nevertheless a good example of the application of the emerging science of nutrigenomics in the evaluation of a prebiotic.

Prebiotics have recently been proposed as ingredients to encourage satiety or, more specifically, to enhance satiety. It is not clear whether this effect is mediated via the microbiota or some other mechanism so this may or may not be a prebiotic effect *sensu stricto*. King et al. (2005) studied the impact of xylitol and polydextrose on satiety in 20 human subjects. Xylitol (25 g), xylitol:polydextrose (50:50; 25 g) or polydextrose (25 g) in yoghurt ingested as a preload consumed 90 min before lunch suppressed combined calorie intake by 5–8% versus a sucrose control. This study demonstrated that xylitol and/or polydextrose, under these specific conditions, mediates a satiety effect. Whilst the dose of polydextrose and/or xylitol was relatively high the effect was sustained over a 10 day period, which is encouraging.

Vasankari and Ahotupa (2005) found that the ingestion of 12.5 g of polydextrose along with a hamburger meal reduced the total postprandial hypertriglyceridemia by 25%. It has been postulated that the polydextrose passing through the lumen of the intestine interferes with fat uptake in some way. Further investigations are required to confirm the effect and clarify the mechanism.

11.5 Regulatory Considerations

As noted above, there is no universally adopted definition of a "prebiotic". Although further studies are certainly needed, polydextrose seems to comply with most current scientific opinion on what constitutes a "prebiotic" and, where formal opinions have been given they have largely been positive. In the absence of a formal opinion polydextrose may be considered as a prebiotic on the basis that this informs and does not mislead consumers. In Europe, under the auspices of the recently adopted Regulation on Nutrition and Health Claims

made on foods (EC1924/2006), two relevant claims for polydextrose have been forwarded by EU Member States to Brussels and are now on the consolidated list under consideration by the European Food Safety Authority (EFSA). These are:

Polydextrose improves bowel function
Conditions of use: 4g/day
Nature of evidence: Authoritative bodies, reviews, human studies, animal studies
Examples of wording: Polydextrose promotes good intestinal health;
Polydextose improves bowel function and gut comfort; Polydextrose stimulates metabolic activity
Polydextrose is a prebiotic with bifidogenic properties
Conditions of use: 4g/day
Nature of evidence: Human studies, *in vitro* studies
Examples of wording: Polydextrose stimulates the growth of beneficial bacteria in the gut; Polydextrose stimulates the growth of bifidobacteria in the colon; Polydextrose stimulates the growth of lactobacilli bacteria in the gut; Prebiotics promote healthy/well-balanced gut bacteria/flora;

Time will tell which claims will finally be consolidated into the "Article 13" health claims list due for publication in January 2010. Local authorities should always be consulted before finalizing a food or beverage claim.

11.6 Future Developments

Further studies are currently underway to elucidate the prebiotic properties of polydextrose. The emphasis is mainly on human intervention studies and subgroups of the population will be targeted in some of these. Mechanistic data are also considered important to explain observations in humans and to support claims. The impact on novel biomarkers will be included in the evaluation where relevant.

11.7 Conclusion

A wide variety of studies have been undertaken to evaluate the prebiotic properties of polydextrose. The following attributes have been demonstrated:

- Slow fermentation rate
- Sustained fermentation throughout the colon

- Enhancement of bifidobacteria
- Increase in butyrate production
- No lactic acid accumulation
- Decrease in putrefaction (protein fermentation)
- Stimulation of the gastric immune system – IgA (Rat model)
- Reduction of colon cancer risk (based on rat and *in vitro* models)
- Attenuation of postprandial triglycerides

The technological versatility of polydextrose, combined with the interesting range of physiological benefits it mediates, makes polydextrose an excellent choice for those seeking to enhance the nutritional profile of foods and beverages. However, it should be noted that the prebiotic status of polydextrose is still under debate, with further studies in progress and questions over data generated using more traditional techniques. It is important when intending to label a food or beverage as 'prebiotic' to consider the totality of the evidence and to always seek local opinions in respect of legislative requirements.

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12 Prebiotics in Companion and Livestock Animal Nutrition

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12.1 Introduction

Prebiotic supplementation of animal diets began in an attempt to increase concentrations of beneficial intestinal microbiota. It was understood that prebiotics inhibited growth of intestinal pathogens and decreased concentrations of stool odor-causing metabolites. Since the use of prebiotics began, several countries have banned the use of antimicrobials in livestock animal feeds, and several more have placed restrictions on the quantity of antimicrobials that can be used. Prebiotic supplementation has become increasingly popular as the body of evidence supporting its use continues to grow. As this literature expands, the number of potential prebiotic substances has grown beyond those that are naturally occurring, such as those found in chicory and yeast products, to include a large number of synthetic or chemically/enzymatically manufactured prebiotics.

Definition and types of prebiotics. To be considered a prebiotic, a compound must conform to the following guidelines: first, it must be resistant to digestion in the upper gastrointestinal tract (remain unaltered through hydrolytic-enzymatic digestion); second, it must selectively stimulate one or a limited number of beneficial microbiota; and third, it must benefit host health by improving colonic microbiota composition (Roberfroid et al., 1998). Fructans are one of the most popular prebiotic supplements available, comprising 61% of the publications on the topic of prebiotic supplementation. While fructans occur naturally in feeds, hydrolytic and enzymatic methodologies have produced fructans of varying chain lengths. Examples of fructans include fructooligosaccharides (FOS), short-chain FOS (scFOS), oligofructose (OF), and inulin, as well as several blends of varying chain lengths. Generally, fructans

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contain a linear chain of fructose, which can vary in length from 2 to 60 units, with or without a glucose unit on the end of the chain (Flickinger et al., 2003). Fructans escape hydrolytic digestion due to their β-(2,1) bonds (Flickinger et al., 2003).

Mannanoligosaccharide (MOS) research comprises 33% of the reports about prebiotics. Similar to fructans, several forms of MOS are available. These forms can differ greatly in their ability to influence intestinal microbiota. The crudest form of MOS is unmodified yeast (*Saccharomyces cervisiae*), which appears to act as a prebiotic due in part to the MOS contained in its cell wall. In addition, purified yeast cell wall has been extracted and utilized in animal feeds. Mannanoligosaccharides are able to bind with certain intestinal microbiota due to the mannan component in its structure and thereby prevent attachment of microbiota to the intestinal cells (Spring et al., 2000).

Soy oligosaccharides also have received attention as prebiotic supplements, comprising 9% of prebiotic literature. These oligosaccharides have been fed intact as a component of the whole soybean, or as purified raffinose and stachyose to determine their individual and synergistic effects. Raffinose and stachyose are noted for gas production which occurs when these oligosaccharides are fed intact in soybeans or as purified oligosaccharides. Additionally, several guar fractions, namely gum, germ, hull, and meal, have been investigated as potential prebiotics due to their fermentative characteristics.

Galactooligosaccharide (GOS) and trans-galactooligosaccharide (TGOS/TOS) research comprises 9% of prebiotic literature. Galactooligosaccharides are produced by subjecting lactose to microbial galactosyltransferases (Tzortis et al., 2005). Trans-galactooligosaccharides are water-soluble, contain galactose units with β-linkages that prevent hydrolytic digestion, and are rarely found in feeds (Houdijk et al., 1998). Gluconic acid, a GOS-like prebiotic, also has been investigated (Biagi et al., 2006).

In addition to naturally occurring prebiotic substances, several synthetic prebiotics have been investigated. Lactosucrose, also called 4G-β-D-galactosylsucrose or 0-β-D-galactopyranosyl-(1–4)-0-α-D-glucopyranosyl-(1–2)-β-D-fructofuranoside, contains galactose, glucose, and fructose and is produced by *Arthrobacter* spp. K-1 (Fujita et al., 1990). Isomaltooligosaccharide (IMO) is produced by the enzymatic transgalactosylation of glucose and contains isomaltose, panose, isomaltotriose, and other oligosaccharides with four or five glucose residues (Zhang et al., 2003). Pullulan consists of glucose units linked as α-1,6-maltotriose subunits (Spears et al., 2005). γ-Cyclodextrin is a ring-shaped oligosaccharide composed of α-1,4-linked glucose units (Spears et al., 2005). Both pullulan and γ-cyclodextrin are

partially hydrolyzed in the upper digestive tract of non-ruminants, but the majority of each compound is believed to pass into the large intestine.

Digestive strategies of companion and livestock species. Prebiotic efficacy in any animal species is closely correlated to its digestive strategy. Non-ruminant animals, such as the dog and cat, employ post-gastric fermentation. The dog and cat have a very small or nonexistent cecum and a tubular colon with little sacculation (NRC, 2006). Due to these features, they typically have a short (approximately 12 h) intestinal residence time and digest approximately 8% of food in the colon (NRC, 2006). Other non-ruminants, such as poultry and swine, also employ post-gastric fermentation but are capable of a higher level of colonic fermentation than cats and dogs. These animals have one cecum (swine) or two ceca (poultry) adapted to microbial fermentation. In addition, the colon of swine is highly sacculated and allows for an increased residence time. Fermentation in the colon of swine accounts for 5–28% of their maintenance energy requirement (NRC, 1998). Herbivorous non-ruminants like the horse have a very large cecum and highly sacculated colon that allows for up to 60% of the animal's energy needs to be met through fermentation (NRC, 2007a). Unlike other non-ruminants, herbivorous non-ruminants are able to consume and utilize cellulose for energy.

Similar to non-ruminant animals, the young ruminant, also referred to as a pre-ruminant, employs post-gastric fermentation until the ruminal microbiota are established. Ruminal microbiota stabilize after 6–8 wk, and these animals are fed as ruminants after this point (Damron, 2006). Adult ruminant animals are pre-gastric fermenters. The majority of fermentation in adult ruminants occurs in the reticulorumen, which can provide 50–70% of the energy requirements of the animal through short-chain fatty acid (SCFA) production (Damron, 2006). While these animals are both pre- and post-gastric fermenters, hindgut fermentation has not been the focus of prebiotic research in ruminants.

Microbiota methodologies. One of the key outcomes of prebiotic supplementation is a beneficial alteration in the microbiota composition of the host animal, so identification of specific microbial communities is necessary to determine efficacy. For many years, researchers have investigated alterations in microbiota using culturing techniques (e.g., plating on selective media). While plating can quantify microbial communities, there are several drawbacks to its use. First, only viable microbiota can be cultured. If the species of interest is an anaerobic colony, this task can be quite challenging as viable members will begin to expire shortly after the sample is collected, even under conditions of proper storage and transport. Second, selective media must be employed to ensure that only a specific microorganism is cultured. However, several microbes can be grown on

the same media, which can lead to inaccurate quantification (Rastall, 2004). Additionally, it is now known that several microbial species are dependent upon one another for growth and maintenance of the colony in question (NRC, 2007b). To culture such microbial species individually is impossible using current plating methods.

Fluorescence *in situ* hybridization (FISH) initially was developed as a qualitative tool to identify two or more species in a growth medium. With FISH, a fluorescent marker is hybridized to a specific region of microbial DNA. The microbe then is fixed to a microscope slide (Harmsen and Welling, 2002). The accuracy of FISH counting varies greatly, as general practice is to count the number of microbiota in a specific area and multiply by the entire area to arrive at a conclusion. Further advancements in FISH technology allowed separation and, thus, quantification of microbiota by joining FISH with flow cytometry.

To address the problems associated with plating, molecular methods have been developed to more clearly understand the makeup of the intestinal microbiome. Quantitative PCR (qPCR) has provided insight into the microbial community of the intestine. Primers and probes specific to a microbial genus or species are prepared, and then added to a mixture of DNA and buffer to quantify the genus or species based on their DNA. This method, like the others, is not without its problems. First, the numbers of microbes quantified do not strictly represent viable microbiota, as the primers and probes amplify DNA of all the cells present. This may lead to an inaccurate count of changes in microbiota due to prebiotic activity (Middelbos et al., 2007a). Second, the primers and probes may not be specific enough to the species in question. While the primers and probes selected for each organism strive to specify an exact microorganism, there can be cross-reactivity among species and genera of microorganisms with similar genetic codes. Therefore, each primer/probe pairing should be checked against a "negative control" or a pure culture of microbial DNA that should not react with the primer/probe pairing. While several issues remain unsolved as regards current methods in quantifying microbial communities, technologies continue to improve.

12.2 Prebiotic Use in Companion Animal and Livestock Species

Canines. Several types of prebiotics have been utilized in canine nutrition research. Details of the experiments are presented in *Table 12.1*. As microbiota

Table 12.1

***In vivo* experiments, listed in chronological order beginning in 2005, reporting effects of prebiotics in dogs[a,b,c]** (*Cont'd* p. 358)

Reference	Outcome variables quantified	Animals/ treatment (age, initial BW)	Dietary information; time on treatment	Daily prebiotic dose; source	Major findings
Jeusette et al. (2005)	Plasma leptin	12 Obese dogs (between 1 and 9 yr; 21.9 ± 0.8 kg)	Basal diet (Obesity veterinary diet, Royal Canin, France) fed to promote weight loss	Control (0%)	No effect of scFOS supplementation on leptin, insulin, ghrelin, or gluose
	Plasma insulin		Chemical composition 34% CP, 10% fat, 19.8% dietary fiber, 1% scFOS	2% scFOS (Beghin-Meiji Industrie)	
	Plasma ghrelin		Study continued until ideal BCS obtained (5 out of 9)		
	Blood glucose				
Spears et al. (2005)	Food intake	Five purpose-bred adult dogs (3.7 yr; 28.9 kg)	Basal diet: Hill's prescription diet d/d- Rice and Duck	No supplement (0 g)	↑ γ-cyclodextrin:
	Apparent ileal and total tract digestibility			1 g high molecular weight pullulan	Linear ↓ food intake (control: 352 g; 4 g/d treatment: 305 g)***

Table 12.1 (*Cont'd* p. 360)

Reference	Outcome variables quantified	Animals/ treatment (age, initial BW)	Dietary information; time on treatment	Daily prebiotic dose; source	Major findings
Spears et al. (2005) (con't.)	Microbial populations		Chemical composition 17% CP, 14% fat, 4.0% TDF	2 g high molecular weight pullulan	Quadratic ↑ ileal bifidobacteria (control: 9.46 CFU/g DM feces; 2 g/d: 10.20 CFU/g DM feces; 4 g/d: 9.83 CFU/g DM feces) and lactobacilli (control: 9.12 CFU/g DM feces; 2 g/d: 10.11 CFU/g DM feces; 4 g/d: 9.42 CFU/g DM feces)***
	Fecal characteristics		14 d study	1 g γ-cyclodextrin	Quadratic ↓ fecal *Clostridium perfringens* (control: 9.75; 2 g/d: 9.44; 4 g/d: 9.76 CFU/g DM feces)**
				2 g γ-cyclodextrin	↑ pullulan:
					Linear ↑ ileal bifidobacteria (9.46 CFU/g DM feces, control; 10.12 CFU/g DM feces, treatment) and lactobacilli (9.12 CFU/g DM feces, control; 10.02 CFU/g DM feces, 4 g/d treatment)***
Vanhoutte et al. (2005)	Fecal population banding patterns (DGGE)	Seven adult dogs	Basal diet: see Propst et al. (2003)	Baseline	Inulin:
			Chemical composition not provided	4.5 g/d OF	↑ *Streptococcus lutetiensis*
			10 d study	5.6 g/d inulin	

Gouveia et al. (2006)	Number of leukocytes, neutrophils, and lymphocytes	Eight dogs (2–6 mo) all suffering from gastroenteritis	Basal diet not provided	Control (0 g)	MOS:
	Fecal microbiota		Chemical composition not provided	2 g MOS (Bio-MOS)	Elimination of *E. coli* in 85.71% of animals
			10 d study		
Verlinden et al. (2006)	Nutrient digestibility	Four adult beagle dogs (2–11 yr; 6–15 kg)	Basal diets: hydrolyzed protein diet (Hill's z/d ultra, allergen-free); intact protein source (Hill's d/d with duck and rice)	0% Supplementation	Inulin:
	Fecal characteristics		Chemical composition:	3% Inulin (Raftifeed IPS, DP 2–60; Orafti, Tienen, Belgium)	↓ Fecal DM (12%)***
	Hematology		Hydrolyzed-18% CP, 3% TDF		↓ Apparent CP digestibility in intact protein + inulin diet (4.6%)***
	Serum and fecal IgA, IgG, IgE, IgM				↑ Estimated bacterial protein content in feces (% of fecal DM, 16%; and % of CP intake, 33%) in intact protein + inulin diet*
			Intact protein-14% CP, 5% TDF		
			21 d study		

Table 12.1 (*Cont'd* p. 362)

Reference	Outcome variables quantified	Animals/ treatment (age, initial BW)	Dietary information; time on treatment	Daily prebiotic dose; source	Major findings
Adogony et al. (2007)	Mammary, nasal, and blood immunoglobulin concentrations	Eight primiparous female beagles (10–12 kg)	Control diet: 31% CP, 16% crude fat, 7.8% TDF	0% Supplementation	scFOS:
	Diarrhea incidence		Test diet: 30% CP, 15% crude fat, 6.6% TDF	1% scFOS (Profeed, Beghin-Meiji, France)	↑ IgM in colostrum and milk (40%)**
			Gestation d 35 through weaning		↑ Blood IgM concentrations (60%)***
Apanavicius et al. (2007)	Food intake	Six hound-cross puppies (12 wk)	Control diet: 32% CP, 19% fat, 3% TDF	0% Supplementation	scFOS and Inulin:
	Gastrointestinal tract histopathology		scFOS diet: 32% CP, 19% fat, 3% TDF	1% scFOS	↓ Change in food intake following infection (26%)**
	Body temperature after infection		Inulin diet: 30% CP, 19% fat, 5% TDF	1% Inulin	↓ Enterocyte sloughing severity (9%)**
	Ileal and colonic nutrient and ion transport				Maintenance of ileal Na + -dependent glucose transport (400% ↓ in control, no change in supplemented puppies)
			14 d study		Inulin:

Apanavicius et al. (2007) (con't.)	Microbial populations				↑ Change from baseline in fecal acetate (control: −37.7 μmol/g; inulin 85.5 μmol/g) and SCFA concentrations (control: −53.5 μmol/g; inulin: 145.5 μmol/g)**
					↑ Change in fecal lactobacilli concentrations (7%)**
Middelbos et al. (2007a)	Nutrient digestibility	Six purpose-bred adult female dogs (4.5 yr; 23 kg)	350 g/d	Control – no supplemental fermentable carbohydrate	Supplemented diets:
	Fecal microbial populations		14 d study	Control + 2.5% cellulose	↓ CP digestibility (15%)**
	Fecal fermentative end-products			Control + 2.5% beet pulp	↑ Fecal bifidobacteria concentrations (14%)**
					↑ Fecal lactobacilli concentrations (8%)***
	Immunological indices			Control + 1.0% cellulose + 1.5% scFOS (Nutraflora P-95)	
				Control + 1.0% cellulose + 1.2% scFOS + 0.3% yeast cell wall (YCW, Safmannan)	↑ Fecal butyrate concentrations (67%)**
				Control + 1.0% cellulose + 0.9% scFOS + 0.6% YCW	

Table 12.1

Reference	Outcome variables quantified	Animals/ treatment (age, initial BW)	Dietary information; time on treatment	Daily prebiotic dose; source	Major findings
Middelbos et al. (2007b)	Apparent ileal and total tract nutrient digestibility	Five purpose-bred adult female dogs (4 yr; 23 kg BW)	280 g basal diet	0 g supplementation	YCW:
	Serum IgA, IgM, and IgG		Chemical composition 30% CP, 21% fat, 4% TDF	Control+0.07 g YCW (Safmannan)	↑ ileal nutrient digestibility (10% DM, 11% CP)***
	Fecal microbial populations		14 d study	Control+0.35 g YCW	Linear ↓ in monocyte counts (control: 1.0 thousands/μL; 0.65% supplementation: 0.7 thousands/μL)**
				Control+0.63 g YCW	Linear ↓ in fecal *E. coli* populations (control: 9.1 CFU/g DM feces; 0.65% supplementation: 8.2 CFU/g DM feces)**
				Control+0.91 g YCW	

[a]For research published prior to 2004, please refer to the review of Swanson and Fahey (2006)

[b]*BW* body weight, *CFU* colony forming units, *CP* crude protein, *d* day, *DGGE* denaturing gradient gel electrophoresis, *DM* dry matter, *g* gram, *IgA* immunoglobulin A, *IgE* immunoglobulin E, *IgG* immunoglobulin G, *IgM* immunoglobulin M, *kg* kilogram, *mo* month, *MOS* mannanoligosaccharide, *OF* oligofructose, *SCFA* short-chain fatty acid, *scFOS* short-chain fructooligosaccharide, *TDF* total dietary fiber, *yr* year, *YCW* yeast cell wall, *wk* week

[c]*$P < 0.001$, **$P < 0.05$, ***$P < 0.10$

factor significantly into the general health and well being of all animals, the majority of canine prebiotic research has focused on monitoring changes in gut microbiota. Microbiota reside in all areas of the intestinal tract, but are generally studied in feces rather than using invasive techniques to obtain colonic contents. Fifty-five percent of authors observing changes in microbiota reported increased bifidobacteria (Middelbos et al., 2007a; Swanson and Fahey, 2006), while 32% reported increased lactobacilli (Apanavicius et al., 2007; Middelbos et al., 2007a; Swanson and Fahey, 2006). Potentially pathogenic microbiota generally decreased in response to prebiotic supplementation. Thirty-six percent of authors reported decreased clostridia (Spears et al., 2005; Swanson and Fahey, 2006); however, two authors reported increased clostridia in response to scFOS and OF supplementation (Swanson and Fahey, 2006). *Escherichia coli* was reported to decrease in response to MOS-supplementation (Gouveia et al., 2006; Middelbos et al., 2007b; Swanson and Fahey, 2006), while streptococci increased in response to fructan supplementation (Swanson and Fahey, 2006; Vanhoutte et al., 2005). Overall, prebiotic supplementation appears to benefit the microbial ecology of the dog.

Commensurate with changes in microbiota populations, changes in fermentative end-products occur with prebiotic supplementation. Increased concentrations of fecal SCFA, particularly butyrate, were reported in 46% of studies observing changes in fecal metabolites (Apanavicius et al., 2007; Middelbos et al., 2007a; Swanson and Fahey, 2006). Concentrations of acetate and propionate increased in 23 and 31% of studies, respectively (Apanavicius et al., 2007; Swanson and Fahey, 2006). Most SCFA are generated via carbohydrate fermentation. Conversely, branched-chain fatty acids (BCFA), ammonia, phenols, and indoles are generated via protein fermentation. In general, concentrations of BCFA, phenol, and indole decreased in response to prebiotic supplementation (Swanson and Fahey, 2006). Ammonia concentrations responded inconsistently to prebiotic supplementation: 23% of studies reported increased ammonia (Swanson and Fahey, 2006) while 15% of studies reported decreased ammonia in feces (Swanson and Fahey, 2006).

Prebiotics have demonstrated clear effects on nutrient digestibility. Crude protein digestibility is negatively impacted by inulin consumption (Middelbos et al., 2007a; Verlinden et al., 2006). However, ileal dry matter and crude protein digestibilities increased in dogs fed diets supplemented with yeast cell wall (Middelbos et al., 2007b). Although no differences in nutrient digestibility were observed, dogs consumed less food in a linear fashion when provided diets with increasing concentrations of γ-cyclodextrin (Spears et al., 2005).

Closely tied to nutrient digestibility, fecal quality and consistency are affected by prebiotics. As reported in 50% of published studies, higher concentrations of supplemental prebiotic compounds led to decreased fecal dry matter (Swanson and Fahey, 2006; Verlinden et al., 2006). Three percent supplementation of inulin resulted in a decrease in fecal dry matter in dogs consuming both hydrolyzed and intact protein diets (Verlinden et al., 2006). Also affected by prebiotic supplementation are fecal volume and fecal score. Thirty-five percent of studies reported an increase in fecal volume with prebiotic supplementation (Swanson and Fahey, 2006). Fecal scores were reported to increase in three studies (decreased consistency) while decreased fecal score was reported in two studies (Swanson and Fahey, 2006).

Increased immune responses also have been observed in dogs consuming prebiotics. When fed to primiparous beagles, scFOS increased IgM concentrations in colostrum, milk, and blood (Adogony et al., 2007). Under a *Salmonella typhimurium* challenge, puppies consuming either scFOS or inulin experienced decreased enterocyte sloughing and maintenance of sodium-dependent glucose transport (Apanavicius et al., 2007). While feed intake decreased, it appeared that supplementation of prebiotic fructans allowed for normal metabolism to occur. When fed diets supplemented with increasing concentrations of yeast cell wall, a linear decrease in monocyte concentration was observed (Middelbos et al., 2007b).

Felines. Five studies involving prebiotic supplementation of felines have been published (*Table 12.2*). Increased bifidobacteria and lactobacilli, along with decreased clostridia, staphylococci, and *E. coli* have been reported (Swanson and Fahey, 2006). Supplementation with fructans also increased fecal nitrogen of microbial origin in supplemented cats (Hesta et al., 2005; Swanson and Fahey, 2006). Fecal fermentative end-products – ammonia, phenol, and indole – decreased with lactosucrose supplementation (Swanson and Fahey, 2006), whereas inulin increased total SCFA concentrations (Swanson and Fahey, 2006).

Nitrogen and crude protein digestibilities decreased in response to supplementation with fructans (Swanson and Fahey, 2006). This likely would be observed in response to other prebiotics; however, these effects remain open to investigation in the cat. Fructan supplementation also increased fecal nitrogen excretion and decreased fat digestibility (Hesta et al., 2005; Swanson and Fahey, 2006). With respect to fecal characteristics, fructan supplementation decreased fecal dry matter and fecal score while increasing dry fecal output, fecal volume, and number of defecations per day (Hesta et al., 2005; Swanson and Fahey, 2006).

Table 12.2

***In vivo* experiments, listed in chronological order beginning in 2005, reporting effects of prebiotics in cats[a,b,c]**

Reference	Outcome variables quantified	Animals/ treatment (age, initial BW)	Dietary information; time on treatment	Daily prebiotic dose; source	Major findings
Hesta et al. (2005)	Urea metabolism	Four female cats (>7 yr; 2.2 and 4 kg)	Basal diet fed for ME requirement of ideal BW	0% Supplementation	FOS:
	Fecal odor components		Chemical composition 29% CP, 37% crude fat, 1% CF	3.11% FOS (Raftilose), DMB	↑ Fecal moisture (6%)***
			3 wk study		↑ DM fecal output (27%)***
					↑ Fecal N excretion (36%)***
					↓ Urinary N excretion (48%)***
					↑ Fecal bacterial N (% of N intake; 125%)***

[a]For research published prior to 2004, please refer to the review of Swanson and Fahey (2006)

[b]*BW* body weight, *CF* crude fiber, *CP* crude protein, *DM* dry matter, *DMB* dry matter basis, *FOS* fructooligosaccharide, *kg* kilogram, *ME* metabolic energy, *N* nitrogen, *yr* year, *wk* week

[c]*P < 0.001, **P < 0.05, ***P < 0.10

Poultry. Prebiotic research has been conducted in poultry since 1990 and, as a result, a large database of research is available in this area (*Table 12.3*). Increased bifidobacteria (Cao et al., 2005; Jiang et al., 2006; Sims et al., 2004; Terada et al., 1994; Thitaram et al., 2005; Xu et al., 2003) and decreased clostridia (Biggs et al., 2007; Butel et al., 2001; Cao et al., 2005; Sims et al., 2004; Terada et al., 1994) have been reported in 30% of studies that investigated the microbial effects of prebiotic supplementation. Fifteen percent of studies also reported increased lactobacilli (Baurhoo et al., 2007a; Jiang et al., 2006; Xu et al., 2003); however, one author reported decreased ileal and cecal lactobacilli with MOS

Table 12.3

***In vivo* experiments, listed in chronological order, reporting effects of prebiotics in poultry[a,b]** (*Cont'd* p. 368)

Reference	Outcome variables quantified	Animals/treatment (age, initial BW)	Dietary information; time on treatment	Daily prebiotic dose; source	Major findings
Coon et al. (1990)	Study 1	Study 1	Study 1	Study 1	Study 1
	TME_n	Eight Leghorn roosters	30 g (precision-fed) test diet	SBM	ESBM:
			Chemical composition 44% CP	ESBM	↑ TME_n (21%)**
	Study 2	Study 2	Study 2	Study 2	Study 2
	Ileal and total excreta CHO digestibilities	30 Leghorn roosters	Same as study 1	Same as study 1	ESBM:
	Intestinal transit time				↓ Ileal sucrose (42%), cellulose (0.5%), total water-soluble CHO (78%) digestibilities**
					↓ Excreta sucrose (54%), raffinose (35%), stachyose (37%), total water-soluble CHO (90%) digestibilities**
					↑ Excreta hemicellulose (52%), cellulose (35%) digestibilities**

Coon et al. (1990) (con't.)					↑ Excreta true DM digestibility (25%)**
					↑ Intestinal transit time (62%)**
					↑ Cecal pH (9%)***
Leske et al. (1993)	Study 1	Study 1	Study 1	Study 1	Study 1
	Nutrient digestibility	6–9 Leghorn roosters	30 g (precision-fed) soy protein concentrate	Control (0%)	1% raffinose:
	TME_n		Chemical composition 73.1% CP, 0.36% stachyose, 0.05% raffinose	Control + 1% raffinose	↓ TME_n (10%)**
			48 h study	Control + 5% stachyose	5% stachyose:
				Control + 1% raffinose + 5% stachyose	↓ TME_n (12%)**
				Control + 6% sucrose	1% raffinose + 5% stachyose:
				Control + 1% raffinose + 5% stachyose + Control + 6% sucrose	↓ TME_n (12%)**
	Study 2	Study 2	Study 2	Study 2	Study 2
	Same as study 1	Same as study 1	Same as study 1	Control (0%)	All concentrations stachyose:
				Control + 1% stachyose	↓ TME_n (13–18%)**
				Control + 2% stachyose	↓ DM digestibility (12–16%)

Table 12.3 (*Cont'd* p. 370)

Reference	Outcome variables quantified	Animals/treatment (age, initial BW)	Dietary information; time on treatment	Daily prebiotic dose; source	Major findings
Leske et al. (1993) (con't.)				Control + 3% stachyose	
				Control + 4% stachyose	
				Control + 5% stachyose	
	Study 3	Study 3	Study 3	Study 3	Study 3
	Same as study 1	Same as study 1	Same as study 1	Control (0%)	0.6% raffinose:
				Control + 0.4% raffinose	↓ TME_n (12%)**
				Control + 0.6% raffinose	↓ DM digestibility (11%)**
				Control + 0.8% raffinose	0.8% raffinose:
				Control + 1.0% raffinose	↓ TME_n (15%)**
					↓ DM digestibility (12%)**
					1.0% raffinose:
					↓ TME_n (16%)**
					↓ DM digestibility (13%)**

Terada et al. (1994)	Performance responses (BW, mortality)	6,360 Cobb broiler chicks (1 d)	Corn-SBM diet	Control (0%)	Lactosucrose:
	Cecal microbiota		Chemical composition (starter–finisher) 23.3–28.3% CP, 1.20–0.91% lys, 0.98–0.87% met + cys, 1.07–1.03% Ca, 0.69–0.75% P	Control + 0.15% lactosucrose	↓ Lecithinase-negative clostridia on d 20 (9%)**
	Cecal metabolites		62 d study		↓ Lecithinase-positive clostridia (31%), bacterioidaceae (2%), total anaerobes (1%) on d 62**
					↑ Bifidobacteria (4%) on d 62**
	Pen ammonia				↓ Pseudomonad occurence (63%) on d 62***
					↓ Staphylococci on d 62 (20%)**
					↓ Cecal ammonia on d 62 (50%)***
					↓ Cecal phenol (38%), *p*-cresol on d 62 (34%)**
					↑ Cecal acetate on d 62 (98%)*
					↑ Cecal butyrate on d 62 (100%)**

Table 12.3 (*Cont'd* p. 372)

Reference	Outcome variables quantified	Animals/treatment (age, initial BW)	Dietary information; time on treatment	Daily prebiotic dose; source	Major findings
Leske and Coon (1999a)	Study 1	Study 1	Study 1	Study 1	Study 1
	TME_n AA digestibility	Seven adult Leghorn roosters	25 g (precision-fed) test diet	SBM	ESBM:
	NSP digestibility		48 h study	ESBM	↑ DM (11%), cellulose (7%), noncellulosic NSP (22%), arabinose (27%), fucose (29%), galactose (25%), glucose (16%), mannose (13%), xylose (39%), uronic acid (18%) digestibility**
					↑ TME_n (14%)**
					↑ Digestible cellulose (414%), noncellulosic NSP (519%)**
	Study 2	Study 2	Study 2	Study 2	Study 2
	TME_n	Nine roosters	Roosters: 25 g (precision-fed) test diet	47% CP SBM	47% CP SBM (10:1 vs. control):
	AME_n	20 Male Ross × Ross broiler chicks	48 h study	47% CP SBM extracted with 80% ethanol, then water (10 parts ethanol/water: 1 part SBM)	↑ TME_n (9%)**
			Chicks: unlimited access to test diet	44% CP SBM	44% CP SBM (8:1 vs. control):
			12 d study		↑ AME_n (14%)**

Leske and Coon (1999a) (con't.)				44% CP SBM extracted with 80% ethanol, then water (8:1)	↑ TME_n (8%)**
					44% CP SBM (water: ethanol vs. control):
				44% CP SBM extracted with water, then 80% ethanol (water:ethanol)	↑ AME_n (14%)**
					↑ TME_n (21%)**
Leske and Coon (1999b)	Hydrogen gas production	Two male Ross × Ross broiler chicks (10 d, 156 g)	6 g (precision-fed) test diet	47% CP SBM	No differences among treatments
			28 h study	ESBM (α-galactoside-free)	
				ESBM with α-galactoside concentrations of standard SBM	
Fernandez et al. (2000)	Study 1	Study 1	Study 1	Study 1	Study 1
	Salmonella challenge	6 or 12 Ross-1 broiler chicks (1 d)	Mash diet (ingredients, chemical compostion not provided)	Control (0%)	All dilutions of HCC dosed prevented cecal *S. enteritidis* colonization (12/12 control birds colonized)
	Cecal microbiota		9 d study – Chicks primed with hen cecal contents (HCC) adapted to designated treatment diet	Control + 2.5% D-mannose	
				Control + 2.5% MOS	
				Control + palm kernel meal	

Table 12.3 (*Cont'd* p. 374)

Reference	Outcome variables quantified	Animals/treatment (age, initial BW)	Dietary information; time on treatment	Daily prebiotic dose; source	Major findings
Fernandez et al. (2000) (con't.)	Study 2	Study 2	Study 2	Study 2	Study 2
	Salmonella challenge	12 or 24 Ross-1 broiler chicks (1 d)	Same as study 1	Same as study 1	MOS:
	Cecal microbiota				↓ Cecal *S. enteritidis* with 10^{-4} HCC (51% vs. mannose group)**
	Study 3	Study 3	Study 3	Study 3	Study 3
	Salmonella challenge	12 or 24 Ross-1 broiler chicks (1 d)	Same as study 1	Same as study 1	MOS:
	Cecal microbiota				↓ Cecal *S. enteritidis* with 10^{-3} (51%), 10^{-4} (67%), and $10^{-3} + 10^{-4}$ HCC (58%; mannose also decreased 37%, 50%, and 42%, respectively)**
	Study 4	Study 4	Study 4	Study 4	Study 4
	Salmonella challenge	12 or 24 Ross-1 broiler chicks (1 d)	Same as study 1	Same as study 1	MOS:
	Cecal microbiota				↓ Cecal *S. enteritidis* with 10^{-3} (38%), 10^{-5} (33%), and $10^{-3} + 10^{-6}$ HCC (24%; mannose also decreased 19% with $10^{-3} + 10^{-6}$ HCC)**

Spring et al. (2000)	Study 1	Study1 (*S. typhimurium* groups)	Study 1	Study 1	Study 1
	Salmonella challenge	Ten line 24 broiler chicks (hatched, groups 1 and 2) plus 26 chicks (hatched, group 3)	Unmedicated corn-SBM diet, all birds' microbiota standardized prior to initial feeding	Control (0 ppm)	MOS:
	Cecal microbiota		Chemical composition not provided	Control + 4,000 ppm MOS (Bio-Mos)	↓ Salmonellae (26%)**
	Cecal metabolites		10 d study		↓ Coliforms (3%)***
	Study 2	Study 2 (*S. dublin* groups)	Study 2	Study 2	Study 2
	Salmonella challenge	Nine chicks (hatched, groups 4 and 5) plus an unspecified number of chicks (hatched, group 6)	Same as study 1	Same as study 1	MOS:
	Cecal microbiota				↓ Salmonella-positive birds (38%)**
	Cecal metabolites				
Butel et al. (2001)	Cecal metabolites	Three "flora" groups (inoculated with human infant fecal flora)	Semi-synthetic diet (ingredients and chemical composition not provided)	Control (6% w/w lactose)	Lactose + OF:
	Cecal microbiota	(1) 11 Germ-free quail (*Coturnix coturnix* subsp. *japonica*, 2 wk) (2) Nine quail (2 wk) (3) 14 quail (2 wk)	28 d study	OF [3% lactose + 3% OF (Raftilose)]	(1) No difference in infection or cecal morphology, metabolites, or microbiota

Table 12.3 (*Cont'd* p. 376)

Reference	Outcome variables quantified	Animals/treatment (age, initial BW)	Dietary information; time on treatment	Daily prebiotic dose; source	Major findings
Butel et al. (2001) (con't.)	Cecal morphology associated with necrotizing enterocolitis				(2) ↓ Cecal wall weight in ill quails (20%)**
					(3) ↓ Cecal *C. perfringens* (19%) and *C. paraputrificum* (31%)**
Fairchild et al. (2001)	Performance responses (BW, feed conversion, [F:G])	56 Young (33 wk) and 56 old (58 wk) male BIG-6 turkey poults (1 d)	Corn-SBM diet	Control (0 g/kg)	MOS with *E. coli*:
	E. coli challenge		Chemical composition 27.9% CP, 5.2% fat, 0.68% met, 1.13% met + cys, 1.71% lys, 1.4% Ca, 0.7% P	1 g/kg MOS (Bio-Mos)	↑ BW in young (13%) and old (15%) chicks at wk 1**
	Intestinal microbiota		3 wk study	2.2 mg/kg flavomycin	MOS with *E. coli*:
				1 g/kg MOS + 2.2 mg/kg flavomycin	
					↑ BW in young chicks at wk 3 (5%)**
					↓ Liver total coliforms on d 7 (42%)**

Parks et al. (2001)	Performance responses (BW, feed consumption, feed conversion)	160 Male hybrid large White turkey poults	Corn-SBM diets	Control (0 g/kg)	MOS:
	Bird mortality		Chemical composition (initial–final) 28.14–16.50% CP, 3.16–5.90% crude fat, 0.59–0.31% met, 1.05–0.61% met + cys, 1.60–0.80% lys, 1.20–0.65% Ca, 0.60–0.32% P	1 g/kg MOS (reduced to 0.5 kg/ton at 6 wk)	↑ BW at wk 20 (3%)**
			140 d study	2 mg/kg flavomycin	↓ F:G from 0–3 wk (4%) **
				20 mg/kg Stafac	MOS + flavomycin:
					↑ BW at wk 12 (4%), 15 (4%), 20 (2%)**
				1 g/kg MOS (reduced to 0.5 g/kg at 6 wk) + 2 mg/kg flavomycin	↓ F:G from wk 0–3 (4%), 0–6 (4%), 0–12 (3%), 0–18 (2%)**
				1 g/kg MOS (reduced to 0.5 g/kg at 6 wk) + 20 mg/kg Stafac	MOS + Stafac:
					↑ BW at wk 15 (3%)**
					↓ F:G from wk 0–3 (6%), 0–6 (4%), 0–12 (3%), 0–18 (3%)**

Table 12.3 (*Cont'd* p. 378)

Reference	Outcome variables quantified	Animals/treatment (age, initial BW)	Dietary information; time on treatment	Daily prebiotic dose; source	Major findings
Fernandez et al. (2002)	Study 1	Study 1	Study 1	Study 1	Study 1
	Salmonella challenge	Five ross-1 broiler chicks (1 d)	Wheat-SBM mash diet (ingredients and chemical composition not provided)	Control (0%)	MOS:
	Cecal Microbiota		2 wk study - chicks (except control) dosed with HCC adapted to designated treatment diet	Control + 2.5% (w/w) MOS	↑ Total anaerobes (4%)**
				Control + 2.5% (w/w) palm kernel meal	↓ Coliforms (17%) and *S. enteritidis* colonization (98%)**
	Study 2	Study 2	Study 2	Study 2	Study 2
	Salmonella challenge	15 Ross-1 broiler chicks (1 d)	Same as study 1 except 4 wk study	Same as study 1	No difference in *S. enteritidis* colonization with MOS
	Cecal microbiota				

Samarasinghe et al. (2003)	Study 1	Study 1	Study 1	Study 1	Study 1
	Performance responses (BW, feed intake, feed conversion)	30 Mixed-sex broilers (unspecified strain, 19 d)	Corn-SBM-rice polish mash diet	Control (0%)	MOS:
	Carcass composition		Chemical composition 24.1% CP, 9.3% crude fat, 5.4% CF, 7.9% ash	Control + 500 ppm virginiamycin	↑ Net protein utilization (31%)**
	Intestinal microbiology		4 wk study	Control + 200 ppm MOS	
				Control + 1 g/kg turmeric root powder	
				Control + 2 g/kg turmeric root powder	
				Control + 3 g/kg turmeric root powder	
	Study 2	Study 2	Study 2	Study 2	Study 2
	Performance responses (BW, feed intake, feed conversion)	36 Mixed-sex broilers (unspecified strain, 21 d)	Same as study 1	Control (0 ppm)	MOS:
	Carcass composition			Control + 500 ppm virginiamycin	↓ Feed intake (5%)**
				Control + 200 ppm MOS	↑ Daily weight gain (8%), feed conversion (13%), energy utilization (6%), protein utilization (5%)**

Table 12.3 (*Cont'd* p. 380)

Reference	Outcome variables quantified	Animals/treatment (age, initial BW)	Dietary information; time on treatment	Daily prebiotic dose; source	Major findings
Samarasinghe et al. (2003) (con't.)	Intestinal microbiology			Control + 1 g/kg turmeric root powder	↑ % Live carcass (2%), liver (25%), abdominal fat weight (55%)**
					↓ Coliforms (58%), yeasts and molds (84%), total viable microbial counts (51%) in intestinal contents**
Shashidhara and Devegowda (2003)	Study 1	Study 1	Study 1	Study 1	Study 1
	Production responses (hatchability, sperm count)	1,560 Cobb broiler breeders (1,440 female, 120 male)	Corn-SBM-rice bran diet Chemical composition (Males) 15.7% CP, 7.6% fiber, 1.0% Ca, 0.34% P	Control (0 g/kg)	MOS: ↑ Egg production on wk 60–62 (3–6%)**
	Immune response		(Females) 16.1% CP, 5.6% fiber, 3.3% Ca, 0.33%P	Control + 0.5 g/kg MOS (Bio-Mos)	↑ Hatchability [total (0.6–5.2%) and fertile egg sets (0.8–5.3%)]**
			8 wk study		↓ Infertile (5–30%) and dead-in-shell eggs (18, 26% on wk 64 and 66)**
					↑ Sperm count (18%)**
					↑ IBDV antibody titer in breeder females (17%)**

Shashidhara and Devegowda (2003) (con't.)	Study 2	Study 2	Study 2	Study 2	Study 2
	Production responses (hatchability, sperm count)	1,440 Cobb breeders (1,284 female, 156 male)	Same as study 1 except 12 wk study	Control (0 g/kg)	MOS:
	Immune response			Control + 1 g/kg MOS	↑ Hatchability (fertile egg set only, 1–3%)**
					↓ Infertile eggs (9–27%)**
					↑ IBDV antibody titer in breeder females (15%) and chicks (47%)**
Xu et al. (2003)	Performance responses (Feed intake, ADG, F:G)	60 Male Avian farms broiler chicks (1 d)	Corn-SBM diet	Control (0 g/kg)	2 g/kg FOS:
	Intestinal microbiota		Chemical composition (initial–final) 22.8–18.2% CP, 0.94–0.81% Ca, 0.85–0.67% P	Control + 2 g/kg FOS (Meioligo-P)	↓ F:G (5%)**
			49 d study	Control + 4 g/kg FOS	↓ Cecal *E. coli* (8%)**
	Enzyme activity			Control + 8 g/kg FOS	↑ Amylase activity (52%)**
	Intestinal morphology				↑ Ileal villus height:crypt depth (31%), microvillus height (25%)**

Table 12.3 (*Cont'd* p. 382)

Reference	Outcome variables quantified	Animals/treatment (age, initial BW)	Dietary information; time on treatment	Daily prebiotic dose; source	Major findings
Xu et al. (2003) (con't.)					4 g/kg FOS:
					↑ ADG (11%)**
					↓ F:G (9%)**
					↑ Intestinal bifidobacteria (12%), lactobacilli(14%)**
					↓ Intestinal *E. coli* (12%)**
					↑ Cecal bifidobacteria (7%), lactobacilli (8%), total anaerobes (3%)**
					↓ Cecal *E. coli* (7%)**
					↑ Protease (27%), amylase (75%) activities**
					↑ Jejunal villus height: crypt depth (26%), microvillus height (21%)**

Xu et al. (2003) (con't.)					↓ Jejunal (17%), ileal crypt depth (25%)**
					↑ Ileal villus height (16%), villus height: crypt depth (55%), microvillus height (39%) **
Yusrizal and Chen (2003)	Performance responses (BW, feed intake, F:G)	32 Ross × Ross broiler chicks (16 male, 16 female; 1 d)	Corn-SBM diet	Control (0%)	Inulin:
	Carcass composition		Chemical composition (initial-final) 21.3–19.8% CP	Control + 1% inulin (Raftifeed IPF)	Males:
	Serum profile		6 wk study	Control + 1% OF (Raftifeed OPS)	↑ Carcass % (2%)**
					↑ BW at wk 2 (10%)**
					↓ F:G at wk 6 (18%)**
	Intestinal morphology				↓ Abdominal fat as % carcass (32%), % live weight (30%)**
					Females:
					↓ Serum cholesterol (17%)**
					↓ Abdominal fat as % carcass (30%), % live weight (30%)**

Table 12.3 (*Cont'd* p. 384)

Reference	Outcome variables quantified	Animals/treatment (age, initial BW)	Dietary information; time on treatment	Daily prebiotic dose; source	Major findings
Yusrizal and Chen (2003) (con't.)					OF:
					Males:
					↑ BW at wk 2 (5%)**
					Females:
					↑ BW (10%)**
					↑ Carcass weight (13%), carcass % (3%)**
					↑ Gut length (8%)**
					↓ F:G at wk 2 (4%), 5 (13%), 6 (18%)**
					↓ Serum cholesterol (20%)**
Zhang et al. (2003)	Performance responses (BW, feed intake, feed efficiency)	72 Male Arbor acres broiler chicks (1 d)	Corn-SBM diet	Control (0%)	0.3% IMO:
	Immune organ weight		Chemical composition (initial–final) 22.1–18.0% CP, 1.1–0.8% Ca, 0.52–0.40% P	Control + 0.3% IMO (IMO-900)	↑ ADG (7%)***
			7 wk study	Control + 0.6% IMO	↑ Thymus weight (49%)**
	Intestinal and cecal microbiota			Control + 0.9% IMO	↓ Crop isobutyrate (61%)**

Zhang et al. (2003) (con't.)					↓ Duodenal isovalerate (61%)*
				Control + 1.2% IMO	↑ Jejunal butyrate (102%), isobutyrate (85%)**
	Intestinal metabolites				0.6% IMO:
					↓ Duodenal isovalerate (59%)*
					All concentrations IMO:
					↑ Feed efficiency (3–6%) ***
					↓ Crop isobutyrate (35–61%)**
					↓ Duodenal acetate (30–64%)**
Sims et al. (2004)	Performance responses (BW; feed consumption, conversion)	180 tom Hybrid turkey poults (1 d)	Ingredient composition not provided	Control (0%)	MOS:
	Intestinal microbiota		Chemical composition (initial-final %) 29–17% CP, 6.1–10.2% crude fat, 1.4–0.9% Ca, 0.75–0.48% P	Control + bacitracin (BAC; 55 mg/kg until 6 wk, 27.5 mg/kg after)	↑ BW at 18 wk (6%)**
			18 wk study	Control + MOS (Bio-Mos; 0.1% until 6 wk, 0.05% after)	↑ Feed conversion at 12 (6%), 15 wk (6%)**

Table 12.3 (*Cont'd* p. 386)

Reference	Outcome variables quantified	Animals/treatment (age, initial BW)	Dietary information; time on treatment	Daily prebiotic dose; source	Major findings
Sims et al. (2004) (con't.)	Intestinal histology			Control + BAC + MOS (same doses)	↑ Bifidobacteria (21%), total anaerobes (9%)**
					↓ Clostridia (29%)**
					MOS + BAC:
					↑ BW at 15 (5%), 18 wk (8%)**
					↑ Feed conversion at 18 wk (11%)**
Zdunczyk et al. (2004)	Performance responses (BW, feed consumption, conversion)	45 BUT-9 turkey chickens (3 d)	Wheat-corn-SBM mash diet with or without an antibiotic	Control (0.0%)	0.1% MOS:
	Cecal enzyme activity		Chemical composition (initial-final) 28.8–25.9% CP, 4.4–5.5% crude fat, 3.4–3.7% CF, 1.3–1.1% Ca, 0.73–0.64% P	Control + 0.1% MOS (Bio-Mos)	↓ Cecal tissue weight (19%)**
	Cecal metabolites		8 wk study	Control + 0.25% MOS	↓ Molar ratio of cecal propionate (60%)**
				Control + 0.5% MOS	0.25% MOS:

Zdunczyk et al. (2004) (con't.)					↑ Cecal DM (19%), pH (11%)**
					↑ Cecal propionate (72%)**
					↑ Cecal α-glucosidase (68%), β-glucosidase (200%), α- galactosidase (89%), β-galactosidase (219%), and β-glucuronidase (65%)**
					0.5% MOS:
					↑ Cecal acetate (40%), butyrate (72%), total SCFA (42%)**
					↓ Molar ratio of cecal propionate (60%)**
Cao et al. (2005)	Performance responses (BW, feed intake, mortality)	1,500 Arbor acres commercial broiler hens (28 d)	Isolated soy protein-cornstarch diet	Control (0%)	FOS:
	Cecal microbiota		Chemical composition 18% CP, 79.8 g/kg Ca, 40.0 g/kg P	Control + 4.1 g/kg green tea polyphenols	↓ Mortality (42%)**
	Cecal metabolites	14 d study		Control + 4.1 g/kg FOS	↑ Cecal bifidobacteria (9%), eubacteria (5%)**

Table 12.3 (*Cont'd* p. 388)

Reference	Outcome variables quantified	Animals/treatment (age, initial BW)	Dietary information; time on treatment	Daily prebiotic dose; source	Major findings
Cao et al. (2005) (con't.)					↓ Cecal bacilli (28%), lecithinase-positive clostridia (30%), *Peptococcaceae* spp. (11%), *Streptococci* spp. (25%), *Staphylococci* spp. (43%)**
					↑ Cecal valerate (54%)**
					↓ Cecal phenol (31%), cresol (48%), ethyl phenol (35%), and indole (36%)**
Cetin et al. (2005)	Blood metabolites	24 White hybrid converter turkey poults (15 d)	Corn-SBM diets	Control (0 g/kg)	MOS:
	IgG and IgM		Chemical composition (initial–final) 27.52–19.45% CP, 4.89–5.25% CF	1 g/kg MOS (Bio-Mos; reduced to 0.5 g/kg after wk 8)	↑ IgG (37%), IgM (44%)**
			15 wk study	1 g/kg Probiotic (Primalac 454; reduced to 0.5 g/kg after wk 8)	↓ α-naphthyl acetate esterase positive T lymphocytes (21%)**

Lee et al. (2005)	Study 1	Study 1	Study 1	Study 1	Study 1
	Performance responses (BW, feed consumption, F:G, mortality)	90 Mixed-sex Ross × Ross broiler chicks	Corn-SBM diet containing no guar	0, 2.5, 5, 7.5, or 10% of Guar gum, Guar germ, OR Guar hull	Guar gum:
	Carcass and deboned breast muscle yield		Chemical composition (starter-finisher) 22.8–17.7% CP, 0.96–0.77% Ca, 0.46–0.36% P		↑ BW (8–9%), F:G (10–14%)**
			6 wk study		5% treatments (vs. 2.5%):
					↓ Feed consumption (9%)**
					↓ Carcass weight (16%), deboned breast weight (14%) and yield (5%)**
					7.5% treatments (vs. 2.5%):
					↓ Carcass weight (16%), carcass yield (2%), deboned breast weight (18%) and yield (8%)*
					10% Treatments (vs. 2.5%):
					↓ Feed consumption (12%)**

Table 12.3 (*Cont'd* p. 390)

Reference	Outcome variables quantified	Animals/treatment (age, initial BW)	Dietary information; time on treatment	Daily prebiotic dose; source	Major findings
Lee et al. (2005) (con't.)					↓ Carcass weight (35%), carcass yield (5%), deboned breast weight (43%) and yield (19%)**
	Study 2	Study 2	Study 2	Study 2	Study 2
	Performance responses (BW, feed consumption, F:G, mortality)	100 Mixed-sex Ross × Ross broiler chicks	Same as study 1	All treatments with or without Hemicell (endo-beta-mannanase):	Guar meal:
				Control (0%)	↓ BW (14%)**
				Control + 5% guar meal	↑ F:G with (8%) and without Hemicell (20%)**
					Guar hull:
				Control + 5% guar germ	↓ BW (14%)**
				Control + 5% guar hull	↓ feed consumption (6%)**
					↑ F:G with (5%) and without Hemicell (15%)**
					Guar germ:
					↑ F:G with Hemicell (6%)**

Parks et al. (2005)	Performance responses (BW, feed consumption, feed conversion)	160 Female large white hybrid converter turkey poults (1 d)	Corn-SBM diets	Control (0 mg/kg)	MOS + virginiamycin:
	Mortality		Chemical composition (initial–final) 28.00–20.45% CP, 4.61–7.22% crude fat, 0.71–0.35% met, 1.20–0.73% met + cys, 1.80–1.03% lys, 1.60–0.85% Ca, 0.75–0.38% P	22 mg/kg Virginiamycin	↓ BW at 3 wk (5%)**
	Production responses		98 d study	500 mg/kg MOS (reduced to 22 mg/kg after 6 wk) + 22 mg/kg virginiamycin	↑ BW at 9 (4%), 12 wk (4%)**
					↑ BW gain at 6–9 wk (8%)**
					↓ Feed conversion rates at 6–9 wk (7%), overall (1%)**
Thitaram et al. (2005)	Performance responses (BW, feed conversion, feed efficiency)	Ten Ross × Ross broiler chicks (1 d)	Standard unmedicated corn-soy diet	Control (0%)	All doses IMO:
					↑ Bifidobacteria (10–11%)**
					1% IMO:
					↓ BW gain (10%)**
					↓ *Salmonella enterica* ser. Typhimurium (31%)**
				Control + 1% IMO	

Table 12.3 (*Cont'd* p. 392)

Reference	Outcome variables quantified	Animals/treatment (age, initial BW)	Dietary information; time on treatment	Daily prebiotic dose; source	Major findings
Thitaram et al. (2005) (con't.)	Cecal microbiota		Chemical composition 22.5% CP, 5.3% crude fat, 2.5% CF, 0.45% P	Control + 2% IMO	
			21 d study	Control + 4% IMO	
Zaghini et al. (2005)	Performance responses (feed consumption)	24 Warren laying hens (44 wk, 2.2 kg)	Corn-SBM diet	Control (0%)	MOS:
	Egg production and quality		Chemical composition 14.79% CP, 3.23% crude fat, 4.54% CF, 3.53% Ca, 0.86% P	2.5 ppm aflatoxin B1	↓ Egg weight at wk 2 (6%), 3 (4%)**
	Liver histology		4 wk study	0.11% MOS (Bio-Mos)	↓ Yolk a* color (6%)**
				0.11% MOS + 2.5 ppm aflatoxin B1	↑ Albumen CP at wk 2 (3%)*, 4 (3%)**
					↑ Albumen ash at wk 4 (14%)*
					MOS + aflatoxin:
					↓ Egg weight at wk 2 (8%)** ↑ Albumen ash at wk 4 (12%)*

Zaghini et al. (2005) (con't.)					↑ Yolk L (5%)*, b* (6%)** color at wk 4
					↑ Albumen CP at wk 2 (3%)*, 4 (3%)**
Zdunczyk et al. (2005)	Cecal microbiota	39 Male BUT-9 turkey poults (3 d)	SBM-wheat-corn diet	Control (0%)	All concentrations MOS:
	Cecal metabolites		Chemical composition (initial–final) 28.77–18.78% CP, 3.4–3.0% CF, 4.4–6.6% crude fat, 1.8–1.2% lys, 1.16–0.73% met + cys, 1.3–1.0% Ca, 0.72–0.50% P	0.1% MOS (Bio-Mos)	↓ Colon weight (16–30%)**
	Cecal enzyme activity		16 wk study	0.4% MOS (decreased to 0.2% after 8 wk)	↑ Cecal ammonia as % BW (49–64%)**
				1.0% MOS (decreased to 0.4% after 8 wk)	0.1% MOS:
					↓ Cecal pH (14%)**
					↑ Cecal ammonia (30%)**
					0.4/0.2% MOS:
					↑ BW at 16 wk (5%)**
					↓ Cecal acetate (45%), total SCFA (34%)**
					↓ Cecal *E. coli* (13%)**
					1.0/0.4% MOS:
					↑ BW at 16 wk (3%)**

Table 12.3 (*Cont'd* p. 394)

Reference	Outcome variables quantified	Animals/treatment (age, initial BW)	Dietary information; time on treatment	Daily prebiotic dose; source	Major findings
Zdunczyk et al. (2005) (con't.)					↓ Cecal acetate (46%), valerate (35%), total SCFA (34%)**
					↓ Cecal *E. coli* (15%)**
Jiang et al. (2006)	Performance responses (BW, mortality, feed consumption, feed efficiency)	72 Male Arbor acres broiler chicks (1 d)	Corn-soy protein isolate diet	Control (0%)	4 g/kg stachyose:
	Cecal metabolites		Chemical composition (starter-grower) 220.5–200.0 g/kg CP, 10.2–9.6 g/kg Ca, 6.7–6.4 g/kg P, 12.3–10.9 g/kg lys, 7.0–5.7 g/kg met, 10.7–9.1 g/kg met + cys	Control + 4 g/kg stachyose	↑ Cecal butyrate on d 21 (23%)**
	Cecal microbiota		42 d study	Control + 8 g/kg stachyose	12 g/kg stachyose:
				Control + 12 g/kg stachyose	↓ 6 wk BW (4%)*
					↓ Overall ADG (4%)*
	Nutrient digestibility			Control + 16 g/kg stachyose	16 g/kg stachyose:
				Control + SBM (10.5 g/kg raffinose, 32.1 g/kg stachyose; no soy protein isolate)	↓ 3 (5%), 6 wk BW (6%)*

Jiang et al. (2006) (con't.)					↓ 3 (5%) and 6 (7%) wk, overall ADG (7%)*
					↑ Stachyose concentrations:
					Quadratic ↓ ADG (control: 49.4 g/d; 4 g/kg: 49.4 g/d; 8 g/kg: 48.8 g/d; 12 g/kg: 47.4 g/d; 16 g/kg: 46.1 g/d)*
					Quadratic ↓ feed intake (control: 92.9 g/d; 4 g/kg: 93.5 g/d; 8 g/kg: 93.3 g/d; 12 g/kg: 91.0 g/d; 16 g/kg: 90.1 g/d)*
					Linear ↓ DM digestibility on d 42 (control: 76.32%; 4 g/kg: 76.36%; 8 g/kg: 74.44%; 12 g/kg: 74.65%; 16 g/kg: 72.17%)*
					Linear ↓ OM digestibility on d 21(control: 81.36%; 4 g/kg: 81.09%; 8 g/kg: 79.69%; 12 g/kg: 79.02%; 16 g/kg: 78.70%)***
					Linear ↓ OM digestibility on d 42 (control: 81.76%; 4 g/kg: 81.57%; 8 g/kg: 80.30%; 12 g/kg: 80.51%; 16 g/kg: 78.46%)**

Table 12.3 (*Cont'd* p. 396)

Reference	Outcome variables quantified	Animals/treatment (age, initial BW)	Dietary information; time on treatment	Daily prebiotic dose; source	Major findings
Jiang et al. (2006) (con't.)					Linear ↓ CP digestibility on d 42 (control: 63.22%; 4 g/kg: 62.48%; 8 g/kg: 60.21%; 12 g/kg: 62.21%; 16 g/kg: 57.69%)***
					Quadratic ↑ bifidobacteria on d 21 (control: 8.09 log CFU/g; 4 g/kg: 8.58 log CFU/g; 8 g/kg: 8.18 log CFU/g; 12 g/kg: 8.02 log CFU/g; 16 g/kg: 7.72 log CFU/g)***
					Quadratic ↑ lactobacilli on d 21 (control: 7.63 log CFU/g; 4 g/kg: 8.69 log CFU/g; 8 g/kg: 8.20 log CFU/g; 12 g/kg: 8.00 log CFU/g; 16 g/kg: 7.67 log CFU/g)***
					Quadratic ↑ cecal butyrate on d 21 (control: 0.48 mmol/g; 4 g/kg: 0.59 mmol/g; 8 g/kg: 0.52 mmol/g; 12 g/kg: 0.54 mmol/g; 16 g/kg: 0.43 mmol/g)**

Jiang et al. (2006) (con't.)					SBM:
					↓ 3 wk (4%), 6 wk BW (4%)*
					↓ 3 wk (4%), overall ADG (4%)*
Kim et al. (2006)	Egg production	Six single comb White Leghorn hens (84 wk)	Complete layer ration	Control (0%)	0.75% FOS:
	Bone measurements		Chemical composition not provided	Full feed	↓ First day out of egg production (20%)**
			51 d study	Feed withdrawal	
				100% Alfalfa	
				0.375% FOS with alfalfa	
				0.75% FOS with alfalfa	
Ma et al. (2006)	Performance responses (BW, feed consumption)	50 Male Arbor Acres broiler chicks (1 d)	Corn-SBM diet	Control (0 g/kg)	MOS + *Ligustrum lucidium*:
	Enzyme activity		Chemical composition (initial–final) 220–200 g/kg CP, 11–10 g/kg lys, 4.5–4.4 g/kg met, 10–9.5 g/kg Ca, 7.2–7.0 g/kg P	10 g/kg *Ligustrum lucidium*	↓ Serum (21%), thigh malondialdehyde concentration (35%)**
				10 g/kg *Schisandra chinensis*	↑ Serum (41%), heart glutathione reductase activity (39%)**

Table 12.3 (*Cont'd* p. 398)

Reference	Outcome variables quantified	Animals/treatment (age, initial BW)	Dietary information; time on treatment	Daily prebiotic dose; source	Major findings
Ma et al. (2006) (con't.)	Serum antibodies		49 d study	50 g/kg MOS + 10 g/kg *Ligustrum lucidium*	↑ Antibody titer against Newcastle disease virus on d 21 (21%), 28 (25%)**
	Spleen lymphocyte proliferation			50 g/kg MOS + 10 g/kg *Schisandra chinensis*	↑ Spleen lymphocyte proliferation (33%)**
					MOS + *Schisandra chinensis*:
					↓ Serum (21%), thigh malondialdehyde concentration (36%)**
					↑ Serum glutathione reductase activity (50%)**
					↑ Antibody titer against Newcastle disease virus on d 21 (18%), 28 (24%)**
					↑ Spleen lymphocyte proliferation (28%)**

Baurhoo et al. (2007a)	Performance responses (BW; feed intake, conversion)	160 Male Cobb broiler chicks (1 d)	Corn-SBM based diet	Control	MOS:
	Cecal microbiota		Chemical composition (starter-grower) 22.0–20.0% CP, 1.0–0.9% Ca, 0.50–0.47% P	Control with antibiotic	↓ Feed intake (9%), BW (9%)**
	Intestinal morphology and histology		42 d study	Control + 0.2% MOS (Bio-Mos; 0.1% in grower diet)	↑ Villus height on d 28 (7%)**
				Control + 1.25% lignin	↑ Goblet cells per villus on d 28 (79%), 42 (94%)**
					↑ Cecal lactobacilli on d 42 (5%)**
				Control + 2.5% lignin	↓ *E. coli* in litter on d 28 (76%), 42 (30%)**
Baurhoo et al. (2007b)	Performance responses (BW; feed intake, conversion)	208 Male Cobb broiler chicks (1 d)	Corn-SBM based diet	Control	MOS:
				Control with antibiotic	↑ Cecal lactobacilli on d 38 (3%)**
				Control + 0.2% MOS (Bio-Mos; 0.1% in grower diet)	↓ *E. coli* on d 9 post-challenge (18%)**
				Control + 1.25% lignin	
				Control + 2.5% lignin	
	Cecal microbiota		Chemical composition not provided		
	E. coli challenge		38 d study (*E. coli* challenge at d 21–12 birds/treatment)		

Table 12.3 (*Cont'd* p. 400)

Reference	Outcome variables quantified	Animals/treatment (age, initial BW)	Dietary information; time on treatment	Daily prebiotic dose; source	Major findings
Biggs and Parsons (2007)	AA digestibility	Four cecectomized roosters plus four conventional roosters	30 g Corn-isolated soy protein diet (precision-fed)	Control (0 g/kg)	Cecectomized roosters:
	TME_n		Chemical composition 160 g/kg CP	4 g/kg inulin	8 g/kg OF:
			48 h study	4 g/kg OF	↑ met digestibility (3%)**
				4 g/kg scFOS	4 g/kg scFOS:
				4 g/kg MOS	↑ met digestibility (3%)**
				4 g/kg TOS	4 g/kg MOS:
				8 g/kg Inulin	↑ met digestibility (5%)**
				8 g/kg OF	8 g/kg MOS:
				8 g/kg scFOS	↑ ile (6%), lys digestibility (6%)**
				8 g/kg MOS	4 g/kg TOS:
				8 g/kg TOS	↑ ile digestibility (5%)**
					8 g/kg TOS:
					↑ ile (8%), lys (8%), met (5%), val digestibility (9%)**

Biggs and Parsons (2007) (con't.)					Conventional roosters:
					4 g/kg Inulin:
					↓ met digestibility (5%)**
					8 g/kg Inulin:
					↓ met digestibility (3%)**
Biggs et al. (2007)	Study 1	Study 1	Study 1	Study 1	Study 1
	Performance responses (BW, feed intake, G:F)	40 Male Hampshire × Columbian chicks (1 d)	Corn-SBM diet	Control (0 g/kg)	Inulin:
	Nutrient digestibility		Chemical composition 23% CP, 1.27% lys, 0.53% met, 0.90% met + cys	8 g/kg Inulin	↓ Metabolizable energy on d 7 (8%), 14 (4%), 21 (4%)**
	Metabolizable energy		21 d study	8 g/kg OF	↓ ile (4%), met (4%) digestibility on d 3**
				8 g/kg scFOS	↓ arg (2%), cys (7%), his (4%), ile (4%), leu (4%), lys (3%), met (4%), phe (3%), thr (6%), val (7%) digestibility on d 7**
				8 g/kg MOS	
				8 g/kg TOS	
					↓ arg (5%), cys (13%), his (7%), ile (8%), leu (6%), lys (5%), met (5%), phe (6%), thr (10%), val (8%) digestibility on d 21**

Table 12.3 (*Cont'd* p. 402)

Reference	Outcome variables quantified	Animals/treatment (age, initial BW)	Dietary information; time on treatment	Daily prebiotic dose; source	Major findings
Biggs et al. (2007) (con't.)					scFOS:
					↓ Metabolizable energy on d 7 (10%), 14 (3%)**
					↓ ile (4%), met (4%) digestibility on d 3*
					↓ arg (3%), cys (9%), his (5%), ile (4%), leu (4%), lys (4%), met (5%), phe (4%), thr (8%), val (10%) digestibility on d 7**
					↓ arg (4%), cys (11%), his (5%), ile (7%), leu (5%), lys (5%), met (5%), phe (5%), thr (8%), val (6%) digestibility on d 21**
					MOS:
					↓ Metabolizable energy on d 14 (4%)**
					↓ met digestibility on d 3 (5%)**

Biggs et al. (2007) (con't.)					↓ arg (2%), cys (5%), his (3%), ile (3%), leu (3%), lys (2%), met (4%), phe (3%), thr (5%), val (3%) digestibility on d 7**
					TOS:
					↓ arg (2%), cys (4%), his (2%), ile (3%), leu (3%), lys (2%), met (3%), phe (3%), thr (5%), val (4%) digestibility on d 7**
					↓ arg (2%), cys (5%), his (2%), ile (2%), leu (2%), lys (2%), met (2%), phe (2%), thr (4%), val (3%) digestibility on d 21**
	Study 2	Study 2	Study 2	Study 2	Study 2
	Performance responses (BW, feed intake, G:F)	40 Male Hampshire × Columbian chicks (1 d)	Same as study 1	Control (0 g/kg)	Inulin:
	Nutrient digestibility		21 d study	4 g/kg inulin	↑ Metabolizable energy on d 7 (7%), 14 (3%), 21 (5%)**
				4 g/kg OF	↓ phe (3%), thr (7%) digestibility on d 3–4**
	Metabolizable energy			4 g/kg scFOS	↓ cys (3%), lys (2%), thr (2%) digestibility on d 21**

Table 12.3 (*Cont'd* p. 404)

Reference	Outcome variables quantified	Animals/treatment (age, initial BW)	Dietary information; time on treatment	Daily prebiotic dose; source	Major findings
Biggs et al. (2007) (con't.)	Cecal microbiota			4 g/kg MOS	OF:
				4 g/kg TOS	↑ Metabolizable energy on d 7 (6%), 21 (3%)**
					↓ ile (4%), leu (4%), phe (3%), thr (7%), val (6%) digestibility on d 3–4**
					↓ cys (2%), met (1%) digestibility on d 21 **
					scFOS:
					↓ Metabolizable energy on d 3–4 (8%)**
					↑ Metabolizable energy on d 21 (2%)**
					↓ arg (5%), cys (8%), his (7%), ile (7%), leu (7%), lys (8%), met (10%), phe (6%), thr (13%), val (11%) digestibility on d 3–4**
					↓ arg (2%), his (1%), ile (2%), leu (1%), lys (3%), met (3%), phe (1%), thr (4%), val (2%) digestibility on d 21**

Biggs et al. (2007) (con't.)					MOS:
					↓ Metabolizable energy on d 3–4 (8%)**
					↑ Metabolizable energy on d 7 (2%), 14 (2%), 21 (5%)**
					↑ arg (1%), cys (6%), his (2%), ile (2%), leu (2%), lys (1%), met (1%), phe (2%), thr (3%), val (4%) digestibility on d 21**
					TOS:
					↓ Metabolizable energy on d 3–4 (12%)**
					↑ Metabolizable energy on d 21 (2%)**
					↓ arg (6%), cys (14%), his (7%), ile (8%), leu (7%), lys (8%), met (5%), phe (7%), thr (13%), val (10%) digestibility on d 3–4**
					↓ arg (2%), cys (1%), his (1%), ile (3%), leu (1%), lys (4%), met (2%), thr (3%) digestibility on d 21**

Table 12.3 (*Cont'd* p. 406)

Reference	Outcome variables quantified	Animals/treatment (age, initial BW)	Dietary information; time on treatment	Daily prebiotic dose; source	Major findings
Biggs et al. (2007) (con't.)	Study 3	Study 3	Study 3	Study 3	Study 3
	Cecal microbiota	20 Male Hampshire × Columbian chicks (1 d)	Dextrose-isolated soy protein diet	Same as study	scFOS:
			Chemical composition 23% CP, 1.41% lys, 0.54% met, 0.90% met + cys		↓ Cecal *Clostridium perfringens* (5%)**
			21 d study		MOS:
					↓ Cecal *Clostridium perfringens* (6%)**
Elmusharaf et al. (2007)	Performance responses (BW; feed intake, conversion)	64 Male Ross 308 broiler chicks (1 d)	Corn-SBM-wheat based pelleted diet	Control (0 g kg)	MOS:
	Coccidiosis challenge (oocyst count, lesion scoring)		Chemical composition 215.6 g/kg CP	Control + 10 g/kg MOS (Bio-Mos) with or without coccidial challenge	↓ oocyst shedding on d 5 (55%), 12 (70%)**
			19 d study		↓ Lesions from *Eimeria acervulina* (72%)**

Lan et al. (2007)	Cecal lactic acid bacteria	60 Male Arbor acres broiler chicks (1 d)	Corn-SBM diet	Negative control (0% SBM, 0% antibiotics)	SBM oligosaccharides:
	Intestinal morphology		Dietary composition 202.1 g/kg CP, 10.0 g/kg Ca, 6.4 g/kg P	Positive control (SBM, antibiotics)	↑ Lactic acid bacteria (determined via threshold cycle values, 17%)**
			15 d study	Negative control + 10 g/kg SBM oligosaccharides	
Rehman et al. (2007)	Performance response (BW)	20 Ross 308 broiler chicks (1 d)	Corn-SBM-wheat diet	Control (0%)	Inulin:
	Intestinal histomorpho-logy		Dietary composition 24.9% CP, 3.5% crude fat, 1.5% CF, 1.1% Ca, 0.87% P	Control + 1% inulin (Raftiline-GR)	↑ Jejunal villus height (20%)**
	Nutrient transport activity		35 d study		↑ Crypt depth (31%)*
					↑ Short-circuit current with glucose (669%) and glutamine (1,571%)*
					↓ Basal values of transmucosal tissue resistance with glucose (47%) and glutamine (37%)*

Table 12.3 (*Cont'd* p. 408)

Reference	Outcome variables quantified	Animals/treatment (age, initial BW)	Dietary information; time on treatment	Daily prebiotic dose; source	Major findings
Solis de los Santos et al. (2007)	Performance response (BW)	18 Hybrid converter tom turkey poults (1 d)	Standard unmedicated corn-soybean starter diet	Control (0%)	All concentrations MOS:
	Intestinal morphology		21 d study	Control + 0.455 g/kg MOS (Alphamune, yeast extract)	↑ BW on d 7 (8%)**
				Control + 0.91 g/kg MOS	↑ Duodenal neutral (47–71%), sialomucin (56–64%), sulfomucin (44–56%) goblet cells on d 7**
					↑ Jejunal crypt depth on d 7 (40–48%), 21 (46–76%)**
					↑ Jejunal villus height (57–79%), villus surface area on d 21 (89–127%)**
					↑ Jejunal neutral (40–51%), sulfomucin (23–66%) goblet cells on d 21**
					↑ Ileal villus height (31–33%), villus surface area (56–57%) on d 21**

Solis de los Santos et al. (2007) (con't.)					↑ Ileal crypt depth on d 7 (30–49%), 21 (41–57%)**
					↑ ileal neutral (60–92%), sialomucin (72–116%), sulfomucin (32–72%) goblet cells on d 7**
					0.455 g/kg MOS:
					↑ Duodenal villus height (21%), surface area (38%) on d 7**
					↑ Duodenal crypt depth on d 21 (3%)**
					↑ Duodenal sulfomucin goblet cells on d 21 (53%)**
					↑ Jejunal villus surface area on d 7 (89%)**
					↑ Jejunal lamina propria thickness on d 21 (64%)**
					↑ Jejunal sialomucin (31%), sulfomucin (50%) goblet cells on d 7**
					↑ Jejunal sialomucin goblet cells on d 21 (84%)**
					↑ Ileal lamina propria thickness on d 7 (44%), 21 (38%)**

Table 12.3 (*Cont'd* p. 410)

Reference	Outcome variables quantified	Animals/treatment (age, initial BW)	Dietary information; time on treatment	Daily prebiotic dose; source	Major findings
Solis de los Santos et al. (2007) (con't.)					↑ Ileal villus height on d 7 (49%)**
					↑ Ileal villus surface area (86%)**
					↑ Ileal neutral (120%), sialomucin (58%), sulfomucin (36%) goblet cells on d 21**
Donaldson et al. (2008)	Study 1	Study 1	Study 1	Study 1	Study 1
	Salmonella enterica serovar Enteritidis challenge	12 Laying hens	Corn-soybean mash	Control (nonmolted)	0.75% FOS:
	Performance responses (feed intake, BW, ovarian weight)		Chemical composition 16.5% CP, 3.5% Ca, 0.48% P	Feed withdrawal (molted)	↓ Number of hens positive for *Salmonella enteritidis* in crop (17%)**
	Crop and cecal metabolites		28 d study	90% alfalfa + 10% layer ration	

Donaldson et al. (2008) (con't.)	Crop, cecal, and organ *Salmonella enteritidis* colonization			90% alfalfa + 10% layer ration + 0.375% FOS	
	Intestinal *Salmonella enteritidis* shedding			90% alfalfa + 10% layer ration + 0.75% FOS	
	Study 2	Study 2	Study 2	Study 2	Study 2
	Same as study 1	Same as study 1	Same as study 1	Same as study 1	No differences observed
	Study 3	Study 3	Study 3	Study 3	Study 3
	Same as study 1 except no feed intake data	Same as study 1	Same as study 1	Same as study 1	0.375% FOS:
					↓ Number of hens positive for *Salmonella enteritidis* in intestines (60%)**
					↓ Intestinal *Salmonella enteritidis* colonization (118%)**
					↓ Crop lactic acid (28%)**

Table 12.3 (*Cont'd* p. 412)

Reference	Outcome variables quantified	Animals/treatment (age, initial BW)	Dietary information; time on treatment	Daily prebiotic dose; source	Major findings
Donaldson et al. (2008) (con't.)					0.75% FOS:
					↑ Crop lactic acid (320%)**
					↑ Crop *Salmonella enteritidis* colonization (127%)**
					↓ Number of hens positive for *Salmonella enteritidis* in intestines (60%)**
					↓ Intestinal *Salmonella enteritidis* colonization (118%)**
	Study 4	Study 4	Study 4	Study 4	Study 4
	Same as study 3	Same as study 1	Same as study 1	Same as study 1	0.375% FOS:
					↑ Crop lactic acid (295%)**
					0.75% FOS:
					↑ Cecal isobutyrate (250%)**

Yang et al. (2008)	Study 1	Study 1	Study 1	Study 1	Study 1
	Performance responses (BW, feed intake, feed conversion)	96 Male Cobb broiler chicks (1 d)	SBM-sorghum diet	Control (0 g/kg)	1 g/kg MOS:
	Metabolizable energy		Chemical composition (starter-grower) 230.0–210.0 g/kg CP, 40.8–44.0 g/kg CF, 57.3–52.1 g/kg crude fat, 12.5–11.0 g/kg lys, 9.0–8.3 g/kg met + cys, 10.0 g/kg, 4.0–3.7 g/kg P	Control + 50 ppm zinc bacitracin	↑ AME (2%)**
	Intestinal microbiota		5 wk study	1 g/kg MOS	↑ Ileal pH (4%)**
	Intestinal metabolites			2 g/kg MOS	↓ Ileal total SCFA (66%)**
					↓ Cecal lactobacilli (5%)**
					↓ Cecal propionate (50%)**
					↑ Cecal butyrate (92%)**
					2 g/kg MOS:
					↓ Feed conversion during wk 1–3 (4%)***

Table 12.3

Reference	Outcome variables quantified	Animals/treatment (age, initial BW)	Dietary information; time on treatment	Daily prebiotic dose; source	Major findings
Yang et al. (2008)					↑ AME (2%)**
					↑ Ileal pH (5%)**
					↓ Ileal lactobacilli (8%), coliforms (8%)**
					↓ Ileal lactic acid (47%), total SCFA (42%)**
					↓ Cecal propionate (59%)**
	Study 2	Study 2	Study 2	Study 2	Study 2
	Metabolizable energy	Eight male Cobb broiler chicks (1 d)	Same as study 1	Same as study 1	MOS:
	Nutrient digestibility				↓ Soluble NSP digestibility (20%)**

[a]*AA* amino acid, *ADG* average daily gain, AME_n metabolizable energy corrected for nitrogen, *BAC* bacitracin, *BW* body weight, *Ca* calcium, *CF* crude fiber, *CFU* colony forming unit, *CHO* carbohydrate, *CP* crude protein, *cys* cysteine, *d* day, *DM* dry matter, *ESBM* ethanol-extracted soybean meal, *F:G* feed:gain ratio, *FOS* fructooligosaccharide, *g* gram, *h* hour, *HCC* hen cecal contents, *IBDV* infectious bursal disease virus, *IgG* immunoglobulin G, *IgM* immunoglobulin M, *IMO* isomaltooligosaccharide, *kg* kilogram, *lys* lysine, *met* methionine, *mmol* millimole, *MOS* mannanoligosaccharide, *NSP* non-starch polysaccharide, *OF* oligofructose, *P* phosphorus, *ppm* parts per million, *SBM* soybean meal, *SCFA* short-chain fatty acid, TME_n true metabolizable energy corrected for nitrogen, *TOS* transgalactooligosaccharide, *val* valine, *wk* week

[b]*$P < 0.01$, **$P < 0.05$, ***$P < 0.10$

supplementation (Yang et al., 2008). Pathogenic microbiota also decreased with prebiotic supplementation. Five authors reported decreased salmonellae and coliforms (Donaldson et al., 2008; Fernandez et al., 2000, 2002; Spring et al., 2000; Thitaram et al., 2005), while four authors reported decreased *E. coli* (Baurhoo et al., 2007a, b; Xu et al., 2003; Zdunczyk et al., 2005). Streptococci, peptococci, bacilli, staphylococci, bacteriodaeceae, pseudomonad, yeast, and mold populations also have been reported to decrease with prebiotic supplementation (Cao et al., 2005; Samarasinghe et al., 2003; Terada et al., 1994).

Changes in fermentation profiles also occur with prebiotic supplementation of poultry. Thirty-six percent of studies observed increased butyrate concentrations with prebiotic supplementation (Jiang et al., 2006; Terada et al., 1994; Yang et al., 2008; Zdunczyk et al., 2004; Zhang et al., 2003). When supplemented with lactosucrose and FOS, decreased cecal phenol and indole concentrations were observed (Cao et al., 2005; Terada et al., 1994). Although inconclusive, total SCFA and lactic acid concentrations generally decreased while intestinal pH increased with prebiotic supplementation (Donaldson et al., 2008; Yang et al., 2008; Zdunczyk et al., 2005). As fermentative end-products are volatile, they can pose a significant problem not only to the health of the animals but also to the people who work with them on a daily basis. Reducing emissions of volatile components such as ammonia improves air quality in production settings and has the potential to improve the environment in general. However, mixed results have been observed with regard to cecal ammonia concentrations: ammonia decreased in response to lactosucrose supplementation (Terada et al., 1994), but increased in response to MOS supplementation (Zdunczyk et al., 2005). Mixed results also have been reported for acetate, propionate, isobutyrate, and valerate concentrations with prebiotic supplementation (Cao et al., 2005; Donaldson et al., 2008; Terada et al., 1994; Yang et al., 2008; Zdunczyk et al., 2004, 2005; Zhang et al., 2003).

Prebiotic supplementation also affects cell proliferation in poultry. Increased intestinal villus height was reported in 40% of studies measuring intestinal morphology (Baurhoo et al., 2007a; Rehman et al., 2007; Solis de los Santos et al., 2007; Xu et al., 2003). Two studies investigating the effects of MOS supplementation reported increased numbers of goblet cells per villus, as well as increases in several types of goblet cells along the intestinal tract (Baurhoo et al., 2007a; Solis de los Santos et al., 2007). Although inconclusive, crypt depth generally increased with prebiotic supplementation (Rehman et al., 2007; Solis de los Santos et al., 2007). Other intestinal characteristics have been observed in several studies, including increased gut length (Yusrizal and Chen, 2003) and microvillus height (Xu et al., 2003).

Changes in nutrient digestibility are of utmost concern whenever a feed supplement is added to a diet. For young chicks, amino acid (AA) digestibility and metabolizable energy generally decreased in response to prebiotic supplementation (Biggs et al., 2007). Decreased AA digestibility also was observed in intact adult roosters; however, when observed in cecectomized roosters, increased AA digestibility was observed (Biggs and Parsons, 2007). This implies that the observed changes in digestibility are caused by microbial fermentation in the ceca. Soy oligosaccharides decreased dry matter digestibility in four studies (Coon et al., 1990; Jiang et al., 2006; Leske et al., 1993; Leske and Coon, 1999a) and true metabolizable energy in three studies (Coon et al., 1990; Leske et al., 1993; Leske and Coon, 1999a), and decreased digestibilities of other nutrients in one study (Leske and Coon, 1999a). Mixed results in apparent metabolizable energy data were observed by three authors with select prebiotic supplements (Biggs et al., 2007; Leske and Coon, 1999a; Yang et al., 2008).

Performance and production responses have been evaluated as regards prebiotic supplementation. Body weight was measured in 57% of studies and, although inconclusive, increased in the majority of those studies (Fairchild et al., 2001; Lee et al., 2005; Parks et al., 2001; Sims et al., 2004; Solis de los Santos et al., 2007; Yusrizal and Chen, 2003; Zdunczyk et al., 2005). Similarly, body weight gain increased (Parks et al., 2005; Samarasinghe et al., 2003), while average daily gain responses were variable with prebiotic supplementation (Parks et al., 2005; Sims et al., 2004; Xu et al., 2003; Yang et al., 2008). Feed intake and feed:gain ratios (F:G) generally decreased with supplementation of fructans and MOS (Baurhoo et al., 2007a; Parks et al., 2001; Samarasinghe et al., 2003; Xu et al., 2003; Yusrizal and Chen, 2003). Increased carcass weight and abdominal fat weight were observed with MOS and inulin supplementation (Samarasinghe et al., 2003; Yusrizal and Chen, 2003). Egg production and hatchability increased in one study (Shashidhara and Devegowda, 2003), while egg weight and yolk color decreased in another study with MOS supplementation (Zaghini et al., 2005).

Swine. As has been observed in poultry, major changes in intestinal microbiota occur in swine in response to prebiotic consumption (*Table 12.4*). Increased bifidobacteria (Estrada et al., 2001; Howard et al., 1995; Loh et al., 2006; Lynch et al., 2007; Pierce et al., 2006; Smiricky-Tjardes et al., 2003; Tako et al., 2008; Tzortis et al., 2005; Xu et al., 2002) and lactobacilli (Lynch et al., 2007; Oli et al., 1998; Smiricky-Tjardes et al., 2003; Tako et al., 2008; Tzortis et al., 2005; White et al., 2002; Xu et al., 2002) were reported in 65 and 53% of studies observing changes in microbial ecology, respectively. Generally, decreased populations of enterobacteria, clostridia, coliforms, and *E. coli* were observed with

Table 12.4

***In vivo* experiments, listed in chronological order, reporting effects of prebiotics in swine** (*Cont'd* p. 416)

Reference	Outcome variables quantified	Animals/treatment (age, initial BW)	Dietary information; time on treatment	Daily prebiotic dose; source	Major findings
Howard et al. (1995)	Study 1	Study 1	Study 1	Study 1	Study 1
	Performance responses (BW, feed intake)	Ten newborn male pigs (36 h)	Infant formula	Control (0%)	FOS:
	Cecal and colonic microbiota		Chemical composition: 59.4 g/L CP, 56.2 g/L fat, 50.8 g/L CHO	Control + 3 g/L FOS	↑ Cecal cell density (11%)*
	Cecal metabolites		15 d study		↑ Cecal labeled cells (17%)**
	Cecal and colonic morphology				↑ Proximal colonic crypt height (10%), leading edge (21%), labeled cells (42%), proliferation zone (12%), and labeling index (34%)*
					↑ Proximal colonic cell density (5%)***
	Carcass morphology				↑ Distal colonic crypt height (20%), leading edge (42%), cell density (26%), labeled cells (82%), and labeling index (43%)*
					↑ Distal colonic proliferation zone (13%)***
	Study 2	Study 2	Study 2	Study 2	Study 2
	Fecal microbiota	Six newborn male pigs (36 h)	Same as study 1 except 6 d study	Same as study 1	FOS: ↑ Bifidobacteria on d 6 (247%)***

Table 12.4 (*Cont'd* p. 418)

Reference	Outcome variables quantified	Animals/treatment (age, initial BW)	Dietary information; time on treatment	Daily prebiotic dose; source	Major findings
Houdijk et al. (1998)	Performance responses (BW, feed intake, feed conversion)	Ten castrated male Great Yorkshire × Landrace × Great Yorkshire piglets (57 d, 15.6 kg)	Corn-casein-fishmeal-meat meal diet	Control (0%)	FOS:
	Fecal microbiota		Chemical composition not provided	Control + 7.5 g/kg FOS (Raftilose P95)	↓ ADG (low FOS) during wk 2 (7%)***
			6 wk study	Control + 15 g/kg FOS	↓ ADG during wk 1 (10–21%)*
				Control + 10 g/kg TOS (Oligostroop)	↓ ADG during wk 1–3 (11%)**
				Control + 20 g/kg TOS	↓ DMI during wk 1 (4–11%)***
					↓ DMI during wk 1–3 (8%)**
					↑ Feed conversion during wk 1 (6–16%)*
					↓ Feed conversion (high FOS) during wk 2 (11%)**, 3 (2%)***
					↓ Fecal DM from d 0–35 (4%)***
					TOS:
					↓ ADG during wk 1 (19–20%)*
					↓ ADG (low TOS) during wk 2 (16%)***
					↓ ADG during wk 1–3 (11–14%)**
					↓ DMI during wk 1 (6–13%)***
					↓ DMI during wk 1–3 (7–11%)**
					↑ Feed conversion during wk 1 (7–21%)*
					↓ Feed conversion during wk 2 (22%)*, 3 (16%)***
					↓ Fecal DM from d 0–35 (7–13%)***

Oli et al. (1998)	Clinical symptoms of infection	Five crossbred pigs (21 d)	Antibiotic-free early-wean pig pellets plus 15 μg/kg BW cholera toxin to induce diarrhea	Oral electrolyte solution	FOS at 24 h:
	Stool consistency		Chemical composition not provided	Oral electrolyte solution + 0.5% FOS	↑ Redox potential (29%)**
	Intestinal metabolites		72 h study		↑ Small intestinal (26%), cecal (21%) lactobacilli**
					↓ Small intestine mucosal enterobacteria (25%)**
	Intestinal microbiology				↓ Cecal (16%), colonic (27%) enterobacteria**
					FOS at 72 h:
					↓ Cecal enterobacteria (18%)**
Estrada et al. (2001)	Study 1	Study 1	Study 1	Study 1	Study
	Performance responses (BW, ADFI, F:G)	20 Weanling Canabrid × Camborough 15 pigs (8 d, 6.2 kg)	Wheat-SBM diets	Control (0.0%)	FOS:
	Fecal microbiota		Chemical composition not provided	Control + 0.5% FOS (Raftilose P95) + 10^{10} *Bifidobacterium longum* str. 75119 (given on d 1, 3)	↑ ADG from d 0–7 (16%)*
			21 d periods		↑ F:G from d 0–7 (20%)**
					↓ Enterobacteria (7%), clostridia (11%), and total anaerobes (12%) on d 7**
					↑ Bifidobacteria on d 7 (16%)**

Table 12.4 (*Cont'd* p. 420)

Reference	Outcome variables quantified	Animals/treatment (age, initial BW)	Dietary information; time on treatment	Daily prebiotic dose; source	Major findings
Estrada et al. (2001) (con't.)	Study 2	Study 2	Study 2	Study 2	Study 2
	Performance responses (BW, ADFI)	20 Weanling Canabrid × Camborough 15 pigs (8 d, 6.2 kg)	Wheat-SBM diets	Control (0.0%)	FOS:
	Fecal microbiota		Chemical composition not provided	Control + 10^7 *B. longum* str. 75119	↓ BW on d 12 (5%), 19 (9%)*
	Serum IGF-I			Control + 0.5% FOS	↓ ADG on d 5–12 (22%), 12–19 (15%), 0–19 (18%)*
				Control + 0.5% FOS + 10^{10}*B. longum* str. 75119 (given on d 1, 3)	↓ ADFI from d 0–5 (12%)***
					↓ Serum IGF-I on d 12 (56%), 19 (42%)**
Zhang et al. (2001)	Performance responses (ADG, ADFI, F:G)	8 Landrace × Large white × Duroc barrows (12.5 kg)	Corn-soy protein isolate diet	Control (0%)	SBM:
	Nutrient digestibilities		Chemical composition 19.75% CP, 0.97% Ca, 0.50% P, 1.09% lys, 0.37% met, 0.75% met + cys	Control + 1% stachyose	↓ Nitrogen retention (6%)**
	Nitrogen retention		7 d periods	Control + 2% stachyose	
				Control + 23.5% SBM (0.16% raffinose, 0.75% stachyose; no soy protein isolate)	

Davis et al. (2002)	Study 1	Study 1	Study 1	Study 1	Study 1
	Performance responses (ADG, ADFI, G:F)	54 Weanling Hampshire × Duroc × Yorkshire x Landrace barrows (18 d-old, 6 kg)	Corn-soy diet with supplemental Cu at 0 or 175 ppm	Control (0%)	MOS:
			Chemical composition (initial-final) 1.5–1.2% lys, 0.98–0.77% thr, 0.27–0.24% trp, 0.90–0.72% met + cys	Control + 0.2% MOS	↑ ADG with (76%) and without (47%) Cu during phase 1**
			38 d study		↑ G:F with (62%) and without (38%) Cu during phase 1**
					↑ ADG during phase 3 (8%), overall (6%)**
					↑ G:F during phase 3 (7%)***, overall (6%)**
	Study 2	Study 2	Study 2	Study 2	Study 2
	Performance responses (ADG, ADFI, G:F)	36 Crossbred barrows and gilts (20 kg)	Corn-soy diet with supplemental Cu at 0 or 125 ppm in starter and grower phases and 0 or 175 ppm in finisher phase	Starter:	MOS:
			Chemical composition (grower-finisher) 20.2–16.7% CP, 1.10–0.85% lys, 0.78–0.64% thr, 0.24–0.19% trp, 0.67–0.57% met + cys	Control (0%)	↑ ADG without Cu in finisher phase (7%)**
			Study end at 106 kg average BW	Control + 0.2% MOS	↓ ADG with Cu in finisher phase (5%)**
				Grower:	
				Control (0%)	
				Control + 0.1% MOS	
				Finisher:	
				Control (0%)	
				Control + 0.05% MOS	

Table 12.4 (*Cont'd* p. 422)

Reference	Outcome variables quantified	Animals/treatment (age, initial BW)	Dietary information; time on treatment	Daily prebiotic dose; source	Major findings
Houdijk et al. (2002)	Intestinal and fecal metabolites	Four castrated Great Yorkshire-Landrace × Great Yorkshire pigs (38 d, 10.4 kg)	Cornstarch-casein liquid diet	Control (0 g/kg)	40 g/kg FOS:
			Chemical composition 168.4 g/kg CP, 18.7 g/kg crude fat, 17.7 g/kg hemicelluloses, 49.1 g/kg cellulose, 5.4 g/kg lignin	Control + 10 g/kg FOS (Raftilose P95)	↓ Ileal pH (6%)**
			37 d study	Control + 40 g/kg FOS	↓ Ileal acetate (32%)**
				Control + 10 g/kg TOS (Oligostroop)	↑ Ileal isovalerate (405%)**
				Control + 40 g/kg TOS	↑ Fecal pH (11%)**
	Intestinal and fecal microbiota				↑ Fecal isobutyrate (100%)**
					40 g/kg TOS:
					↑ Fecal pH (11%)**
					↑ Fecal isobutyrate (143%)**
White et al. (2002)	Study 1	Study 1	Study 1	Study 1	Study 1
	Performance responses (ADG, ADFI, G:F)	35 Hampshire × Landrace-Yorkshire barrows and gilts (21.8 d, 6.6 kg)	Corn-SBM-whey diet	Control (0%)	Yeast diet:
	Fecal microbiota		28 d study	Control + antibiotic	↓ Feed intake on wk 1 (22%), 3 (13%), overall (11%)**
	Circulating antibodies			Control + 3% brewer's yeast	↓ Overall daily gain (9%)**
	Intestinal morphology			Control + 3% brewer's yeast + 2% citric acid	↑ Lactobacilli on d 28 (4%)**
	Fecal metabolites				↓ Total coliforms on d 14 (3%), 28 (3%)**

White et al. (2002) (con't.)					Yeast + acid diet:
					↓ Feed intake on wk 3 (10%), 4 (14%), overall (10%)**
					↓ Daily gain on wk 1 (23%), 4 (11%), overall (9%)**
					↓ Bifidobacteria (6%), aerotolerant aerobes (7%) on d 28**
					↑ IgG (42%)*
					↓ Isovalerate (32%)**
	Study 2	Study 2	Study 2	Study 2	Study 2
	Performance responses (ADG, ADFI, G:F)	Eight Hampshire × Landrace-Yorkshire pigs (11 d old, 4.1 kg)	Same as study 1 except 39 d study	Control (0%)	Yeast diets:
	Fecal microbiota		*E. coli* K88 challenge on d 29	Control + antibiotic	↓ Coliform shedding (10%)**
	E. coli challenge			Control + 3% brewer's yeast	↓ Coliforms in jejunum (23%)*
	Circulating antibodies				↓ Coliforms in cecum (9%)**

Table 12.4 (*Cont'd* p. 424)

Reference	Outcome variables quantified	Animals/treatment (age, initial BW)	Dietary information; time on treatment	Daily prebiotic dose; source	Major findings
Xu et al. (2002)	Performance responses (ADG, ADFI, F:G)	32 Jiaxing Black × Duroc × Landrace barrows (20.8 kg)	Corn-SBM diet without antibiotic supplementation	Control (0%)	4 g/kg FOS:
				Control + 2 g/kg FOS (Meioligo-P)	↑ ADG (8%)**
					↓ F:G (7%)**
				Control + 4 g/kg FOS	↑ Bifidobacteria (9%) and Lactobacili (11%) in small intestine**
	Digestive enzyme activity		Chemical composition 17.8% CP, 4.8% crude fat, 2.0% CF, 0.94% lys, 0.56% met + cys, 0.7% Ca, 0.55% P	Control + 6 g/kg FOS	↓ clostridia in small intestine (19%)**
	Intestinal and colonic microbiota		42 d study		↑ Bifidobacteria (8%) and lactobacilli (11%) in proximal colon**
	Intestinal histomorphology				↓ *E. coli* (8%) in proximal colon**
					↑ Jejunal villus height (17%)**
					↑ Jejunal villus height:crypt depth (40%)**
					↑ Protease (56%), trypsin (43%), and amylase (30%) activities in small intestinal contents**
					6 g/kg FOS:
					↑ ADG (7%)**
					↓ F:G (8%)**

Xu et al. (2002) (con't.)					↑ Bifidobacteria (8%) and lactobacilli (10%) in small intestine**
					↓ Clostridia in small intestine (22%)**
					↑ Bifidobacteria in proximal colon (8%)**
					↑ Trypsin (50%) and amylase (40%) activities in small intestinal contents**
					↑ Jejunal villus height (15%)** All concentrations FOS:
					↓ Clostridia in proximal colon (12–21%)**
Correa-Matos et al. (2003)	*Salmonella typhimurium* challenge	48 piglets (2 d)	Sow's milk replacer formula (Advance Baby Pig Liqui-Wean)	Control (0 g/L)	Soy polysaccharide:
				Control + 7.5 g/L methylcellulose	↑ Ileal sucrase activity (131%)**
	Performance responses (BW, feed intake)		Chemical composition not provided	Control + 7.5 g/L soy polysaccharide	↓ Ileal transmucosal resistance (39%)**
					↑ Colonic total SCFA (70%)**
	Physical activity		14 d study	Control + 7.5 g/L FOS	↑ Fecal moisture (100%)**
	Stool consistency				FOS:
	Histomorphology				↑ Ileal sucrase activity (152%)**
	Disaccharidase activity Intestinal electro-physiology				↑ Colonic total SCFA (50%)**
	Intestinal metabolites				

Table 12.4 (*Cont'd* p. 426)

Reference	Outcome variables quantified	Animals/treatment (age, initial BW)	Dietary information; time on treatment	Daily prebiotic dose; source	Major findings
LeMieux et al. (2003)	Study 1	Study 1	Study 1	Study 1	Study 1
	Performance responses (ADG, ADFI, G:F) over 3 phases and overall	35 Weanling Yorkshire × Landrace or Yorkshire × Landrace × Duroc barrows, boars, or gilts (20 d, 4.8 kg)	Corn-soy diet with no additional Zn (contained antibiotic)	Control (0%)	No effect of supplementation
				Control + 0.2% MOS (Bio-Mos)	
				Control + 0.3% MOS	
			Chemical composition (phase 1–3) 23.8–18.9% CP, 1.6–1.1% lys, 0.9% Ca, 0.8% P		
			28 d study		
	Study 2	Study 2	Study 2	Study 2	Study 2
	Performance responses (ADG, ADFI, G:F) over 3 phases and overall	25 Weanling Yorkshire × Landrace or Yorkshire × Landrace × Duroc barrows, boars, or gilts (17 d, 5.4 kg)	Corn-soy diet with supplemental Zn at 0 or 3,000 ppm (contained antibiotic)	Control (0%)	0.2% MOS:
				Control + 0.2% MOS	↑ ADG without Zn in phase 2 (11%), phase 3 (14%), overall (18%)***
			Chemical composition same as study 1	Control + 0.3% MOS	↑ ADFI without Zn in phase 2 (22%), overall (14%)***
			28 d study		↓ G:F without Zn in phase 2 (9%)***
					↑ G:F with Zn in phase 3 (17%)***
					↑ G:F with Zn (8%)***
	Study 3	Study 3	Study 3	Study 3	Study 3
	Performance responses (ADG, ADFI, G:F) over 3 phases and overall	25 Weanling Yorkshire × Landrace or Yorkshire × Landrace × Duroc barrows, boars, or gilts (16 d, 4.9 kg)	Corn-soy diet with supplemental Zn at 0 or 3,000 ppm (contained antibiotic)	Control (0.0%)	0.2% MOS:
				Control + 0.2% MOS	↓ ADFI without Zn in phase 2 (5%)***, overall (9%)**
					↑ ADG without Zn in phase 2 (17%), overall (6%)***
					↑ G:F without Zn in phase 2 (24%)***
			Chemical composition same as study 1		
			21 d study		

LeMieux et al. (2003) (con't.)					↓ ADG with Zn in phase 2 (14%), overall (16%)***
					↓ ADFI with Zn in phase 2 (10%)***, overall (11%)**
					↓ G:F with Zn in phase 2 (5%)**
	Study 4	Study 4	Study 4	Study 4	Study 4
	Performance responses (ADG, ADFI, G:F) over 3 phases and overall	20 weanling Yorkshire × Landrace or Yorkshire × Landrace × Duroc barrows, boars, or gilts (18 d, 4.7 kg)	Corn-soy diet with supplemental antibiotic at 0.00 or 0.75% (no supplemental Zn), or with supplemental Zn at 0 or 3,000 ppm (no supplemental antibiotic)	Control (0%)	0.2% MOS:
			Chemical composition same as study 1	Control + 0.2% MOS	↓ ADFI in phase 2 with (4%) and without antibiotic (10%)***
			27 d study		↓ ADG in phase 2 without antibiotic (15%)**
					↓ G:F in phase 2 without antibiotic (6%)*
					↑ G:F in phase 2 with antibiotic (7%)**
					↑ ADG in phase 2 with antibiotic (2%)**
					↓ G:F in phase 2 without Zn (6%)**
					↑ G:F in phase 2 with Zn (7%)**
Mikkelsen et al. (2003)	Performance responses (BW, feed consumption)	28 Mixed-sex Landrace × Yorkshire piglets (4 wk)	Cornstarch-wheat-fishmeal-casein diets Chemical composition not provided	Control	FOS:
	Fecal scores		4 wk study	Control + 4% FOS (Raftilene ST)	↑ Daily feed intake on d 14–28 (12%)***
	Fecal microbiota			Control + 4% TOS (Elix' or)	↓ Fecal valerate (30%) and isobutyrate + isovalerate (23%) on d 3**
	Fecal metabolites				↑ Fecal yeast on d 14 (24%)**
					TOS:
					↑ Daily feed intake on d 14–28 (22%)***
					↑ Fecal DM on d 3 (19%)**
					↑ Fecal valerate on d 28 (51%)**
					↑ Fecal yeast on d 7 (67%), 14 (42%), and 28 (38%)**

Table 12.4 (*Cont'd* p. 428)

Reference	Outcome variables quantified	Animals/treatment (age, initial BW)	Dietary information; time on treatment	Daily prebiotic dose; source	Major findings
Petkevicius et al. (2003)	*Oesophagostomum dentatum* challenge	Eight mixed-sex Landrace × Yorkshire × Duroc pigs (10 wk, 20.4 kg)	Barley flour-SBM diet	Basal + 300 g/kg oat hull meal (Control)	Inulin:
	Performance response (BW)		Chemical composition 17% CP, 2% fat, 19.8% dietary fiber	Basal + 160 g/kg inulin (Raftiline)	↓ Fecal DM (23%), NSP (53%)*
	Fecal egg counts		13 wk study: all pigs maintained on control for 10 wk, but dosed with 6,000 *O. dentatum* infective larvae at 3 wk; treatment diets fed for 3 wk	Basal + 210 g/kg sugar beet fiber	↑ Fecal ash (93%), protein (177%), and fat (129%)*
				Basal + 150 g/kg sugar beet fiber + 60 g/kg inulin	↓ Fecal acetate (13%), butyrate (27%)*
	Fecal metabolites				↑ Fecal propionate (35%), valerate (155%), and total SCFA (49%)*
					↓ Intestinal *O. dentatum* egg counts (100%), worm burdens (100%), and lower worm concentrations in female pigs compared to male pigs (60%)** Sugar beet fiber:
					↓ Fecal DM (27%), NSP (64%)*
					↑ Fecal ash (104%), protein (146%), and fat (167%)*
					↓ Fecal propionate (13%)*
					↑ Fecal total SCFA (35%)*
					↓ Intestinal *O. dentatum* egg counts (38%), worm burdens (72%)**
					Inulin + sugar beet fiber:
					↓ Fecal DM (26%), NSP (60%)*

Petkevicius et al. (2003) (con't.)					↑ Fecal ash (94%), protein (140%), and fat (173%)*
					↓ Fecal propionate (16%)*
					↑ Fecal total SCFA (37%)*
					↓ Intestinal *O. dentatum* egg counts (84%), worm burdens (89%)**
Smiricky-Tjardes et al. (2003)	Nutrient digestibility	12 PIC 326 × C22 pigs (30 kg)	Casein-cornstarch diet	Control (0%)	Soy solubles:
				17% soy solubles (6.9 g raffinose, 27.7 g stachyose, 1.2 g verbascose)	↓ Ileal DM (4%), OM (6%), N (4%) digestibility**
	Intestinal microbiota		Chemical composition 3.02% N	6% TOS	↑ Ileal GOS digestibility (77%)**
					↑ Ileal propionate (52%), butyrate (195%)**
					↓ Total tract N digestibility (4%)**
	Fecal microbiota		7 d periods		↑Fecal bifidobacteria (21%), lactobacilli (7%)**
					TOS:
					↓ Ileal DM (4%), OM (4%) digestibility**
					↑ Ileal GOS digestibility (100%)**
					↓ Total tract DM (2%), OM (3%) digestibility**
					↑ Fecal bifidobacteria (13%), lactobacilli (4%)**
Tsukahara et al. (2003)	Intestinal metabolites	Three castrated male Landrace × Large white × Duroc piglets (40 d, 12 kg)	Standard swine diet No. 1 (Nippon Formula Feed, Yokohama, Japan) without antibiotics or prebiotics	Standard diet (0%)	FOS:
				Standard diet + 10% (w/w) FOS	↑ Digesta water content along large intestine (8–14%)**
	Intestinal morphology		Chemical composition 22% CP, 4.6% crude fat, 0.9% CF, 6.3% ash		↓ pH of digesta samples along large intestine (4–12%)***
					↑ Butyrate (110–243%), total SCFA (13–70%) along large intestine*
			10d study		↑ Acetate (12–54%), valerate (3–444%) along large intestine***

Table 12.4 (*Cont'd* p. 430)

Reference	Outcome variables quantified	Animals/treatment (age, initial BW)	Dietary information; time on treatment	Daily prebiotic dose; source	Major findings
Tsukahara et al. (2003) (con't.)					↑ Crypt depth (22–43%), crypt density (8–26%), epithelial cell count (24–45%), mitotic cell count (113–275%), mitotic zone (48–98%), mitotic index (50–157%), mucin-containing cell count (39–53%) along large intestine*
Davis et al. (2004a)	Study 1	Study 1	Study 1	Study 1	Study 1
	Performance responses (ADG, ADFI, G:F)	54 Weanling Hampshire × Duroc × Yorkshire × Landrace barrows (19 d, 6.2 kg)	Corn-whey-SBM diets supplemented with 200 or 2,500 ppm ZnO	Control (0%)	No performance responses to MOS
				Control + 0.2% MOS	
			38 d study		
	Study 2	Study 2	Study 2	Study 2	Study 2
	Performance responses (ADG, ADFI, G:F)	36 Weanling Hampshire × Duroc × Yorkshire × Landrace barrows (19 d, 4.6 kg)	Corn-whey-SBM diets supplemented with 200 or 2,500 ppm ZnO	Control (0%)	0.2% MOS:
				Control + 0.2% MOS	↑ ADG during d 10–24 with high Zn (15%)**
					0.3% MOS:
				Control + 0.3% MOS	↑ ADG during d 10–24 with high Zn (13%)**
			38 d study		

Davis et al. (2004a) (con't.)	Study 3	Study 3	Study 3	Study 3	Study 3
	Performance responses (ADG, ADFI, G:F)	36 Weanling Hampshire × Duroc × Yorkshire × Landrace barrows (19 d, 5.6 kg)	Corn-whey-SBM diets supplemented with 200, 500, or 2,500 ppm ZnO	Control (0%)	0.3% MOS:
			35 d study	Control + 0.3% MOS	↑ G:F during d 7–21 (4%)**
					↑ Overall G:F (3%)**
					↓ Lymphocyte proliferation with 200 ppm Zn (33%)**
					↑ Lymphocyte proliferation with 500 ppm Zn (29%)**
					↓ Unstimulated lymphocyte proliferation (32%)**
					↓ Phytohemagglutinin-stimulated lymphocyte proliferation (19%)**
Davis et al. (2004b)	Performance responses (ADG, ADFI, G:F)	16 Yorkshire × Landrace × DeKalb EB barrows and gilts (19 d, 5.7 kg)	Corn-whey-SBM diet	Control (0%)	0.3% MOS:
					↑ ADG during d 0–14 (64%), 0-final (31%)**
	Hematologic responses (α1-acid glycoprotein, macrophages, monocytes)		Chemical composition 1.5% lys, 0.90% met + cys, 0.9% Ca, 0.8% P	Control + 0.3% MOS (Bio-MOS)	↑ G:F during d 0-final (20%)**
					↑ BW on d 14 (20%)**, 21 (17%)*
			26 d study		↓ % Neutrophils (14%)***
					↓ α1-acid glycoprotein on d 0 (18%)***
	Intestinal cell immunity				↑ % Lymphocytes (18%)**
					↑ CD14+ in lamina propria on d 19 (96%)***
					↓ CD14+ in blood on d 21 (25%)**
					↓ CD14+MCHII+ in lamina propria on d 21 (82%)***
					↓ CD3+CD4+:CD3+CD8+ in jejunal lamina propria on d 21 (76%)**

Table 12.4 (*Cont'd* p. 432)

Reference	Outcome variables quantified	Animals/treatment (age, initial BW)	Dietary information; time on treatment	Daily prebiotic dose; source	Major findings
Mikkelsen and Jensen (2004)	Intestinal metabolites	Ten piglets (4 wk)	Same as Mikkelsen et al. (2003)	Control (0%)	FOS:
				Control + 40 g/kg FOS (Raftiline ST)	↑ Cecal butyrate (35%)**
	Intestinal microbiota		28 d study	Control + 40 g/kg TOS (Elix' or)	↑ Proximal colonic acetate (11%), butyrate (21%), valerate (136%), and capronic acid (80%)**
	Microbial activity				↑ Intestinal (17%), cecal (28%), and colonic yeast (30%)**
					TOS:
					↑ Distal colonic isobutyrate (29%)**
					↑ Stomach (31%), intestinal (31%), cecal (31%), and colonic yeast (38%)**
Rideout and Fan (2004)	Nutrient digestibility	Six Yorkshire barrows (30 kg)	Corn-SBM diet	Control (0%)	Inulin:
	Ca and P digestibility		Chemical composition 207.3 g/kg CP, 118.3 g/kg NDF, 41.5 g/kg ADF, 6.3 g/kg Ca, 7.6 g/kg P	Control + 50 g/kg inulin	↓ Apparent digestible CP intake (9%)**
			14 d periods		↓ Apparent CP retention (9%), digestibility (5%)***
					↑ Apparent fecal CP loss (29%)***
					↓ Total fecal (8%), overall Ca loss (8%)***
					↓ Total P loss (9%)***
					↓ Urine P loss (61%)*
					↑ TCA-insoluble CP output (9%)***
					↑ Water-soluble Ca fecal output (13%)**
					↓ Water-soluble P fecal output (18%)**

Rideout et al. (2004)	Performance responses (feed intake, water intake)	Six Yorkshire barrows (30 kg)	Corn-SBM diets	Control (0%)	Inulin:
	Fecal metabolites		Chemical composition 20.7% CP, 11.8% NDF, 4.15% ADF, 0.63% Ca, 0.76% P	Control + 5% chicory inulin	↓ Fecal pH (3%)**
			14 d periods		↑ Total fecal N (9%)**
					↓ Fecal skatole (57%)**
Bohmer et al. (2005)	Prececal and total tract nutrient digestibilities	Eight male German Landrace × Pietrain pigs (36 kg)	Corn-wheat-barley-SBM diets	Control (0% inulin, 0 CFU *Enterococcus faecium*)	Inulin:
					↑ Crude ash (19%), CF digestibility (6%) in IRA pigs**
	Intestinal and fecal microbiota	Four normal, four surgically modified with an ileo-rectal-anastomosis (IRA)	Chemical composition 16.6% CP, 5.6% crude fat, 3.6% CF	Control + 8×10^9 CFU *E. faecium*	↑ Crude ash digestibility (22%)**
			12 d periods	Control + 2% inulin (Raftifeed IPS)	*E. faecium* + inulin:
	Intestinal and fecal metabolites			Control + 2% inulin + 8×10^9 CFU *E. faecium*	↑ Crude ash (19%), CF digestibility (12%) in IRA pigs**
					↑ Crude ash (19%),CF digestibility (8%)**
Rozeboom et al. (2005)	Performance responses (ADG, ADFI, G:F)	481 Mixed-sex crossbred piglets (19 d, 6.15 kg)	Corn-whey-SBM diets	Control (0%)	MOS:
				Control + 0.3% MOS (Bio-Mos; reduced to 0.2% in phase 2)	
			Chemical composition (phase 1–4) 1.7–1.15% lys, 0.9–0.8% Ca, 0.8–0.7% P	Control + 110 mg each of tylosin and sulfamethazine	↑ ADG on d 11–42 (8%), overall (7%)**
					↑ G:F on d 0–11 (2%)**
					MOS + antimicrobial:
					↑ ADG on d 0–11 (20%), 11–42 (10%), overall (11%)**
			42 d study	Control + 0.3% MOS (Bio-Mos; reduced to 0.2% in phase 2) + 110 mg each of tylosin and sulfamethazine	↑ ADFI overall (7%)**
					↑ G:F on d 0–11 (18%)**

Table 12.4 (*Cont'd* p. 434)

Reference	Outcome variables quantified	Animals/treatment (age, initial BW)	Dietary information; time on treatment	Daily prebiotic dose; source	Major findings
Shim et al. (2005)	Performance responses (BW, feed intake, feed conversion)	Four male Large white × Landrace pigs (24 d, 8.2 kg)	Wheat-SBM diets	Control (0%)	0.25% FOS:
				Control + 0.25% FOS (Neosugar)	↓ Large intestinal acetate (16%)*
	Intestinal metabolites		Chemical composition 21.9% CP, 1.3% lys, 0.8% Ca, 0.68% P	Control + 3.0% FOS	↑ Large intestinal isobutyrate (140%) and isovalerate (220%)*
	Intestinal morphology		21 d study		↑ Large intestinal butyrate (46%)**
					3.0% FOS:
					↓ pH in cecum (7%)*
	Disaccharidase activity				↓ pH in proximal colon (7%)*
					↓ Large intestinal acetate (9%)*
					↑ Large intestinal valerate (75%)*
					↑ Large intestinal isovalerate (98%)*
Tzortis et al. (2005)	Performance responses (BW, feed intake)	Ten weaned male pigs (35 d, 14.7 kg)	Commercial pelleted diet (Deltawean 15 NGP)	Control (0%)	1.6% GOS:
				Control + 1.6% GOS	↑ Lactobacilli in feces (9%)**
				Control + 4.0% GOS	↑ Lactate in proximal colon (153%)**
				Control + 1.6% inulin	
	Colonic microbiota		Chemical composition 192 g/kg CP, 33 g/kg oil, 13.2 g/kg lys		↓ Propionate in proximal colon (23%)**
	Colonic metabolites		34 d study		4% GOS:
	Fecal microbiota				
					↑ Bifidobacteria in proximal (10%), distal colon (5%) and feces (13%)**
					↑ Lactobacilli in proximal (6%), distal colon (8%) and feces (13%)**

Tzortis et al. (2005) (con't.)					↑ Lactate (68%), acetate (25%) in proximal colon**
					↓ pH in proximal colon (4%)**
					Inulin:
					↑ Bacteroides in proximal colon (5%) and feces (11%)**
					↑ Bifidobacteria in distal colon (8%) and feces (15%)**
					↑ Lactobacilli in distal colon (7%) and feces (10%)**
					↑ Acetate in distal colon (22%)**
Xu et al. (2005)	Performance responses (BW, ADFI, ADG, F:G)	30 Mixed-sex Duroc × Landrace × Large white pigs (33 d, 7.9 kg)	Corn-SBM diet	Control (0%)	FOS:
			Chemical composition 18% CP, 1.24% lys, 0.32% met, 0.68% met + cys, 0.81% Ca, 0.70% P	Control + 75 mg/kg antibiotic	↑ ADG (32%)*
				Control + 0.4% FOS	↑ F:G (24%)*
					↓ Diarrhea rate (71%)**
	Cecal and fecal metabolites				↑ Serum glucose (143%)*
	Intestinal morphology		21 d study		↑ Cecal propionate (56%), butyrate (44%), and total SCFA (31%)*
	Serum chemistry				↑ Fecal acetate (43%), isovalerate (35%), and total SFCA (38%)**
					↑ Jejunal (45%) and cecum villus height (100%)*
					↑ Jejunal (4%)**, ileal (7%)*, and cecal (11%)* goblet cell percentages

Table 12.4 (*Cont'd* p. 436)

Reference	Outcome variables quantified	Animals/treatment (age, initial BW)	Dietary information; time on treatment	Daily prebiotic dose; source	Major findings
Awati et al. (2006)	Performance responses (ADFI)	16 Mixed-sex Hypor × Pietrain pigs (4 wk)	Cornstarch-fishmeal diet	Control (0 g/g)	Inulin + lactulose:
					↓ Fecal DM (9%)**
					↓ Cecal (60%), colonic (43%), Fecal (18%) ammonia**
					↓ Proximal small intestinal (117%), fecal (15%) BCFA**
	Intestinal and fecal metabolites		Chemical composition 179 g/kg CP, 11.5 g/kg lys, 8.3 g/kg Ca, 5.7 g/kg P 10 d periods	Control + 7.5 g/kg inulin + 20 g/kg lactulose	
Biagi et al. (2006)	Performance responses (BW, feed intake, ADG)	12 German Landrace × Pietrain piglets (28 d, 7.4 kg)	Chemical composition (weeks 1–3): 20.7% CP, 3.1% fat, 4.2% CF; (weeks 4–6): 18.5% CP, 3.9% fat, 4.9% CF	Control (0 ppm)	All concentrations Gluconic acid:
				Control + 3,000 ppm 50% Gluconic acid	Quadratic ↑ final BW (0 ppm: 24.5 kg; 3,000 ppm: 26.7 kg; 6,000 ppm: 26.9 kg; 12,000 ppm: 25.3 kg)**
				Control + 6,000 ppm 50% Gluconic acid	
	Intestinal and fecal microbiota			Control + 12,000 ppm 50% Gluconic acid	Quadratic ↑ ADG (0 ppm: 409 g; 3,000 ppm: 464 g; 6,000 ppm: 466 g; 12,000 ppm: 428 g)**
	Intestinal morphology and metabolites		6 wk study		Quadratic ↑ jejunal acetate (0 ppm: 14.6 ppm; 3,000 ppm: 83.2 ppm; 6,000 ppm: 65.4 ppm; 12,000 ppm: 42.7 ppm)**
					Quadratic ↑ total SCFA (0 ppm: 64.5 ppm; 3,000 ppm: 177.1 ppm; 6,000 ppm: 120.7 ppm; 12,000 ppm: 112.1 ppm)***

Krag et al. (2006)	*Trichuris suis* challenge	Seven mixed-sex Landrace × Yorkshire × Duroc pigs	Barley flour-SBM diets	Basal + 300 g/kg oat husk (control)	Inulin groups:
					↓ Number of recovered worms on weeks 7 (69%)* and 9 (52%)**
	Performance response (BW)		Nine wk study: all pigs infected with 2,000 infective *T. suis* eggs at 3 wk; groups N-7, N-9 and N/I-9 fed oat hull meal, group I-7 fed inulin until wk 7; N-7 and I-7 Slaughtered at wk 7, N/I-9 fed inulin for 2 wk	Basal + 160 g/kg inulin (Raftiline)	↓ IgA+ cells in lamina propria on week 7 (25%)*
	Intestinal parasitology				
	Intestinal histology				
					↓ IgG+cells in lamina propria on week 7 (64%)*
					↓ CD3+ cells in lamina propria on week 7 (45%)*
					↓ Mast cells in lamina propria on wk 9 (37%)
					↓ Eosinophils in tela submucosa on wk 9 (55%)**
Loh et al. (2006)	Performance responses (BW, feed intake)	16 German Landrace pigs (42 d)	Wheat-barley-SBM or Corn-wheat gluten meal diets	Control (0%)	Wheat + Inulin:
				Control + 3% inulin (Raftilene ST)	↑ Colonic bifidobacteria (192%)**
					↓ Colonic acetate as molar % (7%)*
	Intestinal microbiota Intestinal meta-bolites		Chemical composition		↑ Colonic butyrate as molar % (26%)*, propionate as molar % (8%)***
			Wheat: 20.3% CP, 4.0% crude fat, 48.0% starch, 0.8% inulin		↑ Colonic pH (5%)*
			Corn: 20.9% CP, 4.6% crude fat, 49.9% starch, 0.9% inulin		Corn + Inulin:
					↑ Colonic bifidobacteria (238%)**
					↓ Colonic acetate as molar % (5%)*
					↑ Colonic butyrate as molar % (28%)*, propionate as molar % (6%)***
					↑ Colonic pH (4%)*

Table 12.4 (*Cont'd* p. 438)

Reference	Outcome variables quantified	Animals/treatment (age, initial BW)	Dietary information; time on treatment	Daily prebiotic dose; source	Major findings
Mountzouris et al. (2006)	Intestinal microbiota	Four castrated growing Duroc × (Large white × Landrace) pigs (12 kg)	Corn-SBM diet	Control (0 g/kg)	FOS:
					↑ Cecal β-galacturonidase activity (161%)**
	Intestinal metabolites		Chemical composition not provided	10 g/kg FOS (Raftifeed OPS)	TOS:
					↑ Cecal (199%), colonic (62%), fecal (119%) β-galacturonidase activity**
	Microbial enzyme activities		30 d periods	10 g/kg TOS (Vivinal GOS 10)	
Pierce et al. (2006)	Intestinal morphology	Five large white × Large white × Landrace piglets (24 d, 7.8 kg)	Wheat-SBM-whey protein isolate diet	Control (0 g/kg)	Low lactose + Inulin:
	Intestinal microbiota		Chemical composition	Control + 15 g/kg inulin	↑ Food intake (24%)**
	Intestinal metabolies		Low lactose: 191.4 g/kg CP, 91.6 g/kg NDF, 14.8 g/kg lys, 5.3 g/kg met		↑ Duodenal (29%), jejunal villus:crypt ratio (53%)**
			High lactose: 191.7 g/kg CP, 63.7 g/kg NDF, 15.2 g/kg lys, 5.2 g/kg met		↓ Ileal pH (1%)**
					↑ Jejunal villus height (50%)**
					↑ Colonic bifidobacteria (8%)**
					↑ Cecal propionate (4%)***, total SCFA (23%)**
					↓ Cecal isobutyrate (50%), isovalerate (53%)**
					↑ Colonic total SCFA (42%)**
					High Lactose + Inulin:
					↓ Ileal pH (3%)**

Pierce et al. (2006) (con't.)					↑ Jejunal villus height (4%)**
					↓ Ileal lactobacilli (50%)**
					↓ Colonic bifidobacteria (9%)**
					↑ Cecal propionate (27%)***
					↓ Cecal isobutyrate (33%), isovalerate (11%), total SCFA (25%)**
					↑ Colonic propionate (27%)**
					↓ Colonic total SCFA (27%)**
Lynch et al. (2007)	Study 1	Study 1	Study 1	Study 1	Study 1
	Nutrient digestibility	Four meat-line boars × large white × Landrace finishing boars (74 kg)	Wheat-SBM diets with high (200 g/kg) CP or low (140 g/kg) CP	Control (0%)	High CP + Inulin:
					↓ Hemicellulose (2%)**, N (3%)* digestibility
	N balance		Chemical composition	Control + 12.5% inulin (Raftiline ST)	↑ ADF digestibility (3%)**
	Ammonia emissions		High CP: 202.4 g/kg CP, 127.8 g/kg NDF, 47.4 g/kg ADF, 20.0 g/kg crude fat		↑ Fecal N excretion (33%)**
					↓ Urinary N:fecal N (25%)**
					Low CP + Inulin:
					↓ NDF (16%), ADF (13%), hemicellulose (18%) digestibility**
			Low CP: 148.2 g/kg CP, 103.1 g/kg NDF, 53.7 g/kg ADF, 14.4 g/kg crude fat		↓ N digestibility (3%)*
					↑ Fecal N excretion (22%)**
			6 d study		↓ Urinary N:fecal N (26%)**
	Study 2	Study 2	Study 2	Study 2	Study 2
	Cecal and colonic microbiota	Six Meat-line boars × Large white × Landrace finishing boars (74 kg)	Same as study 1	Same as study 1	High CP + Inulin:
					↑ Cecal bifidobacteria (6%)*
	Cecal and fecal metabolites				↑ Cecal lactobacilli (9%)***
					↓ Cecal enterobacteria (8%)**
					↓ Colonic propionate (3%)**
					↑ Fresh feces:urine ratio (66%)**

Table 12.4

Reference	Outcome variables quantified	Animals/treatment (age, initial BW)	Dietary information; time on treatment	Daily prebiotic dose; source	Major findings
Lynch et al. (2007) (con't.)					Low CP + Inulin:
					↑ Cecal bifidobacteria (3%)*
					↑ Cecal enterobacteria (10%)**
					↓ Colonic propionate (6%)**
					↓ Fresh feces:urine ratio (22%)**
Yasuda et al. (2007)	Study 1	Study 1	Study 1	Study 1	Study 1
	Digesta inulin content	Six weanling Yorkshire × Hampshire × Landrace pigs (7.7 kg)	Corn-SBM diet	Control (0%)	Inulin:
	Intestinal saccharide concentrations		Chemical composition 18.5% CP, 4.3% CF	Control + 4% inulin (Synergy 1)	↑ Inulin in stomach (0.5%), upper jejunum (3%), and lower jejunum (5%)**
			6 wk study		↑ Fructose in stomach (151%), lower jejunum (156%), and ileum (3,175%)**
					↑ Raffinose in lower jejunum (300%)**
					↑ Stachyose in lower jejunum (6,100%)**
	Study 2	Study 2	Study 2	Study 2	Study 2
	Digesta inulin content	Six weanling Yorkshire × Hampshire × Landrace pigs (11.2 kg)	Same as study 1 except 8 wk study	Same as study 1	Inulin:
					↑ Inulin in stomach (1%), lower jejunum (1%), and ileum (2%)**
					↓ Glucose in stomach (150%)**
					↑ Fructose in stomach (400%) and lower jejunum (600%)**
					↑ Sucrose in ileum (700%)**

Tako et al. (2008)	Blood hemoglobin	Six weanling Yorkshire × Hampshire × Landrace pigs	Corn-SBM diets	Control (0 g/kg)	Inulin:
	Cecal microbiota		Chemical composition 18.1% CP, 4.5% CF, 54.4 mg/kg Fe	Control + 32 g/kg inulin (Synergy 1)	↑ Duodenal expression of ferritin (63%), divalent metal transporter 1 (300%), transferring receptor (67%), cytochrome b reductase (75%), mucin (122%), ferroportin (150%)**
	Intestinal transporter gene expression		6 wk study		↑ Colonic expression of ferritin (50%), divalent metal transporter 1 (133%), and transferring receptor (83%)**
					↑ Cecal lactobacilli (171%) and bifidobacteria (100%)**

[a]*ADF* acid detergent fiber, *ADG* average daily gain, *ADFI* average daily feed intake, *BW* body weight, *Ca* calcium, *CHO* carbohydrate, *CF* crude fiber, *CFU* colony forming unit, *CP* crude protein, *Cu* copper, cys cysteine, *d* day, *DM* dry matter, *DMI* dry matter intake, *Fe* iron, *F:G* feed:gain ratio, *FOS* fructooligosaccharide, *g* gram, *G:F* gain:feed ratio, *GOS* galactooligosaccharide, *h* hour, *IgA* immunoglobulin A, *IgG* mmunoglobulin G, *IGF-I* insulin-like growth factor-I, *IRA* ileo-rectal anastomosis, *kg* kilogram, *L* liter, *lys* lysine, *met* methionine, *MOS* mannanoligosaccharide, *N* nitrogen, *NDF* neutral detergent fiber, *NSP* nonstarch polysaccharide, *OF* oligofructose, *OM* organic matter, *P* phosphorus, *ppm* parts per million, *SBM* soybean meal, *SCFA* short-chain fatty acid, *thr* threonine, *trp* tryptophan, *TOS* trans-galactooligosaccharide, *μg* microgram, *wk* week, *Zn(O)* zinc (oxide)

[b]*$P < 0.01$, **$P < 0.05$, ***$P < 0.10$

prebiotic supplementation (Estrada et al., 2001; Lynch et al., 2007; Oli et al., 1998; White et al., 2002; Xu et al., 2002). Concentrations of intestinal and fecal yeast increased in response to FOS and TOS supplementation (Mikkelsen et al., 2003; Mikkelsen and Jensen, 2004). When challenged with *Oesophagostomum dentatum*, pigs supplemented with inulin experienced decreased intestinal *Oesophagostomum dentatum* eggs and worms (Petkevicius et al., 2003).

Changes in fermentative end-products as a result of prebiotic supplementation of swine follow patterns similar to that of other non-ruminant species. While some inconsistencies occur within the literature, acetate, butyrate, and total SCFA increase with prebiotic supplementation (Biagi et al., 2006; Correa-Matos et al., 2003; Loh et al., 2006; Mikkelsen and Jensen, 2004; Petkevicius et al., 2003; Pierce et al., 2006; Shim et al., 2005; Tsukahara et al., 2003; Tzortis et al., 2005; Xu et al., 2005). Fecal propionate concentrations increased in 28% of studies (Loh et al., 2006; Petkevicius et al., 2003; Pierce et al., 2006; Smiricky-Tjardes et al., 2003; Xu et al., 2005), whereas decreased propionate was observed in 22% of studies (Lynch et al., 2007; Petkevicius et al., 2003; Tzortis et al., 2005). Isobutyrate and valerate concentrations also increased with prebiotic supplementation (Houdijk et al., 2002; Mikkelsen and Jensen, 2004; Shim et al., 2005; Tsukahara et al., 2003), whereas isovalerate concentrations were variable (Houdijk et al., 1998; Mikkelsen et al., 2003; Pierce et al., 2006; Shim et al., 2005; White et al., 2002; Xu et al., 2005). Lactate and cupronic acid increased with GOS and FOS supplementation, respectively (Mikkelsen and Jensen, 2004; Tzortis et al., 2005).

Changes in intestinal morphology with respect to prebiotic supplementation were observed by ten authors. Villus height and villus height:crypt depth increased in response to fructan supplementation (Pierce et al., 2006; Xu et al., 2002; Xu et al., 2005). Fructan supplementation increased cell density, crypt height, crypt depth, crypt density, epithelial cell count, mitotic cell count, mucin-containing cell count, and number of goblet cells (Howard et al., 1995; Tsukahara et al., 2003; Xu et al., 2005). Cell death (CD) markers, mast cells, eosinophils, IgA, and IgG decreased in the lamina propria when pigs were supplemented with inulin (Krag et al., 2006). While observed in only one study, inulin increased intestinal expression of ferritin, divalent metal transporter 1, transferring receptor, cytochrome b reductase, mucin, and ferroportin (Tako et al., 2008).

Changes in nutrient digestibility occurred in response to prebiotic-supplemented diets. Dry matter digestibility, organic matter digestibility, nitrogen digestibility, and nitrogen retention decreased in response to prebiotic supplementation (Lynch et al., 2007; Rideout and Fan, 2004; Smiricky-Tjardes et al., 2003; Zhang et al., 2001). Fiber digestibility also decreased in response to

prebiotic supplementation (Lynch et al., 2007). In contrast, GOS digestibility increased with prebiotic supplementation (Smiricky-Tjardes et al., 2003). Several enzyme activities increased in response to prebiotic supplementation, including protease (Xu et al., 2002), trypsin (Xu et al., 2002), amylase (Xu et al., 2002), sucrase (Correa-Matos et al., 2003), and β-galacturonidase (Mountzouris et al., 2006).

Growth performance response to prebiotics was the most commonly reported outcome variable in the swine. Excluding MOS (discussed below), ADG increased in 21% of studies (Biagi et al., 2006; Estrada et al., 2001; Xu et al., 2002; Xu et al., 2005), while it decreased in 16% of studies (Estrada et al., 2001; Houdijk et al., 1998). Dry matter intake and body weight were variable (Estrada et al., 2001; Houdijk et al., 1998; Mikkelsen et al., 2003; Pierce et al., 2006). Feed:gain ratios generally increased in response to FOS supplementation (Estrada et al., 2001; Xu et al., 2005).

Mannanoligosaccharides increased ADG, body weight, and G:F of swine (Davis et al., 2004b). When measured in response to MOS plus supplemental copper, ADG increased without supplemental copper but the response was variable with supplemental copper (Davis et al., 2002). These responses appear to depend on the age and production phase of the pig, as a greater increase in ADG occurred in younger pigs with copper while a greater increase in ADG occurred in finisher pigs without copper. Gain:feed increased with MOS with and without supplemental copper (Davis et al., 2002). Average daily gain increased as often with MOS and supplemental zinc (Davis et al., 2004a) as without (LeMieux et al., 2003), although supplemental zinc appeared to be less beneficial to older pigs. Average daily feed intake was variable with MOS but without supplemental zinc (Davis et al., 2004a; LeMieux et al., 2003). Mixed results appear in the literature for G:F; however, diets supplemented with MOS and zinc appeared to increase G:F in older pigs (Davis et al., 2004a; LeMieux et al., 2003). Average daily gain and G:F increased in response to dietary supplementation of MOS and antibiotics while decreasing in response to supplementation with MOS without antibiotics (LeMieux et al., 2003). However, ADFI decreased with MOS consumption regardless of antibiotic supplementation (LeMieux et al., 2003).

Horses. Prebiotic supplements are not well researched in horses, as only five studies have been published in this area (› *Table 12.5*). Generally, studies involving prebiotic supplementation have been performed to simulate or provoke laminitis symptoms. Decreased lactobacilli, lactic acid-utilizing bacteria, *E. coli*, and streptococci were observed in two studies that measured changes in microbial populations in response to fructan supplementation (Berg et al., 2005; Respondek et al., 2008). Fermentative responses varied greatly among studies, as acetate and

lactate concentrations both increased and decreased in response to fructan supplementation (Berg et al., 2005; Respondek et al., 2007). However, butyrate and total SCFA concentrations increased consistently with fructan supplementation in these studies (Berg et al., 2005; Respondek et al., 2007). Fecal pH also decreased consistently with fructan supplementation (Berg et al., 2005; Crawford et al., 2007; van Eps and Pollet, 2006). Two studies observed increased insulin concentrations with increased fructan consumption (Bailey et al., 2007; van Eps and Pollet, 2006).

Pre-ruminants and adult ruminants. As the rumen of a pre-ruminant is sterile at birth and, thus, easily influenced by feed and environmental microbiota and because pre-ruminants are under continued stress from the standpoint of health maintenance, prebiotic supplementation may be an excellent nutritional intervention for them. Two studies have been conducted (› *Table 12.6*). Fructooligosaccharides increased ADG, BW, insulin concentrations, leukocytes, and eosinophils while decreasing lactate, NEFA, and post-prandial glucose concentrations (Kaufhold et al., 2000). Mannanoligosaccharides increased fecal scores and rate of recovery from diet-induced scours (Heinrichs et al., 2003). Clearly, more research is needed in this area.

Prebiotic supplementation of adult ruminants is not commonly performed as they have a relatively stable microbial population; however, several studies have been conducted (› *Table 12.7*). Mannanoligosaccharide supplementation of dry dairy cows increased serum rotavirus neutralization titers, while increasing serum protein concentrations and IgA concentrations, and rotavirus neutralization titers were observed in the calves of dams that had consumed MOS (Franklin et al., 2005). However, supplementation with GOS, a yeast culture, or a combination negatively impacted the microbial nitrogen concentration and nitrogen supply available to the host. These supplements also decreased ruminal propionate, which is used for gluconeogenesis, while increasing acetate, which is used for *de novo* fatty acid synthesis (Mwenya et al., 2005). Together, these results can negatively impact the health of the cow by decreasing the blood glucose concentration on which the cow relies for energy and creating fat mass.

12.3 Special Considerations for Prebiotic Use

Age. While the factor of age remains mainly undeveloped with respect to prebiotic supplementation, certain considerations given to dietary fibers also must be considered when adding prebiotics to animal feedstuffs. Concentrations of

Table 12.5

***In vivo* experiments, listed in chronological order, reporting effects of prebiotics in horses[a,b]** (*Cont'd* p. 444)

Reference	Outcome variables quantified	Animals/treatment (age, initial BW)	Dietary information; time on treatment	Daily prebiotic dose; source	Major findings
Berg et al. (2005)	Fecal metabolites	Nine quarter horses: six male, three female (489–539 d, 400.6 kg)	Pasture grass ad libitum with corn-oat concentrate supplement at 1% BW	Control (0 g/d)	All concentrations FOS:
	Fecal microbiota		Chemical composition (concentrate) 13.44% CP, 3.93% fat, 0.61%Ca, 0.56% P	8 g/d FOS	Linear ↓ pH (control: 6.48; 8 g/d: 6.44; 24 g/d: 6.38)*
			10 d blocks	24 g/d FOS	Quadratic ↓ fecal *E. coli* (control 4.90 $\log_{10}$ population; 8 g/d: 4.75 $\log_{10}$ population; 24 g/d: 4.93 $\log_{10}$ population)*
					Linear ↑ fecal lactate (control: 0.36 mg/g; 8 g/d: 0.41 mg/g; 24 g/d: 0.47 mg/g)**
					Linear ↑ fecal acetate (control: 2.13 mg/g; 8 g/d: 2.18 mg/g; 24 g/d: 2.52 mg/g)**
					Linear ↑ fecal propionate (control: 0.58 mg/g; 8 g/d: 0.64 mg/g; 24 g/d: 0.73 mg/g)*
					Linear ↑ fecal butyrate (control: 0.40 mg/g; 8 g/d: 0.46 mg/g; 24 g/d: 0.54 mg/g)**

Table 12.5 (*Cont'd* p. 446)

Reference	Outcome variables quantified	Animals/treatment (age, initial BW)	Dietary information; time on treatment	Daily prebiotic dose; source	Major findings
Berg et al. (2005) (con't.)					Linear ↑ fecal total VFA (control: 3.47 mg/g; 8 g/d: 3.69 mg/g; 24 g/d: 4.25 mg/g)*
van Eps and Pollet (2006)	Performance responses (temperature, heart rate, fecal pH)	18 Mature Standardbred horses (no age, BW given)	60% Lucerne chaff, 15% micronized barley, 25% commercial feed (Cool Command)	Control (0 g/kg)	All concentrations OF:
	Haematological and biochemical data	Control: 6 horses 7.5 g/kg: 2 horses 10.0 g/kg: 8 horses 12.5 g/kg: 2 horses	Chemical composition: not provided	7.5 g/kg BW OF (Raftilose P95)	Lameness induced
	Laminitis score		48 h study	10 g/kg BW OF	Distal limb edema induced
				12.5 g/kg BW OF	↓ Lymphocytes after 12 h (28%)**
					↑ Plasma L-lactate (188%), D-lactate (275%)**
					↓ Fecal pH (3–42%)**
					↓ Plasma bicarbonate (37%)**
					↑ Plasma glucose (26–80%), cortisol (100–950%)**

van Eps and Pollet (2006) (con't.)					10 g/kg BW OF:
					↑ Heart rate after 4 h (24–70%)**
					↑ Rectal temperature after 8 h (1–6%)**
					↓ Plasma sodium, chloride after 24 h**
					↑ Plasma potassium at 16–32 h (54%)**
					↑ Neutrophils after (56–100%)**
					↑ Plasma fibrinogen (63%)**
					↑ Plasma insulin at 12, 40, 44 h**
					12.5 g/kg BW OF:
					↑ Plasma sodium, chloride at 8 h**
Bailey et al. (2007)	Study 1	Study 1	Study 1	Study 1	Study 1
	Glucose tolerance	Ten native-breed horses (15.1 y)	Pasture (mixed sward with clover) for 2 mo, then mature Timothy hay for 7 d	Sward: 138 g/kg DM fructans (DP $\geq$ 3), Timothy hay: 34 g/kg DM	Grass: ↑ serum insulin concentration (107%)**
	Insulin sensitivity				Hay: ↓ serum insulin vs. grass serum insulin concentrations (34%; similar to hay-fed control horses)**
	Plasma TG concentrations				

Table 12.5

Reference	Outcome variables quantified	Animals/treatment (age, initial BW)	Dietary information; time on treatment	Daily prebiotic dose; source	Major findings
Bailey et al. (2007) (con't.)	Study 2	Study 2	Study 2	Study 2	Study 2
	Glucose tolerance	10 laminitis-prone, 11 control native-breed horses (15.1 y)	Hay for 2 wk, then high-protein grass (15% protein)	Hay: 4.1 g/kg/d; Grass: 6.1 g/kg/d (includes 3 g/kg inulin top-dressed on grass)	Laminitis-prone horses:
	Insulin sensitivity				↑ Serum insulin concentrations with fructan addition (206%)*
	Plasma TG concentrations				
Crawford et al. (2007)	Plasma amine concentrations Hoof wall and coronary band temperature	Six laminitis-prone, 6 control mixed, adult native-breed horses (13.2 y, 337 kg)	Hay for 2 wk, then high-CHO diet (2/3 hay plus 1/3 dried grass)	Control (0 g/kg)	Inulin: ↓ Fecal pH (10%)*
	Fecal metabolites		3 wk study	3 g/kg inulin	↑ fecal tyramine (2.5-fold), tryptamine (2-fold)**

Respondek et al. (2007)	Cecal and colonic metabolites	4 cross-bred, cecal cannulated geldings (7 y, 425 kg)	Commercial pelleted feed (HIPPO 122) and wheat straw; barley fed on d 21 in place of morning concentrate meal	Control (0 g/d)	scFOS:
	Cecal and colonic microbiota		Chemical composition: 11.16% CP, 1.91% fat, 17.24% CF, 40.4% NDF, 20.3% starch	30 g/d scFOS (Profeed P95)	↓ Streptococci 5 h post-barley consumption (6%)**
					↓ Lactobacilli (7%), lactate-utilizing microbiota (6%), streptococci (9%) 29 h post-barley consumption**
					↑ Cecal L-lactate concentrations 5 h post-barley consumption (111%)**
					↓ Cecal L-lactate concentrations 29 h post-barley consumption (61%)**
					↑ Cecal D-lactate concentrations 29 h post-barley consumption (121%)**
			24 d periods		↑ Cecal acetate (21–25%), butyrate (30–49%), total VFA (24–31%)***
					↓ Colonic acetate proportion post-barley consumption (7%)***

[a]*BW* body weight, *Ca* calcium, *CF* crude fiber, *CHO* carbohydrate, *CP* crude protein, *d* day, *DM* dry matter, *DP* degree of polymerization, *FOS* fructooligosaccharide, *g* gram, *h* hour, *kg* kilogram, *mg* milligram, *NDF* neutral detergent fiber, *OF* oligofructose, *P* phosphorus, *scFOS* short-chain fructooligosaccharide, *TG* triglyceride, *VFA* volatile fatty acid, *wk* week, *yr* year

[b]*P $<$ 0.01, **P $<$ 0.05, ***P $<$ 0.10

Table 12.6

***In vivo* experiments, listed in chronological order, reporting effects of prebiotics in pre-ruminants[a,b]**

Reference	Outcome variables quantified	Animals/ treatment (age, initial BW)	Dietary information; time on treatment	Daily prebiotic dose; source	Major findings
Kaufhold et al. (2000)	Performance responses (BW, feed intake, ADG)	Seven Simmental × Red Holstein veal calves (10 wk old)	11 L/d whole milk	Control (0 g/d)	FOS:
	Hematological profile		Chemical composition: 122 g DM/kg, 274 g CP/kg DM, 273 g Crude fat/kg DM, 379 g Lactose/kg DM with supplemental milk replace and vitamin premix to allow ADG of 1.4–1.5 kg	Control + 10 g/d FOS (Profeed P95) with morning meal	↑ ADG (10%)***
					↑ Leukocytes on d 14 (45%)**
					↑ Absolute BW gain (10%)***
					↑ Eosinophils on d 21 (560%)**
					↓ Post-prandial plasma glucose concentrations on d 21 (12–27%)**

					↓ Lactate (37%) and NEFA concentrations (46%) on d 14**
					↑ Insulin concentrations on d 14 (58–63%)**
Heinrichs et al. (2003)	Performance responses (BW, skeletal growth)	24 Holstein calves (2 d)	Milk replacer and calf starter feed	Control (0 g/d)	MOS:
	Blood metabolites		Chemical composition: Milk: 20% CP, 20% fat, 0.75% Ca, 0.70% P Starter: 20.9% CP, 3.03% fat, 1.65% Ca, 0.88% P	1.4 g/kg antibiotic	↑ Rate of recovery from diet-induced scours (41%)*
	Fecal scores		6 wk study	4 g/d MOS (Bio-MOS)	↓ Scour severity score in category 2 (38%), 3 (50%)*
					↑ probability of normal fecal score after wk 3 (13%), overall (86%)*

[a]*ADG* average daily gain, *BW* body weight, *CP* crude protein, *d* day, *DM* dry matter, *FOS* fructooligosaccharide, *g* gram, *kg* kilogram, *L* liter, *MOS* mannanoligosaccharide, *NEFA* non-esterified fatty acid, *wk* week

[b]*P < 0.01, **P < 0.05, ***P < 0.10

Table 12.7

***In vivo* experiments, listed in chronological order, reporting effects of prebiotics in adult ruminants[a,b]** (*Cont'd* p. 452)

Reference	Outcome variables quantified	Animals/treatment (age, initial BW)	Dietary information; time on treatment	Daily prebiotic dose; source	Major findings
Franklin et al. (2005)	Performance responses (BW-calves)	19 Multiparous dairy cows (14 Holstein, 5 Jersey)	Alfalfa silage-concentrate TMR	Control (0 g/d)	MOS:
	Blood metabolites (cows and calves)		Chemical composition 18% CP, 3.1% fat, 37% NDF, 24% ADF	Control + 10 g/d MOS	Dams:
	Colostrum analysis		4 wk study		↑ Serum rotavirus neutralization titer (2%)**
	Serum Ig analysis				Calves:
					↑ Serum protein concentration at 24 h (29%)***
					↓ Serum IgA (13%)**
					↑ Serum rotavirus neutralization titer (5%)***
Mwenya et al. (2005)	Total tract digestibility	Four nonlactating, ruminally cannulated Holstein cows (697 kg)	Alfalfa hay cube-oat straw TMR	Control (0%)	GOS:
	Microbial N supply		Chemical composition 13.9% CP, 3.1% crude fat, 38.6% NDF, 25.6% ADF, 4.8% ADL	Control + 10 g/d yeast culture	↓ Uric acid (33%), allantoin (28%), total urinary excretion (29%)*
	Ruminal metabolites	Rumen emptied and switched over at end of each period	27 d periods	Control + 2% GOS control + 10 g/d yeast culture + 2% GOS	↓ Absorption of purine derivatives (39%)*

Mwenya et al. (2005) (con't.)					↓ Microbial N (38%), N supply (39%)*
	Protozoa enumeration				↓ Ruminal pH (2%)*
					↑ Ruminal acetate (5%)*
					↓ Ruminal propionate (22%)*
					↑ Ruminal acetate:propionate (19%)*
					Yeast:
					↓ Uric acid (35%), allantoin (21%), total urinary excretion (23%)*
					↓ Absorption of purine derivatives (31%)*
					↓ Microbial N (31%), N supply (32%)*
					↑ Ruminal acetate (8%)*
					↓ Ruminal propionate (25%), total VFA (11%)*
					↑ Ruminal acetate:propionate (23%)*
					Yeast + GOS:
					↑ Urine N (18%)**
					↑ N retained (144%)**
					↓ Uric acid (23%), allantoin (19%), total urinary excretion (20%)*

Table 12.7

Reference	Outcome variables quantified	Animals/treatment (age, initial BW)	Dietary information; time on treatment	Daily prebiotic dose; source	Major findings
Mwenya et al. (2005) (con't.)					↓ Absorption of purine derivatives (26%)*
					↓ Microbial N (24%), N supply (27%)*
					↑ Ruminal acetate (9%)*
					↓ Ruminal propionate (29%), total VFA (9%)*
					↑ Ruminal acetate:propionate (34%)*
					↓ Ruminal isoacids (26%)*

[a]*ADF* acid detergent fiber, *ADL* acid detergent lignin, *BW* body weight, *CP* crude protein, *d* day, *GOS* galactooligosaccharide, *Ig* immunoglobulin, *IgA* immunoglobulin A, *kg* kilogram, *MOS* mannanoligosaccharide, *N* nitrogen, *NDF* neutral detergent fiber, *TMR* total mixed ration, *VFA* volatile fatty acids

[b]*$P < 0.01$, **$P < 0.05$, ***$P < 0.10$

prebiotics in the diet of very young and very old animals should remain low so as not to drastically change diet digestibility. Very young animals require highly digestible diets to maintain rapid growth rates. While this is important for all species, it is of particular importance for livestock species as profit is closely tied to optimal animal growth and health. While not as great a concern for production livestock species, some aging animals experience a decline in diet digestibility as they approach senior and geriatric status. A dilution of nutrients at this life stage can lead to loss of muscle mass and stimulate other health concerns.

Health status. As demonstrated by several studies, animals with challenged immunity respond better to prebiotic supplementation as compared to healthy animals. When exposed to pathogenic microorganisms and parasites, poultry and swine exhibited more consistent responses to treatment with prebiotic supplements than control groups. While still important in disease prevention, use of prebiotics may not appear as efficacious in healthy, non-challenged animals. Prophylactic use of prebiotic supplements is important, however, in non-challenged animals as the changes that they induce in intestinal microbial ecology lead to an overall healthier animal.

Species. While supplementation appears to have clear benefits to most animal species, certain species respond poorly to prebiotic compounds. For example, the majority of research published in horses links fructan consumption to laminitis. Indeed, fructans have been used to induce laminitis and rapid intake of carbohydrate has been shown to induce laminitis. In contrast, pre-ruminants appear to benefit from the addition of prebiotic compounds to their diets. These animals do not have a strong microbial population established in their rumen and are highly affected by microorganisms in their environment, particularly due to susceptibility to diet-induced shifts in microbiota. Prebiotic supplementation of these animals can influence the populations of microbiota that establish within the rumen and, potentially, can lead to an improved ruminal environment for the animal.

Animal housing. The way in which an animal is housed can greatly affect its response to prebiotic supplementation. Companion animals, generally accepted as indoor pets, are less likely to exhibit a dramatic response to a prebiotic compound unless ill when compared with some livestock species. Livestock species also respond very differently, which generally can be related to the type of living conditions in which they reside. One would expect a greater response to prebiotic supplementation of poultry housed in a floor pen where the birds have free access to their environment, including excreta of other birds, than would be observed in group-housed swine or possibly even caged poultry.

12.4 Conclusion

As evidenced by the body of literature covered in this essay, prebiotic supplementation of companion and livestock animals demonstrates many benefits. By increasing beneficial microbiota while decreasing populations of potentially pathogenic microbiota, animal owners and producers can improve the health and well being of their animals. Prebiotic compounds also improve fermentation in the intestine of animals, as has been observed in all species to which prebiotics have been fed. Increasing SCFA concentrations provides energy to the colon, allows for proper cell maintenance and turnover, and can contribute to the beneficial microbial environment by decreasing intestinal pH. Fermentation associated with odor- and disease-producing compounds such as phenols and branched-chain fatty acids generally decreased with prebiotic supplementation, adding to the list of benefits of prebiotic supplementation. While some conflicting information exists, benefits generally outweigh costs of supplementation. With proper consideration of health status, living conditions, species to be supplemented, and age, prebiotic supplementation as a nutritional intervention strategy has the potential to improve overall health status of many species.

12.4.1 Future Research

Several areas remain open for further discovery in the area of prebiotics as regards animal nutrition. Prebiotic compounds are used in some companion animal foods currently, but further use might be warranted for young, old, and health-compromised pets. Poultry and swine prebiotic research demonstrates clear benefits to these species in the area of intestinal health. While production and performance responses are inconsistent, the animal industry, particularly in the European Union, has shifted or will shift away from antibiotic supplementation. This opens an avenue for further research into the use of prebiotics as antibiotic proxies. Use of prebiotics by horses, pre-ruminants, and ruminant animals can be expanded. New potential prebiotic compounds are created as new production technologies become more advanced, leading to new and potentially more active compounds that stimulate beneficial microbial population growth and fermentative profiles in the intestinal tracts of companion and livestock animal species.

12.5 Summary

- Prebiotics have been investigated in companion animals and many livestock species with varied, but generally positive, results.
- When prebiotics are fed to non-ruminant companion and livestock animals, similar responses are observed across species. Prebiotics can aid the host animal in fighting microbial infections, improving growth performance responses, improving microbial ecology, and optimizing animal health.
- Prebiotic studies in the equine generally have dealt with induction of laminitis with fructans, implying that horses do not tolerate large concentrations of fructans. However, there are no published studies investigating the effects of other prebiotics or low concentration supplementation strategies.
- Ruminant and pre-ruminant studies with prebiotics are sparse, but measurable changes in fermentative end-products, nutrient digestibilities, and immunological indices have been noted.
- Several considerations must be taken into account prior to prebiotic supplementation with regard to age, species, housing conditions, and health status to obtain optimal results.
- While prebiotics have been widely researched, much research remains to be performed as results with some species are sorely lacking. Improved technologies allow for the creation of novel prebiotics that should be investigated in select animal species.
- Overall, prebiotics have the potential to improve animal health by altering gastrointestinal events that impact host animal metabolism.

List of Abbreviations

AA	amino acid
ADF	acid detergent fiber
ADFI	average daily feed intake
ADG	average daily gain
ADL	acid detergent lignin
AME_n	metabolizable energy corrected for nitrogen
BAC	bacitracin
BW	body weight
Ca	calcium

CF	crude fiber
CFU	colony forming units
CHO	carbohydrate
CP	crude protein
Cu	copper
cys	cysteine
d	day
DGGE	denaturing gradient gel electrophoresis
DM	dry matter
DMB	dry matter basis
DMI	dry matter intake
DP	degree of polymerization
ESBM	ethanol-extracted soybean meal
Fe	iron
F:G	feed:gain ratio
FISH	fluorescence *in situ* hybridization
FOS	fructooligosaccharide
g	gram
G:F	gain:feed ratio
GOS	galactooligosaccharide
h	hour
HCC	hen cecal contents
IBDV	infectious bursal disease virus
IgA	immunoglobulin A
IgE	immunoglobulin E
IgG	immunoglobulin G
IGF-I	insulin-like growth factor-I
IgM	immunoglobulin M
IMO	isomaltooligosaccharide
IRA	ileo-rectal anastomosis
kg	kilogram
L	liter
lys	lysine
ME	metabolic energy
met	methionine
μg	microgram
mmol	millimole
mo	month

MOS	mannanoligosaccharide
N	nitrogen
NDF	neutral detergent fiber
NEFA	non-esterified fatty acid
NSP	non-starch polysaccharide
OF	oligofructose
OM	organic matter
P	phosphorus
ppm	parts per million
qPCR	quantitative polymerase chain reaction
SBM	soybean meal
SCFA	short-chain fatty acid
scFOS	short-chain fructooligosaccharide
TDF	total dietary fiber
TG	triglyceride
thr	threonine
TME_n	true metabolizable energy corrected for nitrogen
TMR	total mixed ration
TOS	trans-galactooligosaccharide
trp	tryptophan
val	valine
VFA	volatile fatty acid
wk	week
YCW	yeast cell wall
yr	year
Zn(O)	zinc (oxide)

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13 Analysis of Prebiotic Oligosaccharides

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13.1 Introduction

Carbohydrates and more specifically prebiotics, are complex mixtures of isomers with different degrees of polymerization (DP), monosaccharide units and/or glycosidic linkages. Many efforts are focused on the search for new products and the determination of their biological activity. However, the study of their chemical structure is fundamental to both acquire a basic knowledge of the carbohydrate and to increase the understanding of the mechanisms for their metabolic effect.

Both the identification of their constituents (qualitative) and the determination of their concentrations (quantitative) are the aims of an analytical process. Selection of an appropriate analytical technique, sample preparation (purification, fractionation, etc.) and optimization of the methodology are necessary for determining prebiotic structure. These steps are clearly dependent on the analytes of interest and the type of sample.

There are two main groups of analytical techniques used for the analysis of prebiotics: separation and spectroscopic techniques. Separation techniques (chromatographic and electrophoretic) give rise to the resolution of the constituents of a sample allowing the obtainment of quantitative information; however, the structural knowledge afforded is usually limited. Spectroscopic techniques are frequently necessary to provide detailed structural data of an isolated compound or a simple mixture. Combination of several techniques is often necessary to achieve all the required information about composition of complex mixtures.

Detailed information about the different analytical techniques required for the characterization of prebiotics as well as the state of the art of their applications has been included in this chapter. Although colorimetric methods such as determination of total carbohydrate or reducing sugar contents are still in use for oligosaccharide characterization, the separation techniques such as planar

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chromatography, gas chromatography (GC), high performance liquid chromatography (HPLC) and capillary electrophoresis (CE), which provide qualitative and quantitative information of independent oligosaccharides, are the most widely used and therefore the main aim of this section. These techniques can be coupled to spectroscopic instruments in order to obtain structural information. Moreover, nuclear magnetic resonance (NMR) and mass spectrometry (MS) are directly used for prebiotic structural analysis. These techniques have experienced exceptional advances in recent years, although application to prebiotics is still in progress.

13.2 Analytical Techniques

13.2.1 Planar Chromatography

This term was proposed in 1983 by the Chromatographic Society. It covers both paper (PC) and thin-layer chromatography (TLC). PC was one of the earliest chromatographic techniques used for carbohydrate analysis, but at present it is scarcely utilised and mainly combined with other techniques to give supporting information.

A combination of PC, HPLC and high performance anion exchange chromatography (HPAEC) was used for the isolation of two octasaccharides, two dodecasaccharides and a tridecasaccharide from human milk (Haeuwfievre et al., 1993). Paper chromatography of milk oligosaccharides was used to purify some fractions eluted from an anion exchanger; migration time was 5 days (Guerardel et al., 1999). It has also been used to isolate the transglycosylation product of sucrose with beta-glycosidase from recombinant *Sulfolobus shibatae*, which was identified as α-D-gal*p*-(1→6)-α-D-gluc*p*-β-D-fructofuranoside (Park et al., 2005).

Planar chromatography also covers modern techniques derived from TLC such as HPTLC (High Performance TLC), OPTLC (Over Pressured TLC) and UTLC (Ultra TLC). These techniques are relatively low-cost, easy to perform and they display simultaneously in the chromatogram the overall components present in the sample.

Especially for saving time, thin-layer plates replaced paper, but the earliest methods have evolved and new modes have been introduced. At present, TLC is a well established technique which offers several additional advantages: it is relatively cheap, automated, allows satisfactory quantification and it can even be coupled to spectroscopic techniques such as MS.

13.2.1.1 Pretreatment of Samples

As TLC plates are not reused, the careful pretreatments necessary to keep the integrity of HPLC columns can be omitted. A carbohydrate solution not excessively turbid can be applied to plates, only avoiding the presence (in high amounts) of those compounds which can interfere the elution of analytes, such as proteins, lipids, certain salts, amines or acids.

13.2.1.2 Sorbents and Eluents

The preferred sorbents for carbohydrates are based on silica gel. This substance basically retains solutes by adsorption; separation thus occurs by solubility. In order to introduce new interaction mechanisms, different approaches have been proposed. Impregnation with inorganic salts allows modulation of the separation through complex formation: boric acid, sodium acetate, sodium bisulphite and phosphate buffers have been used for this purpose. Silica can also be functionalised with different organic groups in order to work in reverse-phase mode: amine and diol groups are preferred for carbohydrate analysis. Amino plates can be buffered with phosphates in order to avoid the reaction of the amino groups with the free carbonyls of the sugars.

Elution is carried out with aqueous mixtures of alcohols (methanol, ethanol, isopropanol, butanol); minor amounts of less-polar solvents (acetonitrile, ethyl acetate, acetone) are frequently added depending on the mixture to separate.

Although classic TLC is carried out in isocratic mode, at present it is feasible to use elution gradients by means of AMD (automatic mode development) which allows the formation of step-to-step gradients. AMD is performed by means of commercial equipment which allows a careful control of the process; in brief, the plate is eluted for a short time with the starting solvent mixture, then the solvent is removed and the layer is dried under vacuum; finally, another run starts in the same direction with another solvent of lower elution strength than that used before, and so on. In this way, a stepwise elution gradient is formed. Resolution is improved since spots are focused through the successive elution steps, which affords very narrow bands.

The introduction of HPTLC has improved both resolution and quantitative measurements. This technique is based on the use of special plates which have been prepared with very thin and uniform particles of silica (5–6 μm average) which allows shorter migration distances (3–6 cm) and reduced elution times

(3–20 min). Resolution can be improved by the use of plates with spherical particles such as LiChrospher™ (Merck).

OPTLC is based on a pressured chamber in which the vapor phase above the sorbent is almost eliminated. The eluent is pushed through the layer by a pump; continuous development can be performed.

UTLC was introduced in 2002 and it is based on monolithic structures created on the plate without particles; the sorbent layer is a continuous bed about 10 μm thickness containing macropores (1–2 μm) and mesopores (3–4 nm) (Hauck and Schulz, 2003). This geometry allows faster separations (1–6 min), lower limits of detection and reduced analyte and solvent volumes, but resolution has not yet been totally optimized. This technique seems to be promising for carbohydrate analysis, although applications need to be developed.

13.2.1.3 Visualization and Quantification

There is a broad range of choices for visualization of sugar spots. Both spraying and dipping modes have been used, although the latter is preferred at present, especially with high-performance methods. Chromogenic reagents based on amines and strong acids such as diphenylamine-aniline-phosphoric acid in acetone (Martinez-Castro and Olano, 1981; Reiffova and Nemcova, 2006), urea-phosphoric acid (Bonnett et al., 1997) and *N*-(1-naphthyl)ethylenediamine dihydrochloride dissolved in sulfuric acid-methanol (Bounias, 1980) have been sprayed on silica gel plates. This latter reagent has been shown to be very sensitive (50 ng). 4-Aminobenzoic acid has been used for visualization on diol plates, whereas α-naftol has been used for amino plates. *In situ* reaction of sugars with the amino groups of amino layers also produces visualization, by simply heating the plate at about 170°C; sugars appeared as white-blue fluorescent spots under UV light at 365 nm.

13.2.1.4 Applications

Table 13.1 summarizes some applications of TLC to the analysis of prebiotic carbohydrates.

Chromatographic analysis of lactulose was revised in 1987 (Martínez-Castro et al., 1987). A TLC method was devised for analysis of lactulose in milk using silica gel plates charged with borate to form complexes allowing separation of lactulose from lactose (Martinez-Castro and Olano, 1981).

Table 13.1

TLC applications for the analysis of prebiotics (*Cont'd* p. 470)

Analytes	Bed	Elution system	Visualization	Quantification	Reference
Lactulose in milk	Silica gel G + 0.03M H_3BO_3	ACN:water (5:2)	1 g diphenylamine + 1 mL aniline + 50 mL H_3PO_4 + 50 mL acetone	Semiquantitative detection limit: 0.02% lactulose	Martínez-Castro and Olano (1981)
14 Sugars in biol fluids; maltodextrines in infant formulas	Cellulose	Threefold development (a) butanol/EtOH/w (3: 2: 1) (b) pyridine/ethyl acetate/ AcH/w (5: 5: 3: 1)	3 g $AgNO_3$ + 12 mL+ (a) 500 mL acetone (b) 50 ml of 10N NaOH in 450 mL EtOH	None	Bosch-Reig et al. (1992)
Oligosaccharides in beer	Amino + 0.4M KH_2PO_4	AMD linear gradient ACN: acetone (1:1)/w 40–20% in 15 steps	Thermal "*in situ*" reaction	Scanner in fluorescence mode at 366 nm	Brandolini et al. (1995)
FOS in biological fluids	Silica gel F254 + 0.02M Na Ac	Butanol:ethanol:w (5:3:2)	Diphenylamine-aniline-H_3PO_4 in acetone	Reflectance densitometry at 370 nm	Reiffova et al. (2006)
Microbiologically-produced Dextrans	Silica gel F	Three ascents EtAc: ACN: w propanol (2:7:5.5:5)	0.2%N(1-naphtyl) ethylendiamine HCl in MeOH with 3% H_2SO_4	Scanner in reflectance mode	Coté and Leathers (2005)
Hydrolyzates of polysaccharides from Linghzi	HPTLC silica gel 60 and HPTLC silica gel 50,000	AMD with 2 solutions: propanol:w (6:4) and propanol:w (83:17) in 7 steps	0.5 g 4-aminobenzoic acid, 9 mL AcH, 10 mL w, 0.5 mL H_3PO_4	Scanned at 365 in absorption mode	Di et al. (2003)
FOS in plants	Silica gel	Two developments in butanol:2-propanol: w (3:12:4)	Urea + H_3PO_4	Qualitative purposes	Bonnett et al. (1997)

Table 13.1

Analytes	Bed	Elution system	Visualization	Quantification	Reference
Oligosaccharides in molasses and artichoke leaves	Diol HPTLC	AMD with 3 solutions: ACN: acetone (1.1)/w (85/15), (94/6) and (95/5) in 9 steps	2 g 4-aminobenzoic acid + 36 mL AcH, 40 mL w, 2 mL H_3PO_4 + 120 mL acetone	Scanning in both fluorescence and absorbance (nanomol)	Vaccari et al. (2001)
Xylooligosaccharides	Microcristalline cellulose	EtOAc:AcOH:w (3:2:1)	Aniline + H Phthalate	Qualitative purposes	Katapodis et al. (2003)
Arabinoxylo-oligosachharides	Silica gel 60	Butanol:EtOH:w (3.2.2)	2% orcinol in (EtOH:H_2SO_4: w 8:1:1)	Qualitative purposes	Rantanen et al. (2007)
Dietary fiber	Silica gel	n-propanol:EtOH:w (7:1:2)	5% H_2SO_4 in MeOH		Mc Cleary and Rossiter (2004)
Human milk oligosaccharides	HPTLC silica	Butanol:AcH:w (2.5:1:1) two developments	0.1% orcinol in 20% H_2SO_4	Qualitative purposes	Kunz et al. (1996)

FOS, fructooligosaccharides; *ACN*, acetonitrile; w, water; *EtOH*, ethanol; *MeOH*, methanol

Classic TLC has been used to analyze different mixtures of oligosaccharides. Fructans of different plants from the family *Poaceae* were separated using two successive developments, allowing the separation of fructans belonging to both 2→1 and 2→6 series from DP3 to DP10 (Bonnett et al., 1997). TLC has been successfully used to monitor the formation of dextrans and isomaltooligosaccharides by the action of glucansucrases from *Leuconostoc* (Côté and Leathers, 2005), as well as xylooligosaccharides (XOS) (Katapodis et al., 2003) and arabinooligosaccharides (AROS) (Rantanen et al., 2007) formed by the action of different xylanases.

Prebiotics have been monitored in different parts of the intestinal tract (jejunum, ileum and colon) of monogastric animals by a simple TLC method (Reiffova and Nemcova, 2006).

HPTLC with AMD achieved a good separation and quantification of several oligosaccharides in molasses (Vaccari et al., 2001). This technique was also shown to be useful for the study of fingerprint profiles of hydrolyzates of polysaccharides from some important and popular Chinese medicinal mushrooms commonly known as Lingzhi (Di et al., 2003).

Human milk oligosaccharides (HMOS) are excessively complex to be directly separated by HPTLC. Nevertheless, this technique is very useful for the analysis of fractions obtained by preparative techniques, as carried out by Kunz et al. (1996).

13.2.1.5 Coupling with MS

This field was revised by Wilson in 1999. TLC was firstly used as a preparative technique for MS, thus working off-line: spots can be easily cut, solvent-extracted and injected into the ion source of the mass spectrometer (St-Hilaire et al., 1998). Nevertheless, many efforts have been directed to achieve an effective coupling, analyzing spots on the plate (Wilson, 1999). First couplings were carried out using fast atom bombardment (FAB) and liquid secondary ion (LSI). The spot was cut from an aluminum plate and attached to the MS probe. Interfaces based on motorised probes have been designed, where a strip of the plate can be slowly moved through the ion source, enabling the analysis of all spots from a lane to give a true chromatogram.

At present the more common techniques are those based on laser desorption, as MALDI (Matrix-Assisted Laser Desorption/Ionization) and SALDI (Surface-Assisted Laser Desorption/Ionization); in both cases the time of flight (ToF) analyzer is a good option, as it will be further seen.

As an example of coupling with MS, native milk oligosaccharides were separated on 10 × 10 silica gel plates and developed in *n*-butanol/acetic/water (110/45/45). MALDI-ToF was selected as MS technique. Glycerol was used as matrix, with an infrared laser for MALDI and an orthogonal ToF (o-ToF) for achieving high mass accuracy, allowing a straightforward method with a detection limit of ~10 pmol of individual compounds (Dreisewerd et al., 2006).

13.2.2 Liquid Chromatography

Liquid Chromatography (LC) is a separation technique which uses a liquid as mobile phase. Although HPLC is at present generally utilized for the analysis of prebiotic carbohydrates, the use of traditional open columns packed with ion exchange resins, carbon-celite or size exclusion gels as stationary phases is still widely practiced. Although analysis of oligosaccharides (such as malto-, isomalto-, gentio- and levan oligosaccharide series; Kennedy et al., 1989) has been carried out by traditional LC open columns coupled to mainly refractive index (RI) detectors, this technique has been mainly focused on preparative purposes. The collection of fractions of homologous oligosaccharides (different molecular weights or monosaccharide units) is in many cases, a required step for their further characterization by other analytical techniques such as MS or NMR.

13.2.3 High Performance Liquid Chromatography

HPLC is one of the most widespread techniques for oligosaccharide analysis, both for analytical and preparative purposes. A high number of methodologies for qualitative and quantitative characterization of prebiotic carbohydrates have been developed using different operation modes and detectors.

13.2.3.1 Sample Preparation

Analysis by HPLC commonly requires sample preparation methods to remove interfering compounds or impurities; the analysis of prebiotic oligosaccharides is not an exception. These methodologies are mainly based on dilution, liquid-liquid or liquid-solid extraction and filtration steps (Sanz and Martinez-Castro, 2007). Nevertheless, derivatization is in some cases necessary, mainly to enhance sensitivity in the detection during analysis.

There is a wide variety of derivatization reagents for oligosaccharides; the state the art in the preparation of derivatives being included in different reviews dedicated to sample preparation or chromatographic analysis of carbohydrates (Lamari et al., 2003; Sanz and Martinez-Castro, 2007), therefore, only a summary is mentioned here.

Most methods are based on the condensation of a carbonyl group in carbohydrates with primary amines to give a Schiff base which is then reduced to a N-substituted glycosil amine. The primary amine has to posses the desired chromophore or fluorophore substituent, usually an aromatic ring. Reductive amination has been carried out with 2-aminopyridine, different trisulphonates, esters of p-aminobenzoic acid, 2-aminoacridone, etc. (Sanz and Martinez-Castro, 2007).

Acetylation reactions of oligosaccharides overcome problems of solubility in organic solvents, whereas perbenzoylated derivatives improve the chromatographic properties on reverse phase columns (Kennedy and Pagliuca, 1994).

13.2.3.2 Chromatographic Columns

Chromatographic columns used for HPLC carbohydrate analysis can be divided according to the composition of their stationary phases and their dimensions and design.

13.2.3.2.1 Stationary Phase Composition

Both reverse phase and cation exchange chromatography have been the most common HPLC modes utilized for carbohydrate analysis till Rocklin and Pohl (1983) suggested the use of HPAEC for this aim. Most of the stationary phases used in these modes are available for both analytical and preparative purposes; this section being focused on the analysis of prebiotic oligosaccharides.

Alkyl-Bonded Silica Phases Among the alkylated silica-based stationary phases, those of octadecyl-coated (C_{18}) sorbents are the most commonly utilized, this columns being useful for the separation of oligosaccharides with different DP. Moreover, columns can present different percentages of bonded alkyl chains which could show a wide effect on carbohydrate resolution. The operation mode used for these columns is the reverse phase (RP)-HPLC where the non-polar ligands are covalently bound to a solid support and the mobile phase is mainly composed by aqueous solutions moderately polar. The retention mechanism is based on the interaction of the packing with polar materials; the

most polar compounds elute first whereas those with lower polarity are more retained. The use of low temperatures for the elution improves the resolution (Kennedy and Pagliuca, 1994). General aspects of underivatised and derivatised carbohydrate analysis by RP-HPLC have been reviewed by El-Rassi (2002).

Water is the most common mobile phase chosen for underivatised carbohydrates since these compounds require high surface tension to achieve an appropriate resolution. Nevertheless, gradients with organic solvents are used, although problems of solubility of oligosaccharides can arise.

Aminoalkyl-bonded Silica Gel Phases Aminoalkyl-modified silica gel columns provide good resolution; however, their stability is low and can be easily degraded. The most common ones for oligosaccharide analysis are aminopropyl-bonded columns, although primary and secondary diamines and secondary and tertiary amines can be also found. Non-polar organic solvents or aqueous organic mixtures are used as mobile phases.

Different mechanisms have been proposed for this chromatography such as partition or hydrogen bonding (Herbreteau, 1992). Oligosaccharides are eluted in order of increasing their molecular weight. Carbohydrates up to DP15 can be separated by this technique, although solubility problems can be found for oligosaccharides of high molecular weights. Nevertheless, if a diamine or a polyamine is added to the eluent a dynamic equilibrium is formed and oligosaccharides up to DP25 can be separated (Kennedy and Pagliuca, 1994). In these cases a presaturation column has to be placed before the injector to avoid dissolution of the analytical column packing.

Many researchers have used amino columns to analyze fructooligosaccharides (FOS) of different DP using acetonitrile:water (75:25) as mobile phase (Sangeetha et al., 2005), however, resolution is not as good as that obtained for mono- and disaccharides and solubility problems appear (Herbreteau, 1992). Moreover, the formation of Schiff bases between reducing sugars and amino groups can reduce the lifetime of these columns.

Cyclodextrin-bonded Phases The use of cyclodextrin-based columns for the separation of neutral prebiotic carbohydrates such as those derived from xylan, inulin or mannan, has been also proposed as a substitution of aminoalkyl modified silica gel columns. These columns are particularly useful for the analysis of oligosaccharides since the retention of these compounds is mainly based on the hydrogen bonding interactions of oligosaccharide hydroxyl groups

with the stationary phase which allows the separation of the different molecular weights. Mobile phases are normally constituted by different percentages of acetonitrile and water. Carbohydrates elutes in order of increasing DP (Herbreteau, 1992).

Other Polar Bonded Phases Several stationary phases with highly polar sorbents such as cyano, hydroxyl, diol, derivatives of poly(succinimide), sulfoalkylbetaine, etc. have been also used for carbohydrate analysis (Ikegami et al., 2008). Analysis on these columns has in common that retention increases with the hydrophilicity of the stationary phase and the analytes and with decreasing hydrophilicity of solvents from mobile phase. All of them are therefore grouped under the acronym HILIC (hydrophilic interaction chromatography). The first generations of HILIC were based on the amino-silica stationary phases and mixtures of acetonitrile:water mobile phases that has been previously described. HILIC belongs to normal phase liquid chromatographic (NPLC) modes with the hydrophilic stationary phase but with the mobile phase replaced by an aqueous/organic mixture (typically acetonitrile in water or a volatile buffer). It is very useful for the separation of polar compounds such as oligosaccharides. The hydrophilic groups of the stationary phase attract water molecules from the mobile phase to form water-enriched layers. The chromatographic mechanism is therefore mainly based on partition equilibrium between both mobile and stationary phases facilitated by the aqueous layers.

Graphitized Carbon Phases Graphitized carbon columns (GCC) were developed as an alternative to RP columns for the analysis of polar compounds (Koizumi, 2002). Their mechanism is based on the unspecific adsorption of polar compounds such as carbohydrates and interaction is enhanced with increasing molecular size. The effect of temperature is not drastic, although high temperatures can produce an increase in the retention due to the higher adsorptive activity of carbon. Eluents for mobile phases include high percentages of organic modifiers such as acetonitrile with no ion-pairing agents; these eluents being compatible to MS detectors.

Size Exclusion Phases As it has been indicated before, size exclusion chromatography (SEC) is widely utilized in its classical form with open columns. Nevertheless, the use of size exclusion for HPLC (HPSEC) is also commonly applied, although its inability to separate linkage isomers has limited its development.

Oligosaccharides are eluting in order of decreasing molecular size from a stationary phase constituted by cross-linked polysaccharide or polyacrylamide. These packing material are available with a range of pore volumes; separation depends on the ratio of their molecular dimensions and the average diameter of the pores (Churms, 1996). Mobile phases should be carefully chosen to avoid all types of interaction, such as electrostatic interactions; acetate buffers or pyridinium acetates being used among others.

Cation Exchange Phases Cation exchange resins are composed by cross-linked polystyrene and silica-based ion exchangers such as calcium or silver. Oligosaccharides up to DP8 for calcium columns and DP12 for silver columns can be separated (Kennedy and Pagliuca, 1994). Carbohydrates elute in order of decreasing molecular size and the chromatographic mechanism is based on both the size exclusion and ligand-exchange.

These phases can show different disadvantages such as compressibility of the gel matrix, efficiency losses when flow rate is increased, the need of high temperature operation (85°C) and extended analysis times (Kennedy and Pagliuca, 1994). The use of H^+ columns and 0.01M sulfuric acid as mobile phase which allows the regeneration of the H^+ reduces the losses of efficiency.

Anion Exchange Phases The advent of HPAEC in 1983 (Rocklin and Pohl, 1983) for carbohydrate analysis notably improved knowledge about oligosaccharide composition of a wide variety of products. Carbohydrates are negatively charged at high pH (pH $>$ 13) and oligo- and polysaccharides up to DP50 can be separated by anion-exchange chromatography using NaOH as mobile phase; amylopectins up to DP80 have even been separated by this technique (Hanashiro et al., 1996). A gradient of increasing concentration of sodium acetate is normally used to help elution of oligosaccharides. Stationary phases are composed of polymeric, non-porous, MicroBead™ pellicular resins such as polystyrene/divinylbenzene or ethylvinylbenzene/divinylbenzene substrates agglomerated with Microbead™ quaternary amine functionalized latex, which are highly resistant to high pHs. CarboPac PA100 and more recently, CarboPac PA200 are columns mainly designed for oligosaccharide separation, although CarboPac PA1 and PA10 can be also used (Cardelle-Cobas et al., 2008; Splechna et al., 2006).

Carbohydrate elution takes place with increasing the molecular weight for oligosaccharides with the same glycosidic linkage, nevertheless, this order can

change when families of oligosaccharides with different linkage variants are mixed (i.e., isomaltohexaose elutes before maltotriose; Morales et al., 2006). The combination of different effects (charge, molecular size, sugar composition and glycosidic linkages) is implied in the chromatographic separation (Gohlke and Blanchard, 2008). This retention behavior is one of the disadvantages of this technique which requires the use of standards for the identification of complex mixtures of carbohydrates with different DP and glycosidic linkages. Nevertheless, this chromatographic technique coupled to a pulse amperometric detector (PAD), as it will be shown later, presents significant advantages: fast analysis, samples do not require a previous derivatization, low to sub-picomole sensitivity, high resolution, etc.

13.2.3.2.2 Column Dimensions and Design

Recently, there is a trend to develop miniaturized systems which allow reduced solvent consumption and disposal, fast analysis and increased sensitivity. Downscaling of the column dimensions to the capillary- or nano-scale has shown several advantages over the conventional chromatography for carbohydrate analysis. Glycans at femtomole level can be analyzed without derivatization. This miniaturization has been carried out for graphitized carbon stationary phases (Ninonuevo et al., 2005), normal and reverse phases (Wuhrer et al., 2005) and HPAE columns (Bruggink et al., 2005a).

Conventional HPLC phases of between 3 and 10 μm diameter of particles are commonly used for oligosaccharide analysis; 3 μm silica columns (such as amino-bonded silica phases) have been demonstrated to improve the analysis of glucooligosaccharides up to a DP of 30–35 (Herbreteau, 1992). Columns with smaller diameter of particles (sub-2 μm) have recently been introduced in the market and allow faster separations without resolution losses.

The use of columns with small particle diameter and column length produces an increase in pressure. In order to solve this backpressure problem, Ultra Performance Liquid Chromatography (UPLC) systems are employed. However, applications to oligosaccharides are scarce.

Fast separation can be also achieved using monolithic columns constituted by highly porous materials with a network of interconnecting channels. These columns allow the use of very fast flows: a mixture can be resolved at a flow of 9 mL min^{-1} reducing the elution time between 5 and 10 times. A wide range of underivatized or derivatized carbohydrates (mono- and oligosaccharides) can be successfully separated with this kind of columns achieving higher

column efficiencies than with particle-packed columns (Ikegami et al., 2006; Ikegami et al., 2008).

13.2.3.3 Detectors

Not only is the separation of oligosaccharides a problem in HPLC due to their similar structures, but also to achieve a sensitive detection can be a difficult task. Carbohydrates itself do not contain a specific chromophore or fluorophore, being necessary to fall back on universal detectors such as RI detectors, or on electrochemical ones. Nevertheless, ultraviolet (UV) detectors for both derivatised and non derivatised carbohydrates or fluorimetric detectors for derivatised ones are also used.

Refractive Index Detector

RI detectors are the most common detectors used for carbohydrate analysis although a lack of sensitivity is normally associated to them. Their main drawback arises from their dependence on temperature and mobile phase composition changes. Therefore, these detectors are commonly utilized with isocratic mode or, if gradients of mobile phases are used, solvents with the same refractive index must be used (Davies and Hounsell, 1996). These detectors are mainly used in the mM-μM concentration range.

UV Detector

UV detectors at low wavelengths (below 210 nm) show similar sensitivity to RI detectors however, they allow changes in temperature and gradient elution. As has been indicated, different methods to introduce chromogenic groups in saccharide molecules have been proposed. However, post-column derivatization (not considered as sample preparation) also improves UV detection. Carbohydrates can be labeled with different reagents such as tetrazolium blue or cyanoacetamide. Hase (2002) reports a table with all the possible reagents used for post-column derivatization in HPLC.

Fluorometric Detector

Postcolumn derivatization of carbohydrates with fluorescent labels such as 2-aminopyridine or 2-aminobenzoic acid allows their detection in subpicomolar concentrations (Gohlke and Blanchard, 2008). However, most of postcolumn derivatization methods have been applied to monosaccharide analysis, while only few works have been reported about oligosaccharide analysis.

Light Scattering Detectors

Evaporative light scattering detectors (ELSD) utilize a spray which atomizes the column effluent into small droplets. These droplets are evaporated and the solutes as fine particulate matter are suspended in the atomizing gas. These particles diffuse the light originated from a monochromatic or polychromatic source. These detectors are universal, more sensitive than RI and are compatible with elution gradients (Herbreteau, 1992).

Liquid light scattering detectors differ from ELSD in that they respond to the light scattered by a polymer or large molecular weight substance present in the column eluent. The high intensity light source is achieved by the use of a laser. There are two forms of the detector: Low angle laser light scattering (LALLS) and multiple angle laser light scattering (MALLS) which provide an appropriate sensitivity and baseline stability. They have commonly been applied to SEC. Light from a laser is scattered to different degrees by the concentration and size of the analyte passing through the cell flow. The intensity of scattered light is highest at low scattering angles and also increases with the molecular weight of the carbohydrate (Davies and Hounsell, 1996). However, laser light scattering can sometimes give confusing results because of molecule-molecule interactions and associations of oligosaccharides with high DP.

Pulse Amperometric Detectors

PADs are commonly coupled to HPAEC and allow the detection of non-derivatised carbohydrates at very low picomole levels. This detection provides a high selectivity; only compounds oxidizable at the selected voltages being detected. PAD is composed by a working electrode of Au or Pt, a stainless steel auxiliary electrode and a reference electrode of Ag/AgCl or H_2. The Au electrode is able to catalyze the oxidation reactions and is the best choice for detection of carbohydrates. Carbohydrates are detected by measuring the electrical current generated by their oxidation at the surface of the working electrode at the selected potential (E_1). Next, the voltage is increased (E_2) to oxidize the gold detector which causes desorption of the carbohydrate oxidation products. Finally, the potential is lowered ($E_{3;}$ negative potential) to reduce the electrode surface for the next pulse. The three potentials are applied for fixed times. More recently, this potential sequence has been modified, because although good results can be achieved the gold electrode surface is gradually lost which affects reproducibility (Rohrer, 2003). Similar to the previous method, the first potential is applied to oxidise the carbohydrate on the surface of the gold electrode. However, the second potential is in this case a reductive potential to clean the working

electrode surface, whereas the third short potential reactivates the electrode oxidizing its surface. A fourth potential is necessary to achieve the initial conditions of the Au surface.

Mass Detectors

The use of MS detectors coupled to HPLC systems has considerably enriched the field of carbohydrate analysis. MS detectors have been commonly utilised with alkyl- and aminoalkyl-bonded phases, although other columns such as GCC can be also used. Currently, MS (both quadrupole (Q) and ion trap (IT) MS; Bruggink et al., 2005a,b) are also being coupled to HPAEC using automated systems for neutralization and removal of eluent salts to be compatible with electrospray ionization (ESI) MS requirements. These systems also allow the collection of different desalted fractions which are suitable for further enzymatic and chemical digestions or NMR and chromatographic analysis. A splitter to divide the effluent to the PAD and a MS detector is installed after the column and a membrane-desalting device placed before the MS system convert the sodium hydroxide into water and sodium acetate into acetic acid. Lithium or sodium chlorides are added after the membrane desalter to enhance the MS signal by the formation of lithium or sodium adducts of carbohydrates (Bruggink et al., 2005a,b). These couplings are a great advantage for carbohydrate analysis allowing the acquisition of information related not only to carbohydrate retention, but also to structural characteristics.

Electrospray ionization (ESI) is the most common ionization source coupled to HPLC for the analysis of carbohydrates. Analytes (which are in an ionic state) and eluent from HPLC (which is highly volatile) are sprayed at atmospheric pressure from a needle which is subjected to a high potential (3,000–5,000 V) giving rise to small droplets. Desolvation of ions is assisted by a heated inert gas (N_2). As the solvent evaporates, the ionic charges of analytes are closer and repel each other, breaking up the droplets. The ions free from solvents are focused in the analyzer: Q, IT, Q-ToF, etc.

13.2.3.4 Multidimensional HPLC

Multidimensional HPLC has been used to mainly separate fluorescently-labeled oligosaccharides. Combinations of amine-bonded phases which can perform as both hydrophilic interaction media and as an anion exchange

phase and RP-columns have been applied for this purpose (Gohlke and Blanchard, 2008).

13.2.3.5 Applications

The number of applications of HPLC to analyze prebiotic oligosaccharides in recent years is huge and only some of them can be mentioned in this chapter, although it is good to consider that most of the methods here reported for common prebiotic oligosaccharides [FOS and galactooligosaccharides (GOS)] can be applied to the analysis of different carbohydrate sources. *Table 13.2* summarizes some of the applications described below.

The most recent applications of prebiotic analysis have been developed for HPAEC-PAD. This technique is widely used to both determine the oligosaccharide composition of prebiotic carbohydrates and to study the degradation patterns of oligosaccharides during fermentation assays to evaluate their prebiotic effect. Different methodologies, mainly based in the use of eluents indicated above (NaOH and NaOAc) and Carbo-Pac PA100 or Carbo-Pac PA200 columns, have been developed by many researchers who tried to achieve the optimum separation depending on the prebiotic source to be analyzed.

Fructooligosaccharides and Inulin

Sangeetha et al. (2005) reviewed the different methods reported in the literature for the analysis of FOS, most of them being based on the use of polar-bonded phases or resin based ion exchange columns coupled to RI detectors and HPAEC-PAD analyses.

There are many reports on the analysis of FOS by RP-HPLC coupled to a diode array detector at a wavelength of 190 nm (Grizard and Barthomeuf, 1999) or to a RI detector (Mujoo and Ng, 2003) using a C18 column and water as mobile phase. Chromatographic profiles showed coelution of glucose and fructose, whereas kestose, nystose and fructofuranosylnystose appeared as separated peaks.

HPAEC-PAD analysis of fractionated inulin allows the separation of different molecular weight fructooligosaccharides (G-F_n; α-D-gluc*p*-[β-D-fruct*f*]$_{n-1}$-D-fructofuranoside) and inulooligosaccharides (F-F_n; β-D-fruct*p*-[α-D-fruct*f*]$_{n-1}$-α-D-fructofuranoside). Assuming that retention time in HPAEC-PAD of a homologous series of carbohydrates increases with increasing DP, the assignment of these carbohydrates can be feasible. However, the coexistence of both series

Table 13.2

HPLC applications for the analysis of prebiotics

Prebiotic	Chromatographic column	Detector	Mobile phase	Reference
FOS in onion	CarboPac PA1	PAD	NaOH and CH_3CO_2Na	Kaack et al. (2004)
FOS from levan	Cation exchange (Ca^{2+})	RI	Water	Kennedy et al. (1989)
FOS	CarboPac PA200	MS	NaOH and CH_3CO_2Na	Bruggink et al. (2005a)
FOS in nutraceutical and functional foods	CarboPac PA100 CarboPac PA10	PAD	NaOH and CH_3CO_2Na	Corradini (2002)
GOS	CarboPac PA10	PAD	NaOH and CH_3CO_2Na	Cardelle-Cobas et al. (2008)
GOS	Cation exchange (Ca^{2+})	RI	Water	Goulas et al. (2007)
	CarboPac PA1	PAD	NaOH and CH_3CO_2Na	
Human milk oligosaccharides	On chip GCC	o-ToF	Formic acid in acetonitrile/water	Ninonuevo et al. (2005)
Soybean oligosaccharides	HPSEC	RI	$NaNO_3$ with NaN_3	Giannoccaro et al. (2008)
	CarboPac PA10	PAD	NaOH and CH_3CO_2Na	
Sucrose derived oligosaccharides	Amino bonded	ELSD	Methanol: acetonitrile:water	Yin et al. (2006)
XOS	Cation exchange (Na^+)	RI	Water	Ohara et al. (2006)
XOS	Cation exchange (Ca^{2+})	RI	5 mM H_2SO_4 in water	Moura et al. (2008)
Lactulose	Amino bonded	RI	Acetonitrile:water	Paseephol et al. (2008)
FOS and glucooligosaccharides	C18	RI	0.1% trifluoroacetic acid (v/v) in water	Rousseau et al. (2005)

($G\text{-}F_n$ and $F\text{-}F_n$) makes their identification more difficult and coelution of some oligosaccharides can be observed. Schütz et al. (2006) investigated the chromatographic profile of inulin up to carbohydrates of DP79 in artichoke heads and dandelion roots by this technique, although quantitative analysis was only carried out for glucose, fructose, sucrose, kestose, nystose and

fructofuranosylnystose. The lack of higher molecular weight standards is one the limitations of this analysis. Ronkart et al. (2007) developed a method to obtain F-F_n standards and to identify them in a complex inulin chromatogram. F-F_n standards were isolated by semi-preparative HPSEC from inulin from globe artichoke treated with endo-inulinase, and analyzed by HPAEC-PAD. Coelution problems of some G-F_n and F-F_n oligosaccharides after HPAEC-PAD analysis can be solved by the use of a coupled ESI MS detector which allows the unveiling of both series by the extraction of the ion chromatograms at the appropriate m/z ratios (Bruggink et al., 2005a,b). *Figure 13.1a* shows the extracted ion chromatograms of FOS up to DP13 obtained by HPAEC-MS using a CarboPac PA200 capillary column (Bruggink et al., 2005a), the F-F_n series being more retained than G-F_n series. A MS spectrum of $[GF_4 + Na]^+$ is also shown in *Figure 13.1b*. As can be seen in *Figures 13.1c* and *13.1d*, MS/MS spectra of the two series (i.e., for DP5 variants) showed similar fragmentation patterns with different relative intensities of m/z ions.

Wang et al. (1999) quantitatively analyzed FOS from different food matrices and compared these results to those obtained by MALDI-ToF. The PAD response was different depending on DP and oligosaccharide linkage; the use of specific standards being necessary to avoid overestimated results.

Different authors have shown the suitability of HPAEC-PAD to evaluate fermentation properties of FOS. Hartemink et al. (1997) observed a different HPAEC degradation pattern of FOS for the different strains assayed (*Ent. cloacae, E. coli, Salm. infantis* and *Sh. flexneri,* among others). Moreover, depending on the FOS source employed, different behavior was observed; i.e., using Profeed P95 (from Nutreco, Boxeer, The Netherlands) as a substrate, *Ent. Clocae and Salm. infantis* produced very little degradation of FOS, however these bacteria showed degradation, mainly of F-F_n series, using Raftilose P95 (from Orafti). Corradini et al. (2004) optimised a gradient elution program using water, 0.6 M sodium hydroxide and 0.5 M sodium acetate as eluents to selectively separate glucose, fructose, sucrose and fructans with DP from 3 to 60 in microbial cultures, obtaining good resolution during the whole chromatogram. This method allowed the evaluation of FOS and inulin consumption by pure cultures of *Bifidobacterium* spp. and by fecal cultures.

Galactooligosaccharides

GOS are produced by transgalactosylation reactions catalyzed by β-galactosidases using mainly lactose as substrate. As consequence of these reactions, a large variety of structures can be obtained (different glycosidic bonds and

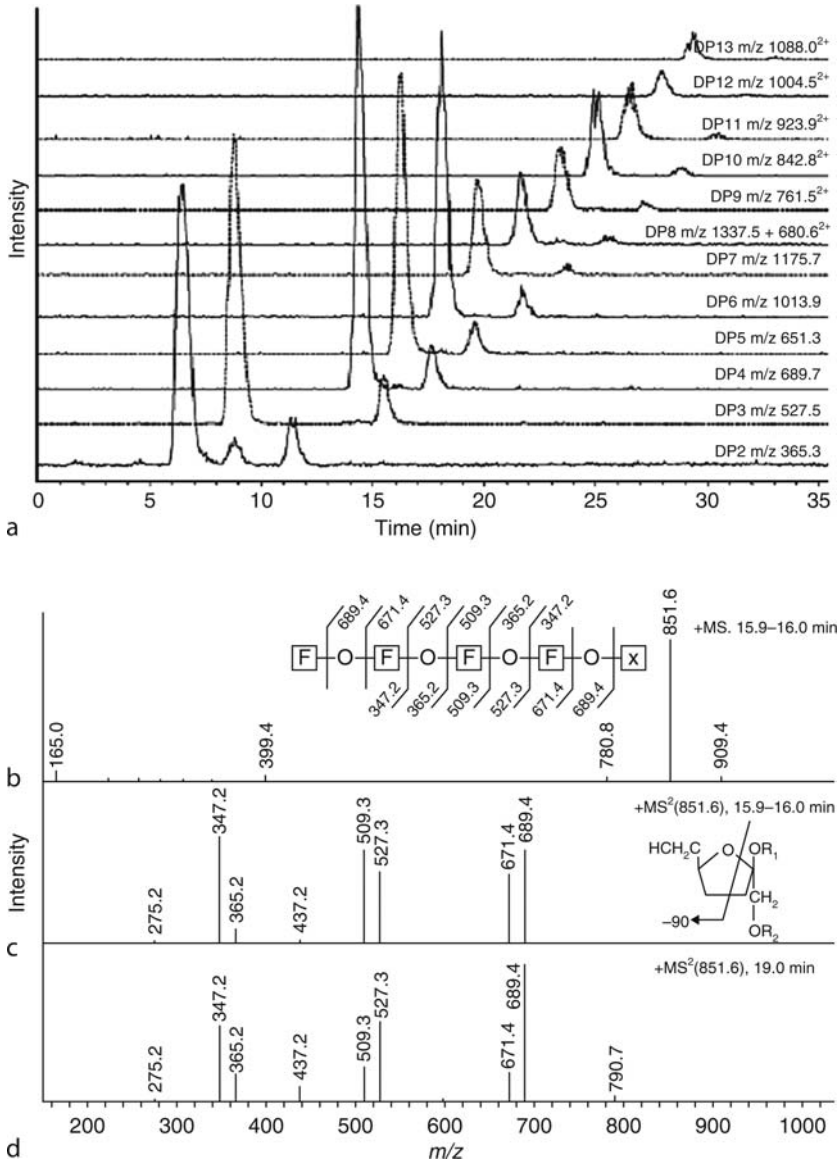

Figure 13.1

HPAEC-on-line-MS analysis of FOS. Extracted ion chromatograms for fructan oligosaccharides of various degrees of polymerization (DP) detected as sodium adducts by capillary HPAEC-on-line-MS (a). Mass spectra of the two isobaric sodium adducts of DP5 fructans: Part (b) shows the MS spectrum of [GF_4 + Na]+; parts (c) and (d) are the MS2 spectra with m/z 851.6 as precursor ion, where (c) represents GF_4 and (d) F_5. In the fragmentation scheme, F stands for fructofuranosyl and X is glucopyranosyl or fructopyranosyl, R_1 and R_2 stand for the rest part of the oligosaccharide chain and R_2 can also be a H. From Bruggink et al. (2005a) with permission from Elsevier.

oligosaccharides of different molecular weights are formed depending on the enzymatic source and the reaction conditions used). Therefore, it is necessary to use high resolution methods to distinguish between structural isomers of oligosaccharides consisting of monosaccharides linked together in various anomeric and positional configurations.

Splechtna et al. (2006) analyzed the composition of GOS (mainly mono-, di- and trisaccharides) by HPAEC-PAD using a Carbopac PA-1 column, although identification of all of the structures could not be completely achieved. Moreover, coelution of some carbohydrates (i.e., glucose and galactose; lactose and allolactose) was observed. These coelution problems have been avoided using a modified method with a CarboPac PA-10 column (Cardelle-Cobas et al., 2008; Martínez-Villaluenga et al., 2008a). Goulas et al. (2007) used a cation exchange column (Ca^{2+}) at 85°C with water as mobile phase and RI detector to determine the synthesis and purification of GOS. Under these conditions, two not well-resolved chromatographic peaks were obtained for oligosaccharides of DP higher than three, whereas disaccharides eluted as one peak and glucose and galactose appeared as separated peaks. HPAEC-PAD with a CarboPac PA1 allowed the separation of the disaccharides obtained in these samples.

Figure 13.2 shows an HPAEC-PAD profile of GOS before (*Figure 13.2a*) and after (*Figure 13.2b*) removal of mono- and disaccharides using activated charcoal (Sanz et al., 2007). The use of a CarboPac PA-100 column allowed the separation of oligosaccharides up to DP7, although a complete resolution of these carbohydrates was not achieved. The lack of standards was the main disadvantage for identification and quantification purposes, necessitating the isolation of oligosaccharides followed by ESI-MS analysis to determine their molecular weights.

Other Prebiotics

Several methods have been also developed for the analysis of other oligosaccharides such as xylooligosaccharides (XOS; Ohara et al., 2006), soybean oligosaccharides (Giannoccaro et al., 2008), lactulose (Paseephol et al., 2008) or glucooligosaccharides (Rousseaua et al., 2005), some of them only tentatively considered as prebiotics. Giannoccaro et al. (2008) have compared two methods using HPSEC-RI (using two analytical Shodex OHpak SB 802HQ columns) and HPAEC-PAD (CarboPac PA10) to analyze soybean sugars. Although both systems gave reproducible results, HPAEC-PAD was more sensitive, faster, and with higher resolution than the HPSEC-RI method.

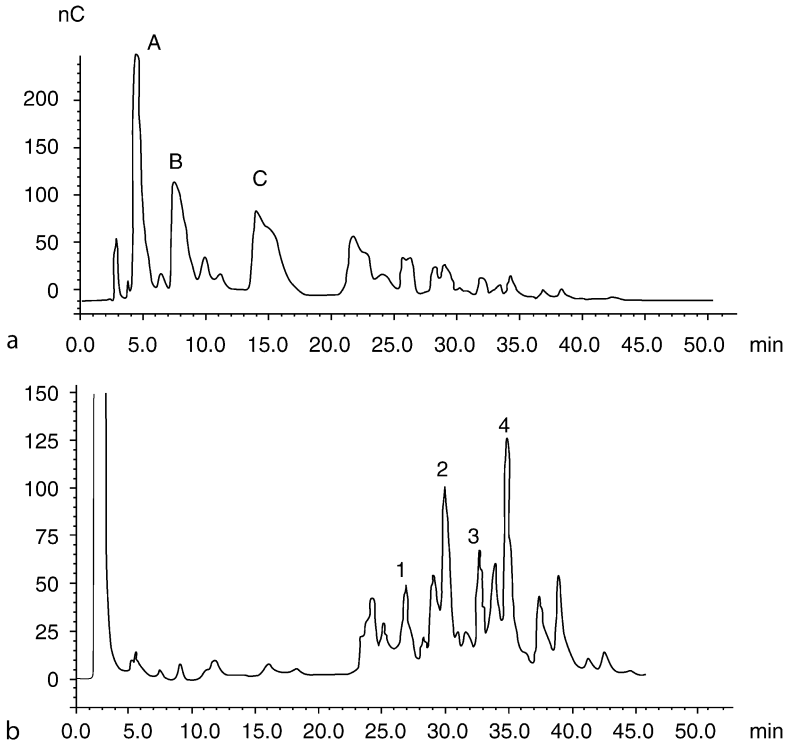

Figure 13.2

HPAEC-PAD analysis of GOS. HPAEC-PAD profiles of: (a) GOS, (b) GOS previously treated with activated charcoal. (A) glucose and galactose, (B) lactose, and (C) unknown disaccharides. From Sanz et al. (2007) with permission from ACS publications.

GCC have recently been used for the separation of carbohydrates in a wide range of applications including coupling to ESI MS. Robust and rapid separations were achieved with these methods. Separation of human milk oligosaccharides has been carried out using on-chip GCC (Ninonuevo et al., 2005) coupled to a MS detector with o-ToF which allows isomeric detection. Consumption of these oligosaccharides by intestinal bacteria has also been evaluated by Ninonuevo et al. (2007) using GCC and UV detection (206 nm) obtaining different chromatographic profiles for the bacteria studied.

Recently, the use of HPLC-ESI MS has allowed the identification of 19 peptides glycated with GOS from 2 to 7 hexose units which prebiotic potential is being studied (Moreno et al., 2008).

13.2.4 Gas Chromatography

Since its first application to carbohydrate analysis by Langer et al. (1958), GC has seen widespread use for sugar determination as it is a relatively cheap, simple and powerful analytical technique. Higher oligosaccharides in foods and diets are often present at low concentrations, thus, the high resolving power, sensitivity and selectivity of GC results is extremely advantageous. The potential of this technique for carbohydrate determination was achieved with the development of capillary columns and their coupling to mass spectrometric detectors; identification and quantification of many prebiotic oligosaccharides as well as structural studies can be performed.

13.2.4.1 Sample Preparation

Purification/Fractionation

As oligosaccharides usually appear in complex matrices, a purification step is required before their analysis as with HPLC determinations. This procedure is often carried out to discard insoluble material, lipids and proteins, desalt the sample or remove impurities.

Soluble carbohydrates in foods are usually extracted with ethanolic or methanolic solutions: oligosaccharides up to DP6 are easily soluble in these solvents while other interfering substances are not, being discarded by filtration or centrifugation. Although these methods are still in use on standard protocols and regulations, other modern procedures such as membrane filtration have been introduced for more complex mixtures.

In those cases where the study of a specific carbohydrate or a group of carbohydrates is required, a fractionation step can be also necessary. This procedure provides an enrichment of the samples in carbohydrates and purifies them before their chromatographic analysis. As an example, nanofiltration, yeast (*Saccharomyces cerevisiae*) treatment, and adsorption onto activated charcoal were used by Sanz et al. (2005) prior to GC analysis in order to remove honey monosaccharides and study the potential prebiotic effect of its oligosaccharides.

Recent methods for the selective extraction of lactulose from a mixture with lactose have been developed by accelerated solvent extraction (ASE; Ruiz-Matute et al., 2007) and supercritical fluid extraction (SFE; Montañés et al., 2007), which allowed the obtainment of a high purity lactulose fraction, using rapid processes with low solvent consumption.

Derivatization

Due to the polar nature of carbohydrates, a derivatization step previous to GC analysis is required. Classical methods are based on the substitution of the polar groups in order to increase their volatility. Acetates, methyl ethers, trifluoroacetates and trimethylsilyl ethers have been the most common derivatives used for carbohydrate determination (Knapp, 1979). Among them, trimethylsilyl derivatives are the most popular, since they present good volatility and stability characteristics. Trimethylsilylation has been recognized as a quantitative rapid derivatization method for a wide range of carbohydrates and related compounds including aldoses, ketoses, aminosugars, alditols, inositols as well as oligosaccharides up to DP4 (Brobst and Lott, 1966; Sweeley et al., 1963).

However, these derivatives give multiple peaks corresponding to the different anomeric forms of carbohydrates. Even though high-resolution capillary columns can adequately resolve complex mixtures, multiple peaks may cause interferences for qualitative identification and quantitative measurement.

Sometimes, the multiple peaks obtained are not considered a disadvantage as they serve as a "fingerprint" for each sugar, aiding their identification. Nevertheless, other derivatives are preferred for analyses of mixtures containing many sugars. Usually the anomeric carbon is modified in order to reduce this effect. Several attempts have been made for this purpose, among them, some possibilities are to: (1) convert the free carbonyl group into an oxime using hydroxylamine chlorohydrate or to an *O*-methyloxime using *O*-methylhydroxylamine chlorohydrate; (2) reduce the aldehyde with sodium borohydride to the corresponding alditol or (3) convert the aldehyde into an oxime and then dehydrate into a nitrile.

Trimethylsilyl oximes can be easily obtained by a two step derivatization procedure (oximation and silylation). They have been widely used for the GC analysis of many oligosaccharides since they produce only two peaks corresponding to the syn (*E*) and anti (*Z*) forms for reducing sugars, and only one peak for non reducing carbohydrates, the derivatives formed having satisfactory GC properties (Molnar-Perl and Horváth, 1997; Sanz et al., 2002).

Alditol acetate derivatives have also been used for sugar GC analysis due to their stability and the simplicity of the resulting chromatograms. The reduction of aldoses to alditols and their conversion to alditol acetates simplifies the chromatograms by producing only one peak for each aldose. Abazia et al. (2003) used these derivatives for the simultaneous GC measurement of lactulose and other sugars in urine. Sugars were reduced with sodium borohydride and acetic acid/methanol 1:9 (v/v) was added to remove the boric acid. Then acetylation was performed by the

addition of dry pyridine and acetic anhydride. Although these derivatives show a high chemical stability and low cost of reagents, they also present some disadvantages. On reduction, ketoses yield a mixture of two sugar alcohols (i.e., fructose produces glucitol and mannitol) and thus, give two chromatographic peaks. In some cases, a significant loss of information may occur in the reduction step as some aldoses and ketoses produce the same alditol and cannot be differentiated (i.e., glucose and fructose both produce glucitol). Although significant improvements have recently been made simplifying the derivatization procedure, some common versions of this method still require tedious evaporations to remove the borate before acetylation. The alditol acetate derivatization has been widely used for monomer analysis of macromolecules by GC (Fox et al., 1989).

An alternative to eliminate the anomeric center is the conversion of sugars to their aldononitrile acetates derivatives. They give a unique peak for every sugar but they cannot be applied for ketoses (Ye et al., 2006). These derivatives have been applied for structural analysis of gums and food samples (McGinnis and Biermann, 1989).

Structural Analysis

Structural analysis of complex carbohydrates requires the characterization of monomer composition and anomeric configuration, as well as the determination of the sequence of monosaccharide residues, branch position, functional groups and glycosidic linkages. The elucidation of structural chemistry of complex carbohydrates requires sophisticated instrumentation such as mass spectrometry (MS) or nuclear magnetic resonance (NMR), but the additional information that GC-MS data provides is essential for carbohydrate characterization.

GC-MS has been applied for either the determination of composition and sequence of oligosaccharides released by partial depolymerization after being converted into proper volatile derivatives or for sugar monomer analysis after complete hydrolysis and derivatization. Partial degradation of polysaccharides to oligosaccharides is achieved by means of enzymatic or mild acidic hydrolysis. The use of acids implies the optimization of conditions to achieve maximum cleavage to oligosaccharides and minimum decomposition of the liberated mono- and/or oligosaccharides.

Methylation analysis is the most widely used method for determining linkage structure of prebiotic oligosaccharides by GC. It basically consists of the following steps: Firstly, the free hydroxyl groups of polymerized sugars are completely methylated, forming their correspondent methyl ethers. Then,

hydrolysis of the polymer is performed releasing the free hydroxyl groups in those places in which previously there were glycosidic linkages. Finally, these hydroxyl groups are converted into more volatile compounds, the most common derivatives being alditol or, aldonononitrile acetates. These samples are analyzed by GC-MS in order to ascertain the original linkages and to obtain quantitative linkage information on complex polysaccharides.

The major drawback of standard methylation analysis is that in certain cases the carbon involved in the cyclic hemiacetal of the monosaccharide is not distinguished from linked positions after hydrolysis of the permethylated polysaccharide. As an alternative, the reductive-cleavage method, which yields partially methylated anhydroalditols while retaining the ring structure, has been successfully used to investigate the structure of different prebiotic oligosaccharides. Rolf and Gray (1984) studied the suitability of the reductive-cleavage method for the study of the linkage positions in D-fructofuranosyl residues of D-fructans of different sources. The same derivatization method was used by Stumm and Baltes (1997) for the structural determination of polydextrose. Previous to methylation, ultrafiltration was applied in order to yield fractions free from monomeric residues. Carbohydrate analysis was performed by GC and GC-MS using both electronic impact (EI) and chemical ionization (CI). EI MS was used to confirm the identity of the carbohydrate derivatives while CI MS with ammonia as reagent gas was useful for the determination of the molecular weight. This method resulted to be useful for the elucidation of the degree of branching, position of the linkages and the type of monomeric compounds involved in the studied samples.

Many novel prebiotic oligosaccharides synthesized either by the use of microbial cells or enzymes have been also characterized by methylation analysis. The determination of carbohydrate structures of neofructo-oligosaccharides produced by *P. citrinum* (Hayashi et al., 2000), oligosaccharides formed by a fructosyltransferase purified from asparagus (Yamamori et al., 2002), and oligosaccharides synthesized by glucosyl transfer from β-D-glucose-1-phosphate to raffinose and stachyose using *T. brockii* kojibiose phosphorylase (Okada et al., 2003), are some examples of the application of methylation analysis.

13.2.4.2 Columns and Stationary Phases

The most significant improvement in GC separation was achieved with the advances in capillary column technology. Although many separations of prebiotic

oligosaccharides have been carried out on packed columns (Farhadi et al., 2003; Karoutis et al., 1992), the use of capillary columns involves an increment on resolution while the analysis time is decreased. The most common liquid stationary phases used for carbohydrate analysis by GC are those based on polysiloxanes (also called "silicones") since they present good thermal stability and high permeability towards solutes. A wide polarity range can be found and depends on the percentage of polar phenyl or cyanopropyl groups in the siloxane chain, the most apolar being 100% methylsilicone while 100% cyanopropyl silicone is the most polar. For high-temperature separations, phases based on a carborane skeleton have been proposed (Joye and Hoebergs, 2000).

Dimensions of capillary columns used for carbohydrate GC analysis can vary in the range of: 1–50 m (length); 0.1–0.5 mm (diameter), and 0.02–2 μm (df).

It has been demonstrated that oligosaccharides with up to 11 monosaccharide units can be analyzed using capillary columns with ultrathin films (<0.05 μm) of thermostable bonded stationary phases at high temperatures (Carlsson et al., 1992).

13.2.4.3 Chromatographic Conditions

As carbohydrates usually appear as a mixture of mono- and oligosaccharides, programed temperature is convenient during the chromatographic run, so that each compound can be analyzed under adequate conditions. The temperature commonly used for carbohydrate analysis range from 60 to 330°C.

GC and high temperature gas chromatography (HT-GC) have been used for carbohydrate analysis by many authors (Joye and Hoebergs, 2000; Karoutis et al., 1992; Montilla et al., 2006). The main differences between these techniques are their operating conditions: GC uses temperatures below 360–370°C whereas HT-GC works above them, but requires special apparatus, high temperature capillary columns and heat resistant fittings for the analysis. Carlsson et al. (1992) developed a HT-GC method for the determination of high oligosaccharides: the temperature program was increased from 40 to 400°C with a linear temperature program of 20°C/min. A conventional GC procedure for the determination of high DP oligosaccharides was developed by Montilla et al. (2006). A maximum temperature program of 360°C allowed the determination of oligosaccharides up to DP 7 in different foodstuff.

High carrier gas velocities and columns coated with thin layers of stationary phase can help to minimize the time spend through the column and, consequently,

reduce analysis time. The use of helium as a carrier gas further decreases analysis time without any substantial loss in column efficiency.

13.2.4.4 Detectors

Flame ionization detection (FID) is the most frequently used for GC analysis of carbohydrates since it possesses good sensitivity for organic products. However, the identification by GC always requires the use of standard compounds, and only few of them are commercially available. The coupling of a MS detector to a gas chromatograph contributes to the identification and quantification of carbohydrates, especially in those cases where complex mixtures of the same DP have to be determined.

Most of GC–MS applications utilize capillary GC with Q MS and EI ionization due to its relatively low cost, high sensitivity, high information content, and the ready availability of commercial instruments. CI with ammonia as reagent gas has also been used in order to confirm the molecular weight in methylation analysis (Stumm and Baltes, 1997).

Certain carbohydrates with the same DP and only differing in the position of their hydroxyl groups show similar mass spectra. In those cases, the combination of retention information provided by GC analysis and spectral data from MS is necessary for carbohydrate identification, although it is not always enough for a proper characterization.

Spectral information of different carbohydrate derivatives such as alditol acetates (Fox et al., 1989), trimethylsilyl oximes (Molnárl-Perl and Horváth, 1997; Sanz et al., 2002), trimethylsilyl O-methyloximes (Laine and Sweeley, 1973) and methyl acetates (Stumm and Baltes, 1997) can be found in literature.

13.2.4.5 Applications

GC applications for prebiotic carbohydrate analysis have been mainly focused on both direct analysis of oligosaccharides with a previous derivatization procedure and methylation analysis for their structural determination.

Many references can be found about the analysis of lactulose by gas chromatography. Lactulose is a disaccharide obtained through lactose isomerization (through Lobry de Bruyn-Alberda Van Ekenstein rearrangement). It is not digested in the small intestine and is fermented by the colonic flora. Some examples are summarized in ◉ *Table 13.3*. Farhadi et al. (2003) developed two

gas chromatographic methods using a packed and a capillary column respectively, for the simultaneous quantitation of urinary lactulose and other carbohydrates (sucrose and mannitol). Columns and chromatographic conditions are summarized in *Table 13.3*. The capillary method was more sensitive, accurate and reproducible for lactulose determination. Moreover, it permitted the use of smaller volumes of urine in the analysis and did not require pretreatment of the samples.

A method was developed for the determination of lactulose in milk (Montilla et al., 2005). This method gave good chromatographic resolution, as well as precise and reproducible results when applied to commercial milk samples submitted to heat treatments of different intensity. In addition, this method provided a good separation among sucrose, lactulose and lactose peaks which allowed the suitable quantification of lactulose in samples containing a high concentration of sucrose.

The degree of polymerization as well as the presence of branches are important in inulin, since they affect to their functionality. Short chain inulin carbohydrates should be separated from their long chain analogues for prebiotic uses. Lopez-Molina et al. (2005) characterized artichoke inulin and demonstrated its health-promoting prebiotic effects. The extraction of artichoke inulin involved several physical steps (see *Table 13.3*); hydrolysis of inulin was also performed. GC-MS analysis of their trimethylsilyl derivatives confirmed that fructose was the main monosaccharide unit in artichoke inulin and its degradation by inulinase indicated that it contained the expected β-2,1-fructan bonds.

Packed column GC was used by Sosulski et al. (1982) for the analysis of oligosaccharides in legumes but long analysis time and poor reproducibility for larger oligosaccharides were the major drawbacks of the method. Karoutis et al. (1992) optimized a methodology by GC for the analysis of raffinose, stachyose and verbascose. Different analytical parameters were assayed: carrier flow-rate, split ratio and nature of derivatization agent (trimethylimidazole or N-methyl-bis (trifluoroacetamide).

Joye and Hoebregs (2000) developed a method for the quantitative determination of oligofructose in foods. The use of high temperature chromatography with an Al-clad capillary column and oven temperatures up to 440°C allowed the determination of carbohydrates up to DP9 in complex matrices in only one chromatographic run. *Figures 13.3a* and *13.3b* show the chromatogram obtained for Raftilose P95 X (Orafti) and an enzymatically synthesized FOS (Actilight®). Malto-, isomalto- and galactooligosaccharides were also analyzed by this method to exclude possible interferences from other sugar compounds. *Figure 13.3c* shows the GC profile of GOS as an example. This method was very accurate and reproducible for the study of these carbohydrates.

Table 13.3

GC applications for the analysis of prebiotics (*Cont'd* p. 496)

Carbohydrates	Derivatives	Pretreatment	Column and dimensions	Cromatographic conditions	References
Lactulose and other sugars in urine	Alditol acetates	Urine purification with Dowex mixed bed resin	ZB-1 capillary column (30 m × 0.25 mm ID)	T programme: 230–300°C Carrier gas: Nitrogen	Abazia et al. (2003)
Lactulose and other sugars in urine	TMSO	Centrifugation and filtration	Glass column packed with 3% SE-30 on 80/100 chromosorb WHP (6-ft × 2 mm ID)	T programme: 220–274°C Carrier gas: Nitrogen	Farhadi et al. (2003)
		None	DB1 capillary column (15 m × 0.53 mm ID × 1.5 μm)	T programme: 220–274°C Carrier gas: Helium	
Lactulose from a sugar mixture in urine	TMSO	None	DB-1701 capillary column (30 m × 0.25 mm ID × 0.25 μm)	T programme: 180–250°C Carrier gas: Helium	Farhadi et al. (2006)
Lactulose in dairy products	TMS	Methanol to remove proteins and fats	SPB-17 capillary column (30 m × 0.32 mm ID × 0.25 μm)	T programme: 235–270°C Carrier gas: Nitrogen	Montilla et al. (2005)
Lactulose from a mixture with lactose	TMS	PLE extraction	SPB-17 capillary column (30 m × 0.25 mm ID × 0.25 μm)	T programme: 250–270°C Carrier gas: Nitrogen	Ruiz-Matute et al. (2007)

Inulin from artichoke	Complete hydrolysis with formic acid and TMS	Aqueous extraction, ultrafiltration, precipitation by ionic-exchange chromatography, low temperature precipitation, centrifugation and lyophilization	HP-5MS capillary column (30 m × 0.25 mm ID)	T programme: 250–280°C Carrier gas: Helium	Lopez-Molina et al. (2005)
FOS, GOS, malto- and isomaltooligosaccharides in food products	TMSO	Extraction with water Foods containing fats: hexane and centrifugation	Al-clad capillary column coated with 5% phenyl polycarborane-siloxane (6 m × 0.25 mm ID)	T programme: 105–440°C Carrier gas: Helium	Joye and Hoebregs (2000)
Oligosaccharides (DP up to 7) in foods (FOS and GOS)	TMSO	Dilution with methanol and centrifugation	CP-SIL 5CB capillary column (8 m × 0.25 mm ID × 0.25 μm)	T programme: 130–360°C Carrier gas: Nitrogen	Montilla et al. (2006)
		Diafiltration of FOS	HT5 capillary column (12 m × 0.32 mm ID × 0.1 μm)	T programme: 130–440°C Carrier gas: Nitrogen	
Soybean oligosaccharides (raffinose, stachyose, verbascose and maltooligosaccharides)	Methyl alditols	Water extraction and clean up with chloroform/ethanol	GC: HT SE-54 capillary column (25 m × 0.32 mm ID × 0.05 μm)	T program (GC): 40–400°C T program (GC-MS): 70–390°C	Carlsson et al. (1992)
			GC-MS: PS264 (10 m × 0.25 mm ID × 0.02 μm)	Carrier gas: Helium	

Table 13.3

Carbohydrates	Derivatives	Pretreatment	Column and dimensions	Cromatographic conditions	References
Pea oligosaccharides (raffinose, stachyose, verbascose)	Trifluoroacetates and TMS	80% aqueous methanol and membrane filtration	DB5–60W capillary column (10 m × 0.32 mm ID × 0.25 μm)	T programme: 188–316°C and 80–250°C Split ratio: 1:50,1:100 and 1:150 Carrier gas: Helium	Karoutis et al. (1992)
Polydextrose	Methyl alditols	Ultrafiltration	SA-5 capillary column coated with 5% diphenyl-95% dimethylpolysiloxane (30 m × 0.25 mm ID)	EIMS: to prove the identity of the carbohydrate derivatives NH_3-CIMS: determination of the molecular weight	Stumm and Baltes (1997)
D-Fructofuranosyl residues of D-Fructans of different sources	Permethylated derivatives		10% SP2401 (1.83 m × 3.18 mm); (3.66 m × 3.18 mm); 3% OV-225 (2.68 m × 3.18 mm); SE-30 (1.83 m × 3.18 mm)	Different temperatures	Rolf and Gray (1984)
Honey oligosaccharides	TMSO	Nanofiltration, yeast (Saccharomyces cerevisiae) treatment, and Adsorption onto activated charcoal	DB1 capillary column (25 m × 0.25 mm ID × 0.25 μm)	T programme: 200–300°C Carrier gas: Nitrogen	Sanz et al. (2005)

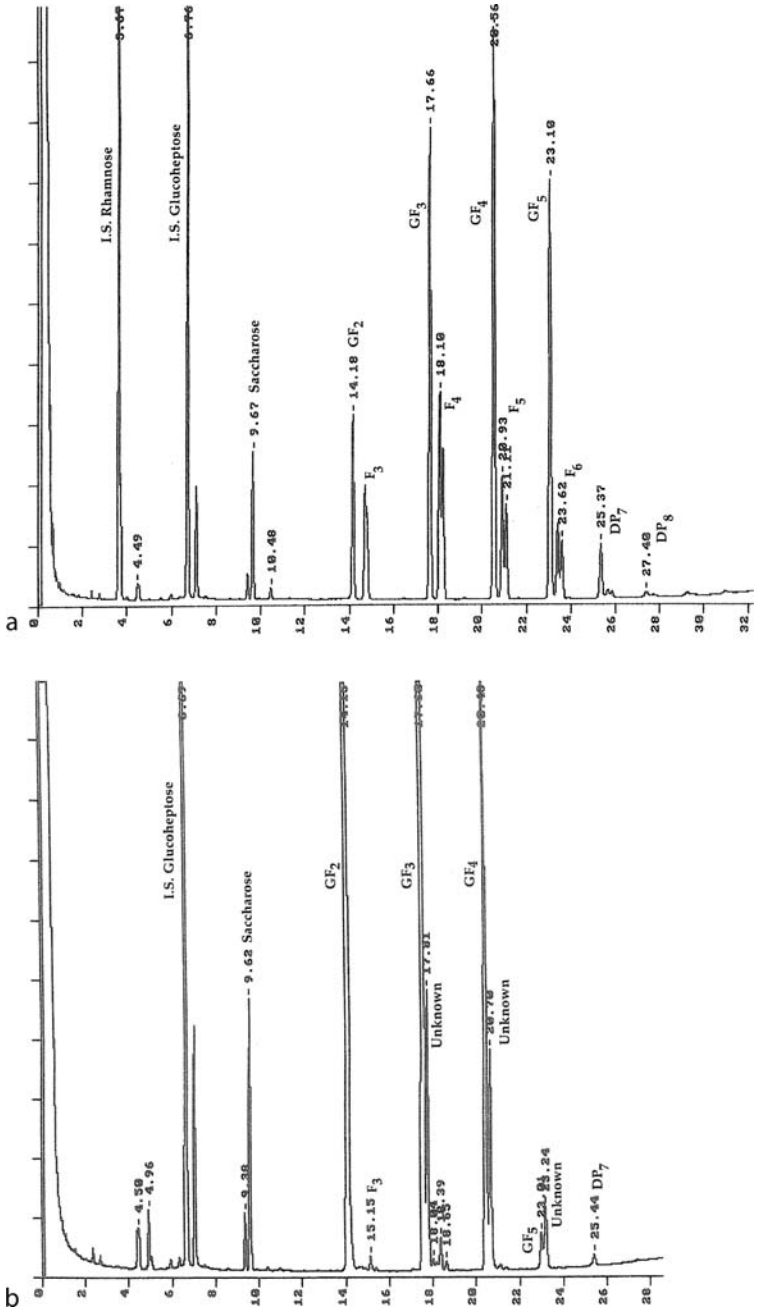

Figure 13.3 (Continued)

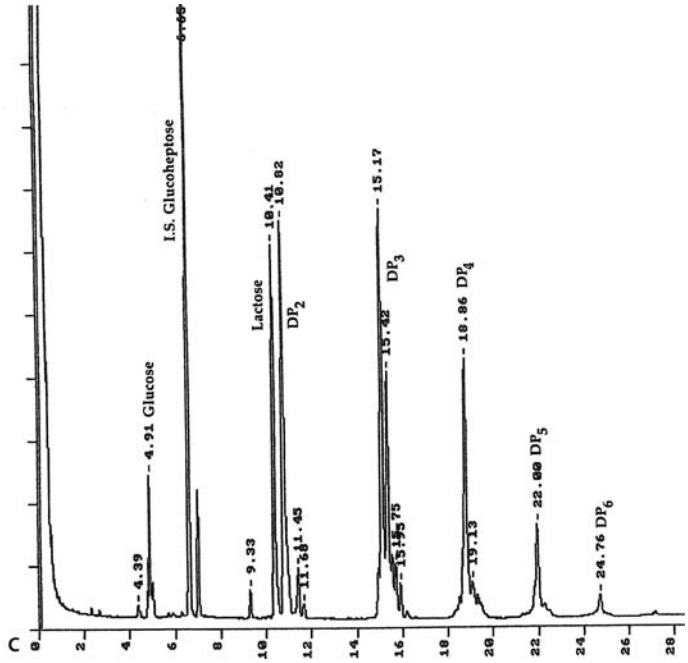

Figure 13.3

GC analysis of FOS and GOS. GC profile of Raftilose P95 X (a), Actilight (b) and galacto-oligosaccharides (c). From Joye and Hoebregs (2000) with permission from AOAC International.

Oligosaccharides up to DP7 were determined in foods and in various pure mixtures using conventional GC (Montilla et al., 2006). The accuracy, repeatability and reproducibility of the method were similar to the results obtained with HT-GC method from Joye and Hoebregs (2000).

Carlsson et al. (1992) developed an HT-GC methodology for the quantitative analysis of oligosaccharides in foods, diets and intestinal contents. Methylation was also performed for the identification of these oligosaccharides by GC-MS, which was able to analyze sugars up to 12 sugar units. Red lentils, soybeans, rapeseed, mung beans and chickpeas were found to contain considerable amounts of the raffinose family of oligosaccharides.

Multiple applications can be found for the structural analysis of oligosaccharides. As an example, the studies about structural determination of D-fructans (Rolf and Gray, 1984) and different oligosaccharides produced from alternansucrases (Côté and Sheng, 2006) can be pointed out. The potential prebiotic properties of these last oligosaccharides are still under study (Sanz et al., 2006).

13.2.5 Capillary Electrophoresis

The high speed of analysis, the minute amounts of analyte required and the high resolution make CE an attractive and powerful microanalytical technique to separate a wide range of charged and uncharged compounds. It is a suitable analytical tool for the analysis of foods and beverages and also has been successfully applied in other fields, such as biochemistry, biotechnology and clinical chemistry (Soga and Serwe, 2000). The advantages of CE over other traditional chromatographic methods include the extremely simple operation and the low consumption of sample and buffers (Bao and Newburg, 2008). The main drawback is the lack of sensitivity when low concentration levels are present.

13.2.5.1 Operation Modes

CE instrumentation includes a high voltage power supply (5–30 kV), buffer reservoirs, a narrow-diameter (50–100 μm) capillary, an automated sampler and detector. Separation is based on migration of compounds in narrow capillaries (length of 0.5–1.5 m) made of fused silica. The two ends of the capillary are immersed in two separated electrolyte reservoirs containing a high voltage electrode.

The mobility of analytes under an electric field depends on several factors, including the analyte charge (neutral, positive or negative), charge to mass ratio, buffer system (pH and ionic strength), presence of buffer additives (surfactants, ion-pairing agents, complexing agents), voltage applied, temperature inside of capillary, length and diameter of the capillary, and nature of the capillary wall (Bao and Newburg, 2008).

Samples can be directly analyzed with minimal sample preparation without a loss of separation performance. After detection of peaks, the hollow capillary is flushed with fresh buffer and is ready for the next injection (Soga and Serwe, 2000).

There are different operation modes of CE: capillary zone electrophoresis (CZE), capillary gel electrophoresis (CGE), micellar electrokinetic chromatography (MEKC), capillary isolectric focusing (CIEF) and capillary isotachophoresis (CITP). In all cases, the separation is achieved due to differences in migration of different solutes, on chosen electrolyte and capillary tube, under an applied electric field.

The most commonly used modes in the analysis of carbohydrates are CZE and MEKC. CZE is also called free solution electrophoresis, and the separation is based on the differences in the charge-to-mass ratio. MEKC is a pseudochromatographic mode of CE whereby separation and analysis of neutral molecules occurs through inclusion into micelle-forming detergents added to the electrophoretic medium (Cheung et al., 2007).

CE has emerged as an alternative to current analytical techniques for carbohydrates. However, carbohydrates analysis can present difficulties such as lack of electric charge and absence of chromophoric/fluorophoric groups in the analyte molecules. To overcome these limitations different procedures have been developed.

Taking into account that sugars are very weak acids (have high ionization constant, pK_a values of 12), different methodologies have been established to ionise them. Electrolyte systems based on borate complexation, metal complexation or highly alkaline pH (e.g., NaOH) have been used. The complexation of carbohydrates with borate-based electrolytes to impart the necessary charge for electrophoresis is the most widely used approach for CE separation of derivatized and underivatized sugars (E-l Rassi, 1999).

13.2.5.2 Detection

Derivatized Carbohydrates

Carbohydrates have high ionization constants (pK_a values of 12 or higher), and therefore they do not carry electrical charges at neutral pH. This, along with the fact that carbohydrates do not absorb UV light above 200 nm hinders its analysis by CE. To overcome both problems different procedures have been developed, such as derivatization with direct detection, using chromophore and fluorescent probes carriers of electrical charges, to facilitate detection via UV-VIS absorbance or laser-induced fluorescence (LIF). However, although derivatization methods lead to improve sensitivity and resolution, several drawbacks are often encountered, such as control problems due to a different reactivity of derivatizing reagents for analytes, formation of several adducts, etc. (Lee and Lin, 1996).

Similarly to HPLC, derivatization of reducing carbohydrates in CE is often performed by reductive amination, between the reducing end and an amino group of the tag reagent, using amines with strong chromophores or fluorophores such as 4-amino benzoic acid and its ethyl ester, 2-aminobenzoic acid, 4-aminobenzonitrile, 2-aminopyridine, etc. (Andersen et al., 2003). A large

variety of derivatization reagents have been suggested for carbohydrate analysis (Campa et al., 2006; Cortacero-Ramirez et al., 2004).

Underivatized Carbohydrates

Indirect Detection An alternative methodology that allows CE analysis of underivatized carbohydrates includes the use of highly alkaline electrolytes, to ionize and ensure an electrophoretic mobility of the saccharides towards the anode and make them suitable for indirect UV detection using chromophore compounds and modifiers (background electrolyte; BGE) to reverse the direction of electroosmotic flow inside the capillary and promote the co-migration of the analytes (Jager et al., 2007; Soga and Serwe, 2000).

Electrochemical Detection Ionization of carbohydrates at high pH values also allows CE analysis with electrochemical detection using copper or gold electrodes. It is an interesting approach because the hydroxyl groups can be partially ionized, which in turn permits their effective separation in CZE mode. Cao et al. (2004) described a simple, reliable and reproducible CE method using NaOH (50 mmol/L) as running buffer and amperometric detection to quantify mono- and disaccharides in rice flour. The detector is composed by an electrode cell system consisting of copper working electrodes, platinum auxiliary and reference electrodes. Likewise, Chu et al. (2005) developed a miniaturized CE method and amperometric detection, with an electrode of copper, to quantify carbohydrates in soft drinks.

Complexation of alternate hydroxyl groups with borate and electrochemical and amperometric detection is another alternative for CE analysis of carbohydrates without derivatization (Cheung et al., 2007).

Refractive Index Detection Refractive index detection has been successfully used in CE to separate carbohydrates considering that they do not possess chromophore groups in their structures. Although the detection limits are relatively low, this type of detector could be used as universal detector in CE of carbohydrates (El Rassi, 1999).

13.2.5.3 Coupling with MS

The coupling of CE–MS can provide important advantages in food analysis because of the combination of the high separation capabilities of CE and the

power of MS as identification and confirmation method (Simó et al., 2005). A detailed review has been written by Campa et al. (2006) which describes the advances in CE-MS of carbohydrates.

13.2.5.4 Applications

Besides the difficulties discussed above with the analysis of carbohydrates by CE, it is also necessary to consider the different structures resulting from heterogeneity in primary sequence, branching and the variety of structural isoforms of oligosaccharides. In order to address these challenges several studies have been focused on finding different separation methods. In this section a description of different CE methodologies found in the literature to analyze oligosaccharides is presented.

Human Milk Oligosaccharides (HMOS)

The difficulty of HMOS analysis is not only due to the low and variable (depending on lactational stage) content but also to the complexity of their structures. Therefore, it is necessary to arrange appropriate and powerful analytical methods to achieve efficient separation and quantification of oligosaccharides and their structural isomers.

Acidic oligosaccharides are not intrinsically strong chromophores, however they can absorb in the low UV range due to the aminoacyl moieties and sialic acid present in the structures of many HMOS (Bao and Newburg, 2008).

Underivatized HMOS

Shen et al. (2000) developed a very reproducible and sensitive CE method (fmol level) for underivatized acidic oligosaccharides with detection by UV absorbance at 205 nm. Eleven oligosaccharides of human milk, ranging from tri- to nonasaccharide (3′-sialyllactose, 6′-sialyllactose, 3′-sialyllactosamine, 6′-sialyllactosamine, disialyltetraose, 3′-sialyl-3-fucosyllactose, etc.) were resolved by MEKC. These oligosaccharides were detected in pooled human milk samples, from different donors, and comparison of oligosaccharides profiles revealed an extensive variation in the structural isomers of sialyllacto-N-tetraose. The running conditions were selected as the best compromise between resolution and running time.

The resolution of structurally similar oligosaccharides, especially those containing chemically labile sialic acid residues is a challenging problem. Shen et al. (2001) employed a CE method and UV detection (205 nm) to separate three sets of structural isomers of sialylated oligosaccharides in human milk and bovine

colostrum. They developed conditions for baseline resolution of specific sets of isomers within a 35 min run. Each set of structural isomers of sialylated oligosaccharides, 3'-silayllactose/6'sialyllactose, sialyllacto-N-tetraose-a (linear), -b (branched) and -c (linear), required a unique running buffer with respect to buffer type, concentration, pH, presence of organic modifiers, and surfactants.

Likewise, Bao et al. (2007) developed a novel method to quantify sialyloligosaccharides from human milk by MEKC and UV detection at 205 nm. As running buffer, they used aqueous 200 mM sodium phosphate (pH 7.05) containing 100 mM sodium dodecyl sulfate (SDS) mixed with 4% (v/v) methanol. The method describes new CE conditions that simultaneously resolve not only separation between pairs of structural isomers of HMOS, 3'-sialyllactose/6'sialyllactose and sialyllacto-N-tetraose-a, -b and -c, but also quantification of the 12 major sialyloligosaccharides of human milk in a single 35 min run. *Figure 13.4* shows a CE separation of sialyloligosaccharide standards and HMOS found in colostrum (b) and in human milk (c). The method allowed finding differences in sialyloligosaccharide concentrations between less and more mature milk from same donors. It is possible to define acidic oligosaccharide expression in milk as function of stage of lactation, genetic variation among lactating mothers, diet, diurnal variation, stress, disease, and geographic origins of a population.

Derivatized HMOS

Major neutral oligosaccharides from human milk, such as 2′-fucosyllactose (2′FL), 3′-fucosyllactose (3′FL), lacto-N-tetraose (LNT), lacto-N-fucopentaose I (LNFP I), lacto-N-fucopentaose II (LNFP II) and fucose, have been quantified by CE using LIF as detection system (λ_{exc} = 488 nm; λ_{em} = 520 nm). Oligosaccharides were derivatized via reductive amination with 2-aminoacridone (AMAC). The CE method allowed to resolve two sets of structural isomers, 2′FL/3′FL and LNFP I/LNFP II. This was rapid, sensitive (2 fmol) and reproducible, and required a simple sample preparation (Song et al., 2002).

Schmid et al. (2002) analyzed using MEKC free oligosaccharides from human milk. They used as derivatization agent various esters of aminobenzoic acid and sodium phosphate (20 mM), pH 7.0, and sodium dodecyl sulfate (SDS) (50 mM) as buffer. Oligosaccharide was detected by UV absorbance at 285 and 310 nm. Previous to their analysis, samples were submitted to a simple clean up of deproteination and defatting before derivatization of oligosaccharides. Major human milk oligosaccharides were detected (FL, LNT, LNFP, lacto-N-difucohexaose).

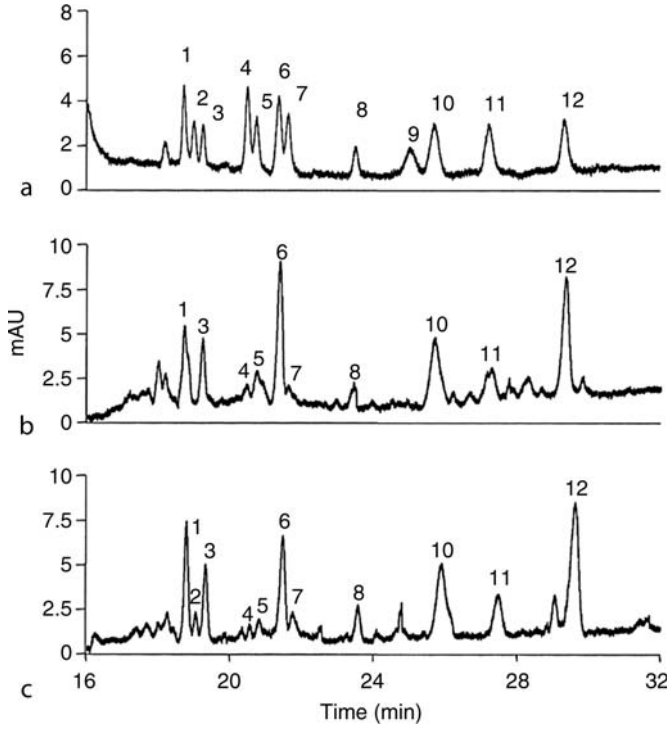

Figure 13.4

CE analysis of sialyloligosaccharides, colostrum sample and mature milk sample. Electrophoregrams of sialyloligosaccharides from a mixture of 12 standard sialyloligosaccharides (a), a colostrum sample (b) and a mature milk sample (c) from a mother in the Boston area. (1) MSMFLNnH (monosialyl, monofucosyllacto-N-neohexaose; (2) MSLNnH I (monosialyllacto-N-neohexaose); (3) MFMSLNH I (monofucosyl, monosialyllactose-N-hexaose); (4) SLNFP II (sialyllacto-N-fucopentaose); (5) SLNT b (sialyllacto-N-tetraose); (6) SLNT c; (7)SLNT a; (8) DSMFLNH (disialyl, monofucosyllacto-N-hexaose); (9) 3′-S-3FL (3′ sialyl-3-fucosyllactose); (10) -6′-SL (6′ sialyllactose); (11) 3′-SL (3′ sialyllactose); (12), DSLNT (disialyllacto-N-tetraose). From Bao et al. (2007) with permission from Elsevier.

Galactooligosaccharides

There is scarce literature on CE methods to analyze GOS. Petzelbauer et al. (2006) separated and quantified the major GOS obtained during lactose conversion at 70°C, catalyzed by β-galactosidases from the archea *Sulfolobus solfataricus* and *Pyrocccus furiosus*. Carbohydrates were analyzed using as running buffer phosphate pH 2.5, derivatized using an aminopyridine solution and detected by UV (240 nm). The authors identified two disaccharides β-D-Gal*p*-(1→3)-D-Glc and β-D-Gal*p*-(1→6)-D-Glc (allolactose); and two trisaccharides

β-D-Gal*p*-(1→3)-lactose and β-D-Gal*p*-(1→6)-D-lactose. As minor compound, β-D-Gal*p*-(1→6)-D-Gal was also identified.

Total GOS and di-, tri-, and tetrasaccharides, derived from lactose hydrolysis with β-galactosidases of *Lactobacillus reuteri* L103 and L461, have been also quantified by CE (Splechtna et al., 2006). The method includes a pre-column derivatization with 2-aminopyridine and detection of derivatives by UV-diode-array detector (DAD). The running buffer was 100 mM phosphoric acid, pH 2.5. The sugars eluted in groups depending on their degree of polymerization and the identified GOS were the same found by Petzelbauer et al. (2006); *Figure 13.5*

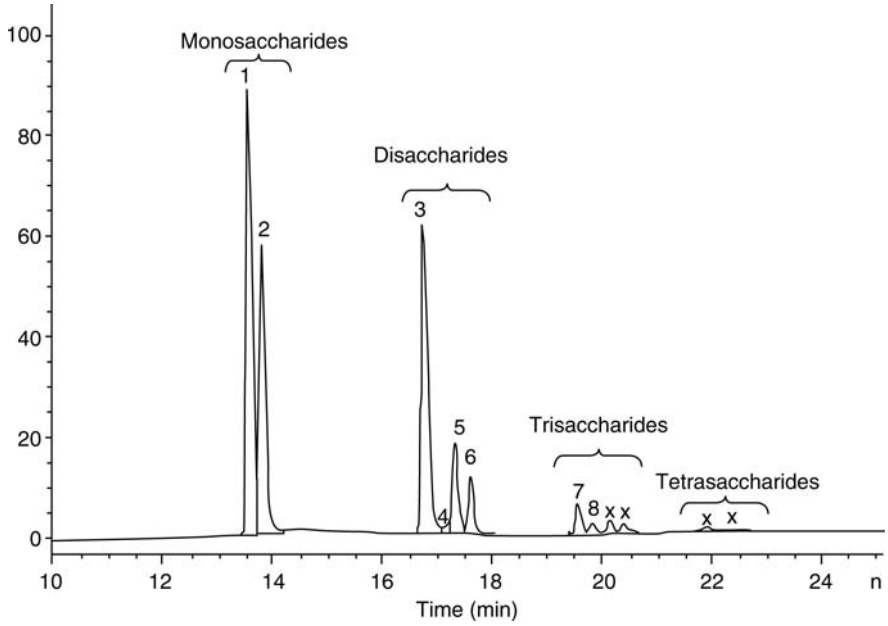

Figure 13.5

CE analysis of GOS. Separation and quantification by capillary electrophoresis of individual GOS produced during the lactose conversion catalyzed by L103 or L461 β-galactosidase. The sample presents a mixture of sugars obtained after the reaction of L103 β-Gal with 205 g/L lactose. The extent of substrate conversion is approximately 67%. The identified compounds are indicated: (1) glucose, (2) galactose, (3) lactose, (4) D-Gal*p*-(1→3)-D-Glc, (5) D-Gal*p*-(1→6)-D-Glc (allolactose) with D-Gal*p* D-Gal*p*-(1→3)-D-Gal, (6) D-Gal*p*-(1→6)-D-Gal, (7) D-Gal*p*-(1→6)-Lac, and (8) D-Gal*p*-(1→3)-D-Lac. Products marked with an x are minor components and were not identified. Peaks appearing at ~ 22 min are tetrasaccharides. From Splechtna et al. (2006) with permission from American Chemical Society.

shows the CE separation attained. Likewise, a quantification of the resulting GOS mixtures obtained from recombinant β-galactosidase of *Lactobacillus reuteri* on lactose hydrolysis has been performed by CE (Maischberger et al., 2007). A comparative study on GOS production was carried out using lactose solutions and whey permeate; and although the initial reaction rate was higher for the latter GOS, the yield was slightly lower.

Lactulose

CE can be used to separate lactulose from mixtures of carbohydrates (Vorndran et al., 1992). This allows a rapid and sensitive analysis of underivatized carbohydrates with indirect UV detection using 6 mM sorbic acid (pH 12.1) as both carrier electrolyte and chromophore. CE analysis has been used to detect carbohydrates in their original form by means of indirect photometry (Oefner et al., 1992). However, direct UV detection of saccharides derivatized pre-column with 2-aminopyridine, ethyl *p*-aminobenzoate or *p*-aminobenzoic acid, allowed a reproducible determination of aldoses and ketoses in fmol range (Oefner et al., 1992).

Underivatized lactulose along with other carbohydrates has been analyzed by CE and amperometric detection using a copper microelectrode. The separation of sugars has been performed in strongly alkaline solutions (LiOH, KOH and NaOH) at pH 13. Among the three studied reagents, the NaOH solution offered good resolution with a suitable time of analysis and it was employed as separation electrolyte. The method is simple, sensitive, and, relatively easy to implement (Colon et al., 1993).

Different CE methods have been used to determine lactulose content in heated milks; thus Guingamp et al. (1999), using an indirect UV detection at 254 nm and sorbate pH 12.04 as running buffer, evaluated heat load of commercial milks. Afterwards, Humbert et al. (2007), by improving milk sample preparation, they determined the lactulose content in pasteurized, indirect and direct UHT, and in-bottle sterilised milk by CE. Lactulose in milk samples was also measured by HPLC and an enzymatic method. The authors found a good correlation between the three methods.

Determination of lactulose along with mannitol is a highly sensitive test for the screening of the diseases that affect intestinal permeability. Paroni et al. (2006) set up a method by CE with indirect UV detection (254 nm) and sorbate, cetyltrimethylammonium bromide and LiOH as background electrolyte to estimate the lactulose-mannitol intestinal permeability in a cohort of patients with type I diabetes.

Fructooligosaccharides (FOS)

FOS are probably the most commonly used prebiotic fibers in the production of functional foods. An evaluation of prebiotic character of FOS has been carried out by Corradini et al. (2004) through short chain fatty acid (SCFA) measurement using CE.

α-Galactosides

The raffinose family of oligosaccharides (ROS) are composed of α-(1→6) galactosides bound to sucrose at C-6 of the glucose. By successive binding of one, two and three additional α-galactoside units to C-6 of the terminating galactose unit, the compounds stachyose, verbascose and ajugose are formed (Andersen et al., 2003). These oligosaccharides are decomposed in the large intestine causing unpleasant effects. However, these effects have been counterbalanced by an increasing interest in non digestible oligosaccharides as functional food ingredients.

Since α-galactosides are non-reducing oligosaccharides, borate complex formation seems to be a promising analytical methodology to analyze them. Arentoft et al. (1993) optimized a high performance capillary electrophoresis (HPCE) method to quantify ROS (raffinose, stachyose and verbascose) based on the formation of borate-carbohydrate complexes and UV detection at 195 nm. Pea seed samples were submitted to a simple extraction procedure and a purification step prior to the determination of individual oligosaccharides. This method could be adapted for the determination of other low-molecular-mass carbohydrates.

Frias et al. (1996) also quantified the ROS family by CZE using disodium tetraborate as running buffer; high-quality electrophoregrams were obtained due to a purification step of pea seeds samples, using Sep-Pak C_{18} cartridges. The ROS family was also quantified by HPAEC-PAD; both methods showed a good linearity and reproducibility and did not show significant differences.

Using indirect UV detection, Andersen et al. (2003) also quantified the ROS family by HPCE. The signal wavelength was set at 350 nm with a reference at 275 nm. As background electrolyte they used pyridine-2,6-dicarboxylic acids, sodium borate decahydrate ($Na_2B_4O_710H_2O$) and hexadecyltrimethylammonium bromide, adjusted to varying pH values (8.0–10.0). The method was applied for the quantification of α-galactosides in a lupine seed sample (*Lupinus angustifolius*) after extraction and purification.

Other Nondigestible Oligosaccharides

There are other oligosaccharides, the so-called "second generation of prebiotics" which could present new physical and chemical properties and different and more specific bioactivities (Joucla et al., 2004).

Homologous of glycoglucans of isomaltose and *Laminaria* have been analyzed by CZE using as running buffer 200 mM borate buffer (pH 9.5) and UV detection at 245 nm. Oligosaccharides were pre-column derivatized with 3-methyl-1-phenyl-2-pyrazolin-5-one (MPP). A good separation was achieved for each oligosaccharide series, which have various types of glycosidic linkages (Honda et al., 1991).

Oligosaccharides derived from partial hydrolysis of dextran were satisfactorily separated by HPCE using coated capillary with a copolymer of hydroxypropylcellulose and hydroxyethyl metacrylate. The running buffer was 100 mM tris-borate buffer, pH 8.8. Derivatization of oligosaccharides was performed using N-(4-aminobenzoyl)-L-glutamic acid and UV detection (Plocek and Novotny, 1997).

Also, efficient electrolyte systems for underivatized carbohydrates based on co-electroosmotic CE can be useful for the separation of derivatized counterparts. A selective separation of derivatized (reductive amination) carbohydrates (xylose, cellobiose, melibiose, maltotetraose, gentiobiose, etc.) using ethyl *p*-aminobenzoate or ethyl *p*-aminobenzonitrile can be obtained using as running buffer electrolyte borate and an organic solvent. Co-directional migration of the anionic analytes with the electroosmotic flow (EOF) was achieved by adding a cationic polymer (hexadimethrine) bromide (HDB). The carbohydrates were detected by UV (280 nm) and the method was applied to the analysis of carbohydrates of plant hydrolyzates (Nguyen et al., 1997).

A comparative study of chromophore response (CZE-UV) and electrochemical signal (HPAEC-PAD) of some model gluco-oligosaccharides (dextrans) with different DP has been carried out. UV detection was performed using 8-aminonaphtalene-1,3,6,-trisulphonic acid (ANTS) as chromophoric dye. Both methods provided similar response for DP 1,000 and 5,000 dextrans (Abballe et al., 2007).

CE with LIF (excitation at 488 nm and emission at 520 nm) and ESI-MS detection has been used to characterise gluco-oligosaccharide regioisomers synthesised by *Leuconostoc mesenteroides* NRRL B-512F with a DP ranging from 2 to 9 (Joucla et al., 2004). Resolution of APTS (9-aminopyrene-1,4,6-trisulfonate) derivatives of gluco-oligosaccharide regioisomers over a wide DP range is more appropriately performed with borate buffer systems (Joucla et al., 2004). The use of combined methods looks promising for profiling mixtures of gluco-oligosaccharides synthesised by glucansucrases.

Xyloglucans belong to the groups of hemicelluloses and are constituted by a β(1→4) linked glucan chain to which different short side chains are attached.

An unambiguous letter code is used for the nomenclature of each segment depending on the side chain, thus they can be classified in few types of structure: XXXG (X = xylose; G = glucose); XXGG and XXXGG. HPAEC-PAD, RP-HPLC and CE (LIF and ESI-MS detection) methods have been compared to determine xyloglucan structures in blackcurrants (Hilz et al., 2006). For CE xyloglucan oligosaccharides were labeled with APTS, separated on a polyvinyl alcohol (N-CHO) coated capillary and detected by LIF (λ_{exc} = 488 nm-λ_{em} = 520 nm). Before analysis, samples were submitted to different extraction steps and xyloglucan material was hydrolyzed with a specific endo-glucanase. The method allowed the identification the structures of xyloglucans as well as the quantification of the main oligomer as XLFG (L = galactose; F = fucose-galactose) present in black currant.

Structural isomers of short oligosaccharides have been also analyzed by CE. In this case, separation of oligosaccharides derived from maltose, cellobiose, xylobiose, and isomaltose has been performed using lithium acetate (pH 5) as running buffer. Oligosaccharides were derivatized using APTS and detected by LIF using wavelengths of excitation and emission of 488 and 520 nm, respectively. The method was applied to the analysis of structural isomers of short oligosaccharides in various plant substrates, and a baseline resolution of three different galactobioses isoforms β (1→4), α (1→4) and α (1→3) was obtained (Khandurina and Guttman, 2005).

13.2.6 Mass Spectrometry

The analysis of prebiotic carbohydrates by different analytical techniques coupled to MS has been reviewed in previous sections. However, a specific mention to this technique has to be done, considering the large number of reported applications where carbohydrates are analyzed directly by MS. Direct infusion ESI and MALDI are the most common ionization sources employed for this purpose. These techniques in combination with tandem MS analyzers have been used to solve structural problems of carbohydrates (Harvey, 1999). Although separation of isomeric oligosaccharides is not possible by MS, identification of their structures based on their different fragmentation patterns has been achieved. Nevertheless, it is not feasible yet to determine the structure of an oligosaccharide just by the study of these patterns. Comparisons to several reference carbohydrates is necessary. Moreover, isomeric oligosaccharides give rise to fragments at the same m/z values, and differences can be observed only in their abundances. Kurimoto et al. (2006) developed a quantitative procedure by quadrupole ion trap (QIT) to solve these problems. Detailed information about

MS of oligosaccharides can be obtained from an exhaustive review written by Zaia (2004), which includes the ionization methods and the mass analyzers generally used for oligosaccharide analysis.

13.2.6.1 Electrospray Ionization

Samples are introduced by direct infusion into the ESI ion source. As indicated above, different analyzers can be coupled to ESI, Q and IT being the most common ones. Nevertheless, in the last years tandem analyzers such as QToF, QIT or triple quadrupole (QqQ) are gaining a significant acceptance and becoming more widespread. While ESI produces a soft ionization the quasi-molecular ion which allows to determine the molecular weight of the analyte, its coupling to IT, QIT, QToF, QqQ or Fourier-transform ion cyclotron resonance (FT-ICR), provides higher structural information by the generation of MS/MS and MS^n. Collision-Induced Dissociation (CID) is the most common method of fragmentation, although other methods such as electron transfer dissociation (ETD), electron capture dissociation (ECD) and infrared multiphoton dissociation (IRMPD), can be applied. In CID the precursor ion is submitted to repeated collisions with a gas and product ions are formed, whereas with IRMPD, photon energy is imparted on both precursor and product ions, resulting in a higher fragmentation (Seipert et al., 2008). ECD and ETD induce fragmentation of positive ions by electron transfer. IT and FT-ICR allow a higher control over CID operation, the possibility of obtaining MS^n and low energy reactions, while QqQ and Q-ToF produce more fragmentation from CID and less operator control, and only MS/MS can be performed.

Table 13.4 shows some recent applications of ESI MS to the analysis of oligosaccharides.

XOS have been analyzed by ESI MS both on positive and negative modes. Whereas the positive ESI MS allowed the identification of neutral and acidic XOS, the negative mode results in simpler MS (Reis et al., 2003a) since there is a lower adduct formation and only acidic XOS ions appear. Isomeric structures of a mixture of arabinoxylooligosaccharides (AXOS) have been also differentiated by analysing their permethylated derivatives by ESI-IT MS upon CID (Matamoros-Fernández et al., 2003); however, the direct analysis of these oligosaccharides using a Q-TOF or IT did not allow the distinction between linear and branched structures.

Table 13.4

Some direct infusion ESI-MS applications for the analysis of prebiotics

Oligosaccharides	Treatment of the sample	Analyzer	Ionization method	Reference
AXOS	Permethylation	IT	CID	Matamoros-Fernández et al. (2003, 2004)
CEOS, MOS, XOS	Addition of ammonium acetate or alkali metal salts	Q-IT FT-ICR	CID	Pasanen et al. (2007)
MOS, CEOS, IMOS	Pyridylamination	Q-IT	CID	Kurimoto et al. (2006)
Sulfated human milk oligosaccharides	Pyridylamination	QqQ	–	Guerardel et al. (1999)
Human milk oligosaccharides	–	Q-ToF	CID	Kogelberg et al. (2004)
XOS		Q-ToF	CID	Reis et al. (2003a)

Fragmentation of cello- (CEOS), malto- (MOS) and xylooligosaccharides (XOS) has been recently studied by ESI MS coupled to QIT and FT-ICR (Pasanen et al., 2007). The effect of different precursor ion types (deprotonated, protonated, ammoniated and alkali metal cationized precursors) and carbohydrate structure (α or β configuration and presence of hexose or pentose units) on the fragmentation of these carbohydrates in CID was evaluated. As an example, *Figure 13.6* shows the scheme of fragmentation of a trisaccharide (*Figure 13.6a*) and the different spectra obtained for deprotonated cellopentaose (*Figure 13.6b*), maltopentaose (*Figure 13.6c*) and xylopentaose (*Figure 13.6d*). Both CEOS and MOS showed similar fragments (A, B and C), however A fragments were more abundant in CEOS. In contrast, the behavior of XOS was completely different; A fragments were the most abundant, and the intensity ratios of C fragments were clearly different from those of CEOS and MOS. In this example, the different fragmentation observed depended both on the anomeric configurations of the glycosidic linkage and on the presence of monosaccharide units. Results of this work also confirmed that the structural information obtained from the CID of oligosaccharides was dependent on the precursor ion type.

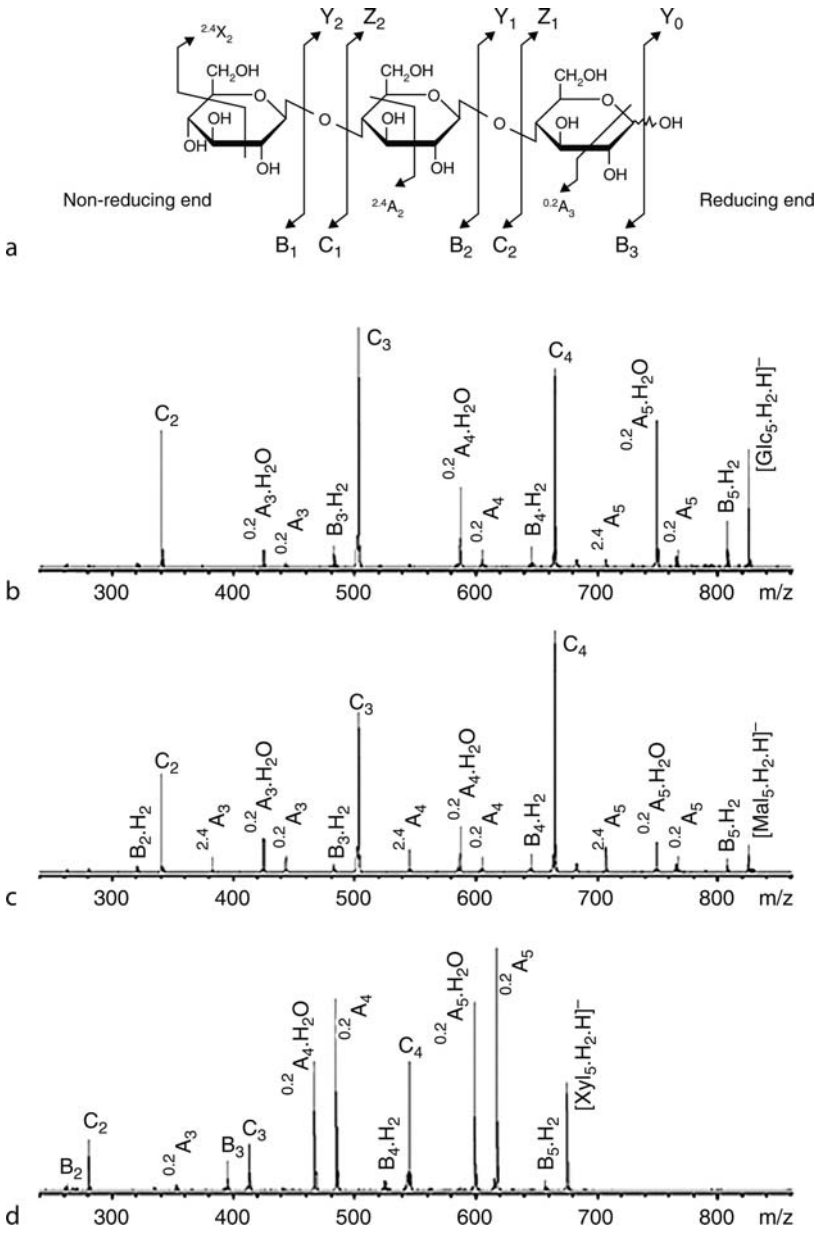

Figure 13.6

CID ESI MS analysis of cellopentaose, maltopentaose, and xylopentaose. Nomenclature for oligosaccharide fragments illustrated for cellotriose (a). CID spectra for deprotonated (b) cellopentaose, (c) maltopentaose, and (d) xylopentaose at a fragmentation amplitude of 0.4 V. From Pasanen et al. (2007) with permission from Elsevier.

13.2.6.2 Matrix-Assisted Laser Desorption/Ionization

An extensive and well written review about the application of MALDI MS to carbohydrate analysis which covers the period 1991–1998 has been reported by Harvey (1999). Although in this section some basic points will be referred to the mentioned study, most recent applications of prebiotic analysis will be discussed.

In MALDI, the sample is mixed with a matrix, allowed to crystallize by evaporation of the solvent and submitted to the laser whose energy is absorbed by the matrix and transferred to the carbohydrate which is ionized.

Nitrogen lasers emitting in the UV at 337 nm are those most commonly used for MALDI analysis, although other lasers that emit in the infrared (IR) have been also assayed.

It has been observed that ionization efficiency by MALDI is similar for neutral carbohydrates of different molecular weights, while efficiency of ESI decreases for carbohydrates of higher DPs (Harvey, 1999). The high sensitivity of MALDI allows the detection of oligosaccharides at picomole levels (Morelle and Michalski, 2005).

MALDI is generally coupled to a time of flight (ToF) analyzer resulting in high sensitivity, because most ions generated by the laser are recorded by the detector (Harvey, 1999), although couplings to other analyzers such as IT (Qin et al., 1996), FT-ICR (Carroll et al., 1996) ToF/ToF (Spina et al., 2004) or Q-ToF (Morelle and Michalski, 2005) have been also described. These couplings provide high-mass accuracy, high resolution, and the possibility of performing multiple methods of tandem MS which can be used to obtain higher structural and complementary compositional information. MALDI-FT-ICR has been recently used to evaluate the consumption of human milk oligosaccharides (Ninonuevo et al., 2007) and FOS (Seipert et al., 2008) by intestinal bacteria.

Harvey (1999) in his review exhaustively explained the different matrices that can be used for the MALDI analysis of free neutral carbohydrates, free acidic carbohydrates, sulfated carbohydrates and glycoproteins. Most recognized prebiotics are neutral carbohydrates, which are commonly analyzed by MALDI using 2,5-dihydroxybenzoic acid (DHB), although other matrices such as 2′,4′,6′-trihydroxyacetophenone (THAP) or mixtures of DHB with different compounds have been also proposed by several authors to obtain finer crystals. One of the problems of DHB matrix is the appearance of multiple matrix peaks at low masses which could interfere with low molecular weight carbohydrates. Therefore, molecular weight of target oligosaccharides should be considered when selecting

a matrix. A good repeatability of spot-to-spot or sample-to-sample, a good crystallization and the production of a spectrum with good signal to noise ratio and good resolution should be also be taken into account for matrix selection (Wang et al., 1999).

Qualitative analyses of prebiotic carbohydrates by MALDI-ToF have been widely carried out (Huisman et al., 2001; Lopez et al., 2003; Reis et al., 2003b; Sanz et al., 2006), however quantitative applications are less common (Wang et al., 1999) since they present difficulties associated to a poor shot-to-shot repeatability and to the crystal's lack of homogeneity. Quantification is normally carried out using an internal standard calibration method and it is desirable in order to obtain single alkali ion adduct peaks to gain peak intensity. Wang et al. (1999) quantitatively analyzed FOS using γ-cyclodextrin as internal standard and KCl to obtain single potassium adduct peaks. However, one of the drawbacks of this technique is the variability of ionization regarding the sample. The medium can show a significant effect on MALDI analysis (Reiffová et al., 2007). A previously proposed method to analyze FOS was used by Wang et al. (1999) to determine FOS content in food extract. Nevertheless, these extracts (i.e., from red onions) suppressed the ions produced from the internal standard although FOS could be detected. Therefore, internal standard should be selected depending on the sample to be analyzed, however, commercial standards are scarce and most of them (such as maltodextrins) posses similar molecular weight to the analytes giving the same response. Seipert et al. (2008) used deuterated reduced maltoheptaose to distinguish its masses from those of FOS with the same molecular weight.

Different comparative studies of prebiotic analyses by HPAEC-PAD and MALDI have been carried out. HPAEC-PAD has been found to be more sensitive in terms of detection limits than MALDI for the analysis of FOS and allowed the separation of linear and branched oligosaccharides. However, MALDI was a faster method, more tolerant to impurities and produced more correct molecular assignments, and was probably in general more accurate for quantitative determinations (Wang et al., 1999).

Table 13.5 shows as an example of some MALDI qualitative and quantitative studies of prebiotic oligosaccharides described in the literature.

13.2.7 Nuclear Magnetic Resonance Spectroscopy

For a long time, NMR has significantly contributed to the knowledge of the structure and conformation of carbohydrates. This technique is especially

Table 13.5

Some examples of MALDI analysis of prebiotics

Prebiotic	Analyzer	Selected matrix	Salts	Analyzes	Calibration	Reference
FOS	ToF	THAP with acetone	0.01 M KCl	Qualitative and quantitative	i.s.	Wang et al. (1999)
FOS and inulin	FT-ICR	0.4 M DHB	0.01 mM NaCl	Qualitative and quantitative	i.s.	Seipert et al. (2008)
Human milk oligosaccharides	FT-ICR	0.4 M DHB	0.01 M NaCl	Qualitative and quantitative	e.s. and i.s.	Ninonuevo et al. (2007)
Human milk oligosaccharides	ToF/ToF	10 mg mL^{-1} DHB	–	Qualitative (CID)	–	Spina et al. (2004)
FOS	ToF	10 mg mL^{-1} DHB	1 mg mL^{-1} CH_3COONa (only for standards)	Semi-quantitative	e.s.	Reiffová et al. (2007)
Fructans	ToF	DHB	–	Qualitative	–	Lopez et al. (2003)
Arabinogalactans	ToF	9 mg mL^{-1} DHB + 3 mg mL^{-1} 1-hydroxy-isoquinoline in water: acetonitrile (70:30)	–	Qualitative	–	Huisman et al. (2001)
XOS				Qualitative	–	Reis et al. (2003b)
XOS	ToF	1% DHB in methanol	–	Qualitative	–	Cano and Palet (2007)
GEOS	ToF	DHB in acetonitrile	–	Qualitative	–	Sanz et al. (2006)
Alternansucrase acceptor products	ToF	DHB in acetonitrile	–	Qualitative	–	Côté and Sheng (2006)

useful for detailed structural analysis of pure products, either in solution or in solid state, but it has been also applied to the study of mixtures of oligosaccharides.

NMR relies on the magnetic properties of the atomic nuclei (spin). The most used nuclei in carbohydrate chemistry are ^{1}H and ^{13}C. NMR operates on a time-scale slower than other spectroscopic techniques (such as IR or UV). This is not a big drawback, since NMR is usually used to elucidate structures, and not as a routine control technique.

13.2.7.1 Sample Preparation

This subject has been described by Bock and Pedersen (1983) and the main features are summarized below.

Samples can be dissolved in deuterium oxide (D_2O) and deuterated solvents, such as dimethylsulfoxide (Me_2SO-d_6), pyridine (Py-d_6) or chloroform (DCl_3). The less polar solvents are usually selected for low-molecular weight sugars, whereas water is necessary for oligosaccharides. It should be taken into account that solvent-induced shifts are low for neutral oligosaccharides when working with ^{13}C-NMR, whereas the effect on ^{1}H-NMR spectra is high. The effect of solvent is always important in acidic or basic carbohydrates. Concentration should be adjusted to avoid high-viscosity solutions, which can cause signal broadenings. Sample clean-up is recommended to suppress soluble paramagnetic impurities.

13.2.7.2 Reference Compounds

The most used reference signals for chemical shifts measurements of carbohydrates are probably tetramethylsilane and acetone; nevertheless, other compounds as DSS (sodium salt of 2,2-dimethyl-2-silapentane-5-sulfonic acid) and TSP ([2,2,3,3-d_4]-3-(trimethylsilyl)-propanoic acid sodium salt) are also used.

13.2.7.3 Methodology

Classic studies about NMR of carbohydrates were published by Vliegenthart et al. (1983) and Rathbone (1985). Whereas the assignment of ^{1}H signals is relatively easy in pure products (specially the anomeric proton), the high amount of

protons when several tautomeric forms and various oligosaccharides are present makes difficult the assignment of all signals. Thus, it becomes necessary to use ^{13}C spectra and different methods including 1D and 2D homo- and heteronuclear experiments, correlation spectroscopy (COSY) including total correlation spectroscopy (TOCSY) and nuclear Overhauser effect spectroscopy (NOESY), etc. The number of different NMR experiments which have been described to elucidate oligosaccharide structures is really high. Experiments such as HSQC (heteronuclear single quantum correlation) and HMQC (heteronuclear multiple quantum correlation) which correlate the chemical shift of proton with the chemical shift of the directly bonded carbon, and HMBC (heteronuclear multiple bond correlation) which uses two or three bonds couplings, are frequently used to assign signals from complex oligosaccharides. A recent review describes several NMR methods for analyzing the structure of oligosaccharides, including assignment of all H-1 NMR signals, NOE experiments, and modification of pulse programs (Kajihara and Sato, 2003).

13.2.7.4 Applications

A series of interesting applications, covering different oligosaccharides with prebiotic properties have been summarized in *Table 13.6*. Saccharides from DP2 (lactulose) and DP3 (kestoses) to higher DP have been included.

Although basic NMR data of many pure sugars, including mono- di- and trisaccharides have been published in the 1980s, fully assigned highly resolved spectra of some prebiotic sugars have been recently reported: lactulose in solution (Mayer et al., 2004) and in crystalline state (Jeffrey et al., 1992); the three natural kestoses (Calub et al., 1999; Liu et al., 1991). α-D-Gal*p*-(1→6)-β-D-Gal*p*-(1→4)-β-D-fructose (three tautomers) and β-D- Gal*p*-(1→4)-D-fructose-(1→1)-β-D-Gal*p* (three tautomers) resulting from enzymatic transgalactosylation during lactulose hydrolysis by the galactosidase from *K. lactis* have also been characterized (Martínez-Villaluenga et al., 2008b). Twelve novel non-reducing oligosaccharides from DP3 to DP6, namely [β-D-Gal*p*-(1→4)]$_n$-α-D-Glc*p*-(1→1)-β-D-Gal*p*[-(4→1)- β-D-Gal*p*]$_m$, with n, m = (1, 2, 3, or 4) and β-D-Gal*p*-(1→2)-α-D-Glc*p*-(1→1)-β-D-Gal*p* were characterized in a mixture produced by β-galactosidase using lactose as a substrate (Fransen et al., 1998). Although the mixture was fractionated, several oligosaccharides were found in the same fraction and several experiments were necessary to achieve complete characterization. A detailed description of signal assignments of two trisaccharides and four tetrasaccharides

Table 13.6

NMR applications for the analysis of prebiotics (*Cont'd* p. 519)

Analyte	Experiments	Solvent	Reference compound	Reference
Related-kestose oligosaccharides in plants	^{1}H, ^{13}C	D_3HCl	Tetramethylsilane	Forshyte et al. (1990)
1-Kestose	2D homonuclear and heteronuclear	D_2O	Acetone	Calub et al. (1990)
6-Kestose, neokestose	2D homonuclear and heteronuclear	D_2O	Acetone	Liu et al. (1991)
Crystalline lactulose	^{13}C CPMAS	–	Adamantane	Jeffrey et al. (1992)
5 Trisaccharides in goat colostrum	^{1}H, ^{13}C, several 2D experiments	D_2O	Expressed relative to DSS, but actually measured by reference to acetone	Urashima et al. (1994)
12 non-reducing oligosaccharides	^{1}H, ^{13}C, several 2D experiments	D_2O	Acetone or acetate for ^{1}H Ext. glucose for ^{13}C	Fransen et al. (1998)
XOS	^{1}H, ^{13}C, several 2D experiments	D_2O	Acetone	Nishimura et al. (1998)
3 Sulfated OS in human milk	^{13}C and ^{1}H	D_2O	Expressed by reference to DSS, but actually measured by reference to acetone	Guerardel et al. (1999)
5 FOS from *Asparagus*	1D ^{1}H and ^{13}C, several 2D experiments	D_2O	TSP	Fukusi et al. (2000)
Neo-FOS produced by a *Penicillium citrinum*	^{13}C	D_2O	Tetramethylsilane	Hayashi et al. (2000)
Kojioligosaccharides	^{13}C	D_2O	TSP	Chaen et al. (2001)
Lactulose	^{1}H, several 1D and 2D experiments		Tetramethylsilane	Mayer et al. (2004)
Inulin-type FOS from *Matricaria maritima*	2D ^{1}H, ^{1}H DQF-COSY/TOCSY and ^{1}H, ^{13}C HMQC/HMBC	D_2O	TSP	Cerantola et al. (2004)
Neutral oligosaccharides from human milk	^{1}H, several 2D experiments	D_2O	Acetone	Kogelberg et al. (2004)

Table 13.6

Analyte	Experiments	Solvent	Reference compound	Reference
Novel oligosaccharides from raffinose and stachyose	^{1}H and ^{13}C 2D-NMR including COSY, HSQC, HSQCTOCSY, HMBC and other	D_2O	TSP and 1,4-dioxane	Takahashi et al. (2005)
Oligosaccharides produced by alternansucrase	^{1}H, ^{13}C, several 2D experiments	D_2O	Acetone	Coté and Sheng (2006)
Cyclic isomaltooligosaccharides	^{13}C	D_2O	DSS	Funane et al. (2007)
IMOS	^{1}H	D_2O		Ao et al. (2007)
GOS from cyanobacterium *Nostoc commune*	^{1}H	D_2O	Acetone	Wienecke et al. (2007)
2 Trisaccharides derived from lactulose	^{1}H, ^{13}C, several 1D and 2D experiments	D_2O	Tetramethylsilane	Martinez-Villaluenga et al. (2008b)
FOS produced by *Aspergillus*	^{1}H, ^{13}C and 2D HMQCT	D_2O	Tetramethylsilane	Mabel et al. (2008)

isolated from *Asparagus* through several NMR experiments has been published by Fukushi et al. (2000).

Among the oligosaccharides with prebiotic properties, natural fructans (FOS) with the two series of inulin and levan with linkages 2→1 and 2→6 respectively, have probably been the most studied (Cerantola et al., 2004; Hayashi et al., 2000).

Most oligosaccharides obtained by enzymatic synthesis such as kojioligosaccharides (Chaen et al., 2001), cycloisomaltooligosaccharides (Funane et al., 2007) are mixtures of similar saccharides with a definite glycosidic linkage and different DP, thus the NMR signal assignment can be performed by comparison with published data.

Three xylooligosaccharides with general structure [*O*-α-D-Glc*p*-(1→2)]$_n$-*O*-α-D-Xyl*p*-(1→2)-β-D-Fru*f* (n = 1,2,3) required several techniques for structural characterisation. The ^{1}H and ^{13}C NMR signals of each saccharide were assigned using two dimension (2D)-NMR including COSY, HSQC, HSQC-TOCSY and HMBC (Takahashi et al., 2007). Similar techniques were applied to the structural

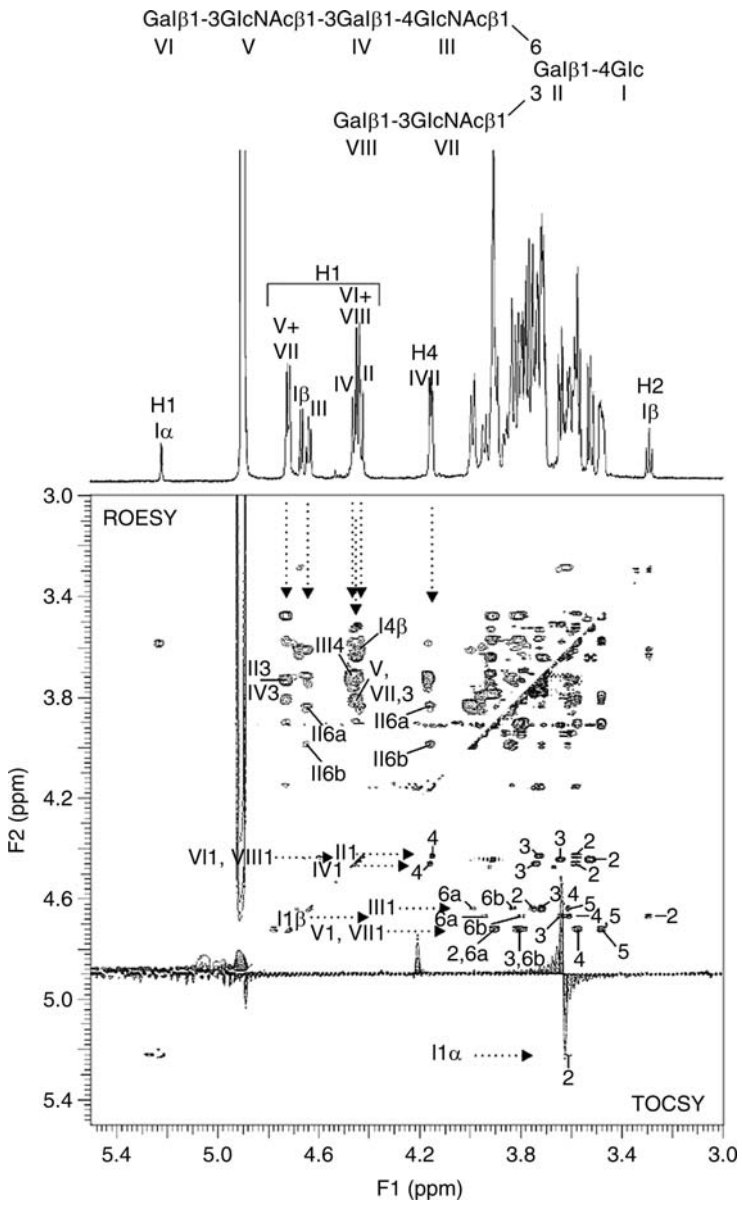

Figure 13.7

^{1}D and ^{2}D ^{1}H NMR spectra of iso-lacto-N-octaose. ^{1}D and ^{2}D ^{1}H NMR spectra (800 MHz) of iso-lacto-N-octaose, region 5.5–3.0 ppm at 15°C. Upper trace, ^{1}H NMR spectrum; top-left half, 300-ms ROESY spectrum and bottom-right half, 140-ms TOCSY spectrum. The structure is shown at the top, depicting the residue labeling. From Kogelberg et al. (2004) with permission from FEBs.

characterization of six novel oligosaccharides (one tetra-, two penta-, two hexa- and one hepta-saccharide) synthesized by glucosyl transfer from β-D-glucose-1-phosphate to raffinose or stachyose by the action of *Thermoanaerobacter brockii* kojibiose phosphorylase (Takahashi et al., 2005).

A special case is that of alternansucrase, which produces oligosaccharides with alternating α-(1→3) and α-(1→6) linkages. When this enzyme was incubated with maltose, one pentasaccharide, two hexasaccharides and one heptasaccharide were isolated as main products, with general structure [α-D-Glc_p-(1→6)-α-D-Glc_p-(1→3)]$_x$-α-D-Glc (Coté and Sheng, 2006). Experiments (gradient-enhanced band-selective HSQC and HSQC–TOCSY and gradient-enhanced band-selective HMBC) were performed at 27°C for the lower DP products and at 50°C for the higher DP ones.

Milk oligosaccharides are extremely complex: about 150–200 oligosaccharides (neutral and acidic) have been described in human milk, whereas a smaller number exist in ruminants' milk. As an example of NMR application to oligosaccharides in goat's milk, four neutral trisaccharides were characterized in goat colostrum: α-L-Fuc*p*-(l→2)-β-D-Gal*p*-(l→4)-Glc, α-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→4)-Glc, β-D-Gal*p*-(l→3)-β-D-Gal*p*-(l→4)-Glc, and β-D-Gal*p*-(1→6)-β-D-Gal*p*-(1→4)-D-Glc (Urashima et al., 1994); NMR spectra were assigned by comparison with those of other previously described oligosaccharides and by several experiments, such as double quantum filtered correlation (DQF-COSY), 1D TOCSY and (^{1}H, ^{13}C) shift correlation.

Human milk neutral oligosaccharides contain galactose, *N*-acetylglucosamine, fucose, and lactose; several anionic oligosaccharides containing *N*-acetylneuraminic acid also exist; a lactose unit at the reducing end is also frequently found (Mehra and Kelly, 2006) but other substituents are possible. As an example, three sulfated oligosaccharides have been analyzed using 2D homonuclear COSY and HMQC (Guerardel et al., 1999). The structural elucidation of very complex milk oligosaccharides has been undertaken by combining two techniques: ESI-MS for determining the branching pattern and ^{1}H NMR for sequence assignment (Kogelberg et al., 2004); *Figure 13.7* shows some spectra of *iso*-lacto-*N*-octaose.

13.3 Summary

- Despite advances in analytical techniques in recent years there is still a lack of accurate and precise methods to characterize and quantify prebiotic oligosaccharides which frequently consist of complex mixtures with similar structural characteristics.

- Traditional analyses such as methylation, TLC or open chromatographic columns with RI detectors are still commonly used. The lack of standards and the similar structure of oligosaccharides are the main drawbacks to achieve a truthful qualitative and quantitative result.
- The selection of the most appropriate methodology for the analysis of prebiotics mainly depends on the nature of the carbohydrate mixture: the complexity of the sample, range of expected molecular weight, etc. No one protocol is able to cover all the possible cases.
- While GC has commonly been used to determine the composition of low molecular weight carbohydrates, oligosaccharides with high DPs are mainly characterised by HPLC.
- Considering the complexity of prebiotic samples their analyses require the use of different techniques, which are combined to obtain useful information.
- HPLC, CE or GC are used for the separation and isolation of the different constituents; methylation analysis and NMR to determine their structures; MS for studying their molecular weight and/or tandem MS systems to complement the structural information.

List of Abbreviations

2′FL	2′-fucosyllactose
2D	two dimensions
3′FL	3′-fucosyllactose
AMAC	2-aminoacridone
AMD	automatic mode development
ANTS	8-aminonaphtalene-1,3,6,-trisulphonic acid
APTS	9-aminopyrene-1,4,6-trisulfonate
ASE	accelerated solvent extraction
AXOS	arabinoxylooligosaccharides
BGE	background electrolyte
CE	capillary electrophoresis
CEOS	cellooligosaccharides
CGE	capillary gel electrophoresis
CI	chemical ionization
CID	collision-induced dissociation
CIEF	capillary isolectric focusing
CITP	capillary isotachophoresis

COSY	correlation spectroscopy (COSY)
CZE	capillary zone electrophoresis
DAD	diode-array detector
DHB	2,5-dihydroxybenzoic acid
DP	degree of polymerization
DQF-COSY	double-quantum-filtered correlation spectroscopy
DSS	2,2-dimethyl-2-silapentane-5-sulfonic acid sodium salt
ECD	electron capture dissociation
EI	electronic impact
ELSD	evaporative light scattering detectors
EOF	electroosmotic flow
ESI	electrospray ionization
ETD	electron transfer dissociation
FAB	fast atom bombardment
FID	flame ionization detector
FOS	fructooligosaccharides
FT-ICR	fourier-transform ion cyclotron resonance
GC	gas chromatography
GCC	graphitized carbon columns
GOS	galactooligosaccharides
HDB	hexadimethrine bromide
HILIC	hydrophilic interaction chromatographic
HMBC	heteronuclear multiple bond correlation
HMOS	human milk oligosaccharides
HMQC	heteronuclear multiple quantum correlation
HPAEC	high performance anion exchange chromatography
HPCE	high performance capillary electrophoresis
HPLC	high performance liquid chromatography
HPSEC	high performance size exclusion chromatography
HPTLC	high performance TLC
HSQC	heteronuclear single quantum correlation
HT-GC	high temperature gas chromatography
IMOS	isomaltooligosaccharides
IR	infrared
IRMPD	infrared multiphoton dissociation
IT	ion trap
LALLS	low angle laser light scattering
LC	liquid chromatography

LIF	laser-induced fluorescence
LNFP I	lacto-N-fucopentaose I
LNFP II	lacto-N-fucopentaose II
LNT	lacto-N-tetraose
LSI	liquid secondary ion
MALDI	matrix assisted laser desorption/ionization
MALLS	multiple angle laser light scattering
MEKC	micellar electrokinetic chromatography
MOS	maltooligosaccharides
MPP	3-methyl-1-phenyl-2-pyrazolin-5-one
MS	mass spectrometry
NMR	nuclear magnetic resonance
NOESY	nuclear overhauser effect spectroscopy
NPLC	normal phase liquid chromatography
OPTLC	over pressured TLC
o-ToF	orthogonal ToF
PAD	pulse amperometric detector
PC	paper chromatography
Q	quadrupole
QIT	quadrupole ion trap
QqQ	triple quadrupole
RI	refractive index
ROS	raffinose oligosaccharides
RP	reverse phase
SALDI	surface assisted laser desorption/ionization
SCFA	short chain fatty acid
SDS	sodium dodecyl sulfate
SEC	size exclusion chromatography
SFE	supercritical fluid extraction
THAP	2',4',6'-trihydroxyacetophenone
TLC	thin layer chromatography
TOCSY	total correlation spectroscopy
ToF	time of flight
TSP	[2,2,3,3-d_4]-3-(trimethylsilyl)-propanoic acid sodium salt
UPLC	ultra performance liquid chromatography
UTLC	ultra TLC
UV	ultraviolet
XOS	xylooligosaccharides

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14 Manufacture of Prebiotics from Biomass Sources

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14.1 Introduction

Biomass from plant material is the most abundant and widespread renewable raw material for sustainable development, and can be employed as a source of polymeric and oligomeric carbohydrates. When ingested as a part of the diet, some biomass polysaccharides and/or their oligomeric hydrolysis products are selectively fermented in the colon, causing prebiotic effects.

This work deals with the chemical structure, manufacture, purification, properties and applications of biomass-derived saccharides (xylans, mannans, arabinogalactans, pectins and/or their respective oligomeric products) which can be employed as food ingredients to achieve prebiotic effects closely related to the ones caused by well known prebiotic oligosaccharides, such as fructooligosaccharides, galactooligosaccharides, and isomaltooligosaccharides.

The colonic fermentation of carbohydrates is a complex problem, because the end metabolic products of given bacterial species can be used as a substrates by others, and some microorganisms may grow upon substrates that they are not able to ferment. The prebiotic behavior of dietary components is mainly related to their ability to modulate the colonic microbiota, enhancing the growth of beneficial bacteria (particularly, bifidobacteria and lactobacilli) selectively.

Prebiotic effects can be achieved by both polymeric and oligomeric carbohydrates. In the chemical processing of biomass polysaccharides, the treatments needed for separation, purification and product tailoring may result in the production of fractions with decreased degrees of polymerization (DP) in respect to the native polymers, making the distinction between "dietary fiber" (with polymeric nature) and "oligosaccharides" difficult. Usually, the term "oligosaccharide" is reserved for DP in the range 3–10 (Tungland and Meyer, 2002),

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whereas fractions of higher molecular weight are considered as "dietary fiber." However, the definition of this latter term has seen several revisions since it was proposed in 1953, and a variety of definitions are available based either on analytical determinations by specific methods or on their biological effects. In fact, no international consensus has been reached on a definition yet. The following definition has been proposed by the AACC Dietary Fiber Definition Committee (2001): "Dietary fiber is the edible parts of plants or analogous carbohydrates that are resistant to digestion and absorption in the human small intestine with complete or partial fermentation in the large intestine. Dietary fiber includes polysaccharides, oligosaccharides, lignin, and associated plant substances. Dietary fibers promote beneficial physiological effects, including laxation, and/or blood cholesterol attenuation, and/or blood glucose attenuation."

On the other hand, the situation has become more complicated by the fact that some "functional fibers," such as inulin-derived fructooligosaccharides, polydextrose, some polyols, and resistant starch, cannot be determined as dietary fiber by the standard AOAC methods, and they could not be confirmed as dietary fiber for labeling purposes. Finally, it has to be taken into account that in many studies, particularly in these where the distribution of molecular weights was unknown, the whole set of soluble fragments from polysaccharide degradation have been considered as "oligosaccharides" (no matter of the DP); and that some DP2 saccharides (such as xylobiose) have been considered as oligosaccharides in food studies (Vázquez et al., 2000). Based on the above ideas, the term "dietary fiber" is employed in this work to denote high molecular weight, polymeric fractions; and the term "oligosaccharides" is reserved for compounds of lower molecular weight, even if they correspond to a broad DP range.

14.2 Xylans and Xylan-Derived Products

14.2.1 Structure of Xylans

Xylans represent an immense resource of biopolymers for practical applications, accounting for 25–35% of the dry biomass of woody tissues of dicots and lignified tissues of monocots, and occur up to 50% in some grasses and tissues of cereal grains. The structure of xylans depends on the source considered. The most

common xylans are made up of a main backbone of xylose linked by β-(1→4) bonds, where the structural units are often substituted at positions C2 or C3 with arabinofuranosyl, 4-O-methylglucuronic acid, acetyl or phenolic substituents (Ebringerova et al., 2005). Xylans are usually named according to the most abundant substituents (arabinoxylans, glucuronoxylans, arabinoglucuronoxylans, etc.). *Figure 14.1* presents structures of typical xylans.

14.2.2 Manufacture of Xylooligosaccharides

The partial hydrolysis of xylans leads to xylooligosaccharides or substituted oligosaccharides (here denoted XO), whose structures depend on the features of native xylans and on the processing conditions employed in their manufacture. Recent studies have been reported on the production of XO by chemical processing of a variety of raw materials, including crop residues, sugarcane bagasse, hardwoods, corncobs, barley and rice hulls, brewery spent grains, almond shells, corn stover and corn fiber, flax, straws, flours, bamboo and fruit wastes.

The breakdown of the xylan chains can be accomplished by different methods (Moure et al., 2006), such as:

- Direct enzymatic hydrolysis of susceptible substrates (for example, isolated xylan or glucuronoxylan, xylan-containing cellulose pulps, fruit wastes, or wheat flour arabinoxylan).
- Chemical processing (for example, by aqueous treatments with water or steam, or in media containing externally-added mineral acids) of native feedstocks or xylan-containing pre-processed solids (for example, sulphite cellulose).
- Combined chemical and enzymatic treatments, where the chemical treatments can be employed either to make the raw material accessible to enzymes or to yield soluble xylan fragments from a native feedstock (for example, alkaline processing of xylan-rich, native substrates followed by xylanolytic hydrolysis).

Our group has paid special attention to the production of XO by treatments with hot, compressed water (also called hydrothermal treatments, autohydrolysis, hydrothermolysis or water prehydrolysis), which cause the acid-catalyzed degradation of xylan. When the aqueous processing of xylan-containing substrates is carried out under suitable operational conditions, the hemicellulosic chains are

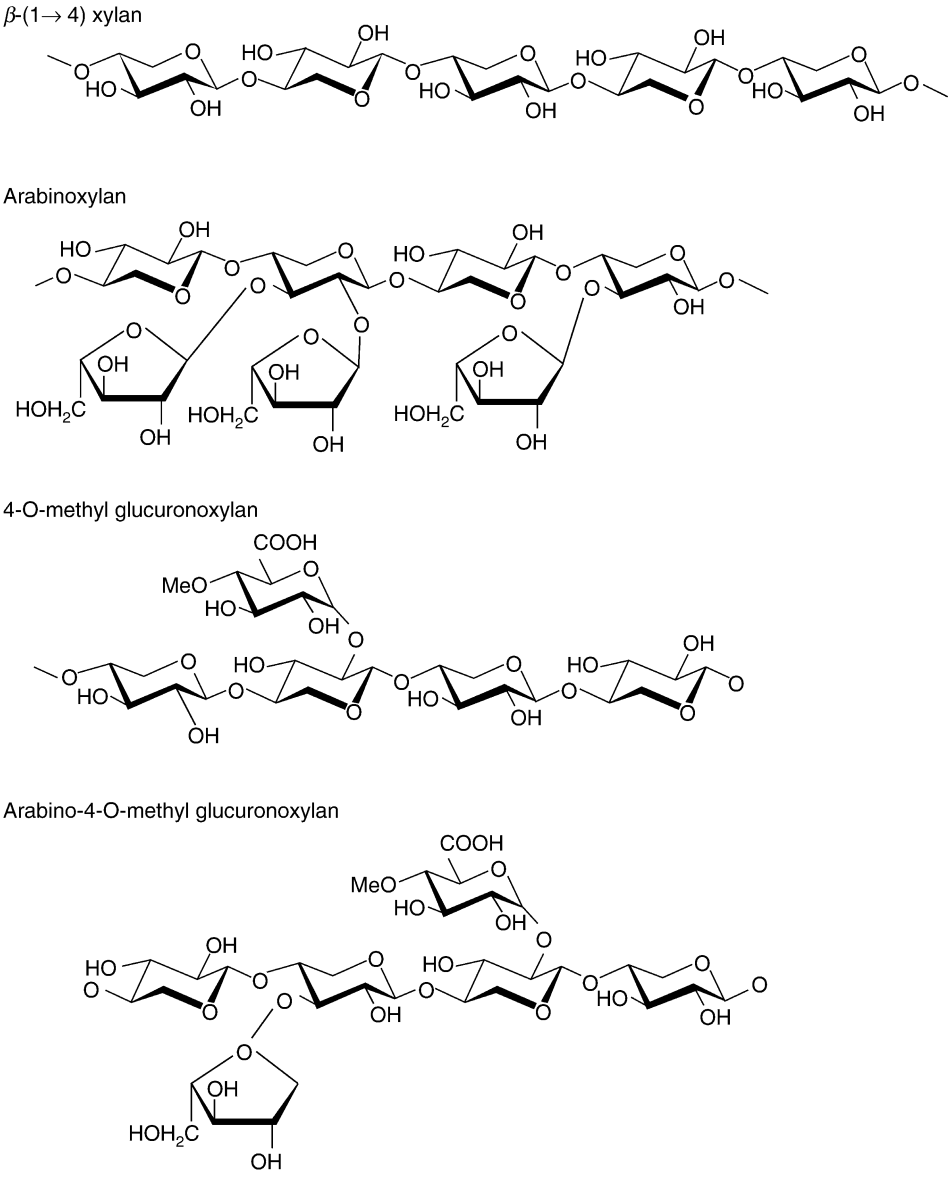


Figure 14.1
Structure of typical xylans.

progressively broken down by the hydrolytic action of hydronium ions (generated from water autoionization and from *in situ* generated organic acids), yielding soluble products (mainly oligosaccharides), and leaving both cellulose and lignin in solid phase with little chemical alteration.

Autohydrolysis presents several advantages, such as:

- Environmentally friendly operation (no chemicals except water and lignocellulosic feedstock are needed, and no sludges are generated in the neutralization stages).
- Compatibility with further fractionation and/or hydrolysis processing of the spent solids to separate cellulose and lignin, enabling the possibility of an integral utilization of the raw material. The autohydrolysis liquors contain XO and soluble reaction byproducts, and the exhausted solids from autohydrolysis are suitable substrates for the enzymatic hydrolysis of cellulose (with or without a previous delignification step), or for applications as feed, fuel, or construction materials. This operational mode follows the "biorefinery approach," which is expected to decrease greenhouse gas emissions and to be compatible with a sustainable development.
- XO generated by autohydrolysis treatments present a rich substitution pattern, conserving the major structural features of the native xylan (Kabel et al., 2002a). For example, only a part of the ester groups are split in hydrothermal treatments, in comparison with the saponification caused by processes involving alkaline stages.

In autohydrolysis treatments, XO behave as typical reaction intermediates, and their maximal concentrations are achieved under medium-severity conditions. Optimization studies for XO production can be carried out using two different approaches:

- Pseudohomogeneous kinetic modeling.
- Empirical assessment based on the severity factor.

The kinetic modeling of lignocellulose autohydrolysis is a complex problem involving the catalytic, heterogeneous reaction of a solid substrate by means of a number of different reactions. In order to simplify the problem, the kinetic modeling of xylan solubilization has been carried out assuming irreversible, pseudohomogeneous, first-order kinetics, of several individual reactions, which are governed by kinetic coefficients following the Arrhenius equation that are assumed to be independent from time, particle size and acidity. This approach has been employed in studies involving a variety of substrates, including corncobs, brewery spent grains, rice and barley husks, hardwoods and almond shells. In some cases, the progressive breakdown of xylan chains has been interpreted through some or all of the following hypotheses:

- Xylan can be made up of two fractions with different susceptibility to hydrolytic reactions
- One or both xylan fractions are decomposed to give high-molecular weight oligomers

- High molecular weight oligomers decompose to give low-molecular weight oligomers
- Xylooligomers are decomposed to give xylose
- Xylose and terminal groups of xylooligomers can be dehydrated to furfural
- Furfural can be converted into degradation products

Additionally, some studies consider the simultaneous breakdown of arabinosyl chains to give arabinose, and the generation of acetic acid from acetyl groups.

Based on experimental data, some studies have reported on the values of preexponential factors, activation energies and mass fractions of susceptible xylan, enabling the calculation of concentration of the various chemical species under defined experimental conditions.

Alternatively to the modeling by pseudohomogeneous kinetics, the severity factor R_0 (a parameter that includes the effects of both time and temperature) has been employed to give a simplified, empirical interpretation of the measured effects (Abatzoglou et al., 1992).

R_0 can be calculated using the equation:

$$R_o = \int_0^t \exp\left(\frac{T - T_r}{\omega}\right) dt$$

where T is temperature (°C), t is time (min), and T_r and ω are parameters with values of 100 and 14.75°C, respectively. In first-order reaction systems, the measured variable and R_0 are expected to have an exponential interrelationship under either isothermal or non-isothermal operation.

R_0 was introduced to measure the hemicellulose conversion, but its ability for comparing on a quantitative basis the effects caused by hydrothermal treatments carried out under different conditions of time and temperature allowed its utilization for assessing other reaction effects (Garrote et al., 2003), including:

- Generation of XO, monosaccharides and furfural
- DP distribution of XO
- Degree of deacetylation and acidity of the reaction media
- Amount, composition, degree of polymerization and enzymatic digestibility of the exhausted solids

- Release of low molecular weight phenolics
- Antioxidant activity of byproducts present in autohydrolysis media

14.2.3 Purification of Xylooligosaccharides

In order to produce food-grade XO (whose usual commercial purity lies in the range 75–95%), the liquors from xylan conversion have to be refined. Refining involves the selective removal of undesired compounds (usually, monosaccharides and non-saccharide compounds) to obtain a concentrate with a XO content as high as possible.

Purification of XO obtained by enzymatic processing of substrates containing susceptible xylan is facilitated by the previous chemical processing of the raw material, as well as by the specific action of xylanases. On the other hand, the purification of XO present in crude autohydrolysis liquors is a complex problem, because the degradation of xylan progresses simultaneously with several side-processes (including extractive removal, solubilization of acid-soluble lignin, acetic acid generation from acetyl groups, sugar degradation to furfural or hydroxymethylfurfural, neutralization of ashes, and participation of Maillard reactions involving either the protein fraction of the feedstock or its hydrolysis products). All of these effects result in the presence of undesired, non-saccharide compounds in liquors from hydrothermal processing, which have to be removed (at least in part) before utilization.

Several strategies have been proposed for refining crude liquors. For example, in single-stage autohydrolysis reactions, a significant part of the dissolved feedstock may correspond to easily extractable compounds (for example, waxes, low molecular weight phenolics and soluble inorganic components). This kind of compound can be removed by a previous extraction stage with solvents or water. When the raw material is pretreated with hot water under mild conditions, the hemicellulosic polymers remain in solid phase almost untouched and ready for further hydrolytic conversion under harsher conditions, whereas the easily extractable compounds are separated in liquors. Ethanol extraction has been carried out before autohydrolysis processing to achieve these objectives. Alternatively, two consecutive aqueous treatments of increasing severity (the first one for removing extractives, and the second one for generating XO from xylan) can be performed. This strategy has resulted in cleaner liquors and/or in XO-containing solutions with enhanced susceptibility to further refining treatments.

However, in most cases, a sequence of different physicochemical treatments may be necessary to achieve XO concentrates of commercial purity. Focusing on the refining of autohydrolysis liquors, the mass ratio XO/total solutes can be improved by means of several separation methods (which can be assayed individually or in combination), including:

- Solvent extraction
- Vacuum evaporation
- Solvent precipitation
- Freeze-drying followed by solvent extraction
- Chromatographic separation
- Adsorption
- Ion exchange
- Membrane technologies

The corresponding objectives and effects are considered in the following paragraphs.

Ethyl acetate extraction of autohydrolysis liquors enables the integrated benefit of the several fractions from autohydrolysis of lignocellulosics, yielding:

- A refined aqueous phase having an increased mass fraction of oligomers
- An organic phase containing antioxidant compounds

Processing of lignocellulosics by sequential stages of autohydrolysis and ethyl acetate extraction leads to:

- An oligosaccharide-containing, refined aqueous phase
- An organic phase, mainly made up of phenolics and extractive-derived compounds, for which antioxidant applications have been proposed
- A solid phase enriched in cellulose, which also contains lignin, that can be fractionated by further treatments, or employed directly as a substrate for the enzymatic hydrolysis of cellulose

One of the most remarkable advantages of solvent extraction (and particularly, of ethyl acetate extraction) is the high selectivity of this method: for example, ethyl acetate extraction of rice husks autohydrolysis liquors resulted in the removal of 38% of the non-saccharide components, whereas just 5% of

the initial XO were lost (Vegas et al., 2004). When the same operational procedure was applied to barley husks autohydrolysis liquors, 33% of the non-saccharide compounds was removed, in comparison with just 14% XO loss (Vegas et al., 2005).

However, the purification effects achieved by solvent extraction are usually too weak for practical purposes, and further physicochemical processing is needed to achieve products fulfilling the market specifications.

Gas chromatography coupled with mass spectrometry (GC-MS) analysis of the acetate-soluble fraction of autohydrolysis liquors from *Eucalyptus* wood showed the presence in extracts of hemicellulose-derived products (sugars and furfural) and non-saccharide components; the latter corresponding mainly to lipophilic extractives. Among them, stearic acid and palmitic acid were the main fatty acids, with minor amounts of oleic acid, 9,12 octadecanedienoic acid and tetradecanoic acid. Dehydroabietic acid was the major resin acid, whereas the phenolic compounds identified were gallic acid, vanillin, 1,2,3 trihydroxybenzene, syringaldehyde, syringic acid and 3,4 dihydroxybenzoic acid (Vázquez et al., 2005). GC-MS analysis of the dichloromethane-soluble fraction of corncob autohydrolysis liquors allowed the identification of sugar-derived compounds (accounting for 35% of the extracted material), lignin-derived compounds (accounting for 59.2% of the extracted material), nitrogen-containing compounds (accounting for 1.1% of the extracted material) and fatty acids (accounting for 4.7% of the extracted material). Furfural was the most abundant compound, followed by vanillin, 4-methylphenol and 4-vinylguaiacol (Garrote et al., 2007).

Vacuum evaporation has been assayed for increasing the XO concentration of autohydrolysis liquors, with simultaneous removal of volatile components (particularly acetic acid). Some flavors and/or their precursors can be also eliminated by evaporation. Depending on the case considered, vacuum evaporation can be the first step of a multistage process, or an intermediate one.

Ethanol precipitation has been assayed for isolating arabinoxylooligosaccharides from enzymatic hydrolysis of wheat flour, whereas precipitation of XO from autohydrolysis liquors has been assayed using ethanol, acetone or 2-propanol. The purification effects achieved and the recovery yields depended on the solvent and on the raw material employed, which controls the XO substitution pattern and the possible presence of stabilizing, non-saccharide components. Solvent precipitation of raw *Eucalyptus globulus* wood autohydrolysis liquors was strongly affected by the presence of even minimal amounts of water, which decreased the

precipitation of hemicellulose-derived products (Vázquez et al., 2005). This behavior was ascribed to the XO substitution pattern (with comparatively high proportion of uronic groups) and/or to the presence of stabilizing non-saccharide components in *Eucalyptus* autohydrolysis liquors. Better results have been reported for the processing of rice husk autohydrolysis liquors (Vegas et al., 2004): ethanol precipitation of ethyl-acetate extracted liquors led to refined products with contents of non-saccharide compounds as low as 9 wt.%, but at a limited recovery yield. Comparatively, 2-propanol resulted in limited removal of non-saccharide compounds (which accounted for 12 wt.% of the concentrate), but the XO recovery yield increased up to 75.8%. Acetone presented a behavior related to the one observed for 2-propanol, with slightly lower recovery yield. Ethanol precipitation was not suitable for the processing of barley husks autohydrolysis liquors (owing to operational problems), whereas acetone and propanol achieved similar effects, with some advantage for acetone, which led to slightly higher recovery yield and provided XO concentrates containing 19 wt.% of non-saccharide products (Vegas et al., 2005).

As the presence of even minimal amounts of water affected the precipitation of hemicellulose-derived products negatively, the possibility of subjecting the autohydrolysis liquors to freeze-drying and extracting the resulting solids with organic solvents has been explored. The freeze drying-extraction of *Eucalyptus* wood autohydrolysis liquors, previously extracted with ethyl acetate and concentrated, has been assessed for operation with ethanol, 2-propanol and acetone (Vázquez et al., 2005). Extraction with 2-propanol resulted in negligible purification effects, but better results were achieved operating with acetone, which led to a concentrate containing 70.6 wt.% of substituted oligosaccharides. These latter compounds presented increased contents of uronic and acetyl substituents compared to the average values determined for the products present in the crude liquors. Ethanol extraction of the freeze-dried solids resulted in moderate recovery yields, and in enhanced removal of non-saccharide compounds (about 50% of the initial amount). The overall purification effect obtained in the sequential processing of autohydrolysis liquors by ethyl acetate extraction, vacuum concentration, freeze drying and solvent precipitation were in the range reported for more sophisticated operational schemes involving two-stage extraction, chromatographic separation and ion exchange. In a related study, the freeze drying-extraction processing of ethyl acetate-treated autohydrolysis liquors from barley husks resulted in high percentages of solute recovery, but limited purification effects were achieved (Vegas et al., 2005).

Chromatographic separation of XO leading to high purity fractions has been carried out at an analytical scale. Samples from hydrothermally treated lignocellulosics have been fractionated by anion-exchange chromatography and size-exclusion chromatography, whereas chromatographic techniques have been employed for refining samples before structural characterization of XO by ^{13}C NMR, MALDI-TOF or nanospray mass spectrometry. In the same field, simulated moving bed chromatography and size-exclusion chromatography have been employed for purification of substituted XO. Other studies reported on chromatographic separation as a part of multistage processes.

Adsorption (using adsorbents such as activated charcoal, acid clay, bentonite, diatomaceous earth, aluminum hydroxide or oxide, titanium, silica and porous synthetic materials) has been used for purification of XO, following two alternative operational modes:

- XO-containing solutions (for example, obtained by enzymatic hydrolysis of either ammonia-pretreated or native corncobs or corn stover) have been adsorbed onto charcoal, and further eluted with ethanol. Interestingly, the DP range of the fractions coming from ethanol elution depends on the alcohol concentration, allowing the fractionation of XO on the basis of their molecular weight.
- Liquors containing XO can be contacted with charcoal to remove non-saccharide compounds. Under selected conditions, adsorption was higher for lignin-related products than for XO. The separation selectivity was dependent on the properties of the adsorbent (distribution of pore sizes and basic or acidic surface groups).

Ion-exchange resins have been employed for refining XO obtained by different reaction technologies (autohydrolysis or enzymatic processing) from a variety of raw materials (including rice and barley husks, crop residues and cellulose pulps). In several cases, the target products have been acidic XO. Ion exchange enables a variety of refining effects, including:

- Removal of charged, inorganic components.
- Removal of organic compounds, either by ion exchange or by sorption in the polymeric matrix.

Ion exchange results in efficient desalination, but its performance for removing colored compounds is limited. The literature has reported on the individual utilization of either cation- or anion-exchange resins, as well as on operation

with mixed resin beds. Usually, ion exchange is employed for XO purification in combination with other separation technologies. A simple refining process (ethyl acetate extraction followed by anion exchange) has been reported for XO manufacture from barley husk autohydrolysis liquors (Vegas et al., 2005). Ethyl acetate extraction was carried out in three sequential stages, and the resulting aqueous phase was treated with Amberlite IRA 400 (a strong anion exchange, quaternary ammonium, gel-type resin) or Amberlite IRA 96 (a weak anion exchange, polyamine, macroreticular resin). This operational mode resulted in remarkable results: for example, treatment with IRA 96 led to a final concentrate containing solutes with the following composition: 11.3 wt.% monosaccharides, 70.8 wt.% of substituted XO, and 17.9 wt.% of non-saccharide compounds. Spectral data confirmed that this latter fraction was mainly made up of phenolic compounds and melanoidins. Processing of rice husk autohydrolysis liquors by evaporation, ethyl acetate extraction and anion exchange with IRA 400, resulted in a final product containing 9.1 wt.% of monosaccharides, 82.1 wt.% of substituted oligosaccharides and 8.8 wt.% of non-saccharide compounds (Vegas et al., 2004). Acid hydrolysis of the concentrate showed the presence of 3.6 wt.% of phenolic compounds linked to the oligosaccharide chains (determined as acid-soluble lignin), which are types of compounds that possess antioxidant activity and that could contribute to the health benefits of the product. Determination of the nitrogen content proved the presence of melanoidins in the final isolate, which were generated in the autohydrolysis media by reaction of proteins and sugars. As melanoidins also present antioxidant activity, they could contribute to the functional properties of the target compounds.

Membrane technologies are gaining importance for XO manufacture, as they can be used for a variety of purposes, including:

- Production of XO from pure xylan or xylan-containing substrates of lignocellulosic nature, operating in enzyme-containing membrane bioreactors
- Simultaneous DP reduction and fractionation of soluble xylan-derived products from autohydrolysis treatments, operating as in the previous point
- Removal of suspended solids from reaction media by ultrafiltration
- Refining of soluble xylan-derived products present in reaction media by ultrafiltration or nanofiltration (depending on the DP of the target compounds)
- Separation of undesired, low-molecular weight components (including monosaccharides and non-saccharide compounds) from XO- containing media by diafiltration through membranes with a suitable cut-off

- Fractionation of XO on the basis of their molecular weight
- Concentration of XO by solvent removal

In this field, ultrafiltration has been employed for refining almond shell autohydrolysis liquors, as an alternative to ethanol precipitation for isolation of arabinoxylooligosaccharides from wheat bran hydrolyzed with enzymes (Swennen et al., 2005), as well as to discard the high-molecular weight fractions coming either from enzymatic treatments of oxygen-delignified pulp slurry in the manufacture of acidic oligosaccharides (Izumi et al., 2004), or from autohydrolysis media of *Eucalyptus* wood (Gullón et al., 2008).

In studies dealing with the ultrafiltration and nanofiltration processing of XO-containing media, several trade-offs have been identified while selecting the most appropriated membrane and when choosing operating conditions (Vegas et al., 2008a). For a feasible commercial application of this technology, an optimization based on economical profit would be needed, since (as it often happens in separation processes) the trade-offs are ultimately between recovery and purity. Yuan et al. (2004) employed nanofiltration membranes for concentrating XO obtained at the pilot plant scale by enzymatic hydrolysis of xylan from steamed corncobs in a multistage process, which led to concentrated solutions containing xylobiose and xylotriose as major products. In the framework of an alternative multistage process, nanofiltration of pretreated rice husk autohydrolysis liquors, concentrated up to a mass ratio of 4.89 kg feed/kg concentrate, resulted in the removal of 20.9–46.9% of total monosaccharides and in the decrease in the mass ratio of non-saccharide compounds compared to the total non-volatile solutes, from 0.3181 to 0.2378 kg/kg, under conditions enabling the recovery of 67.2–92% of XO. Under the same conditions, almost complete recovery of acetylated oligosaccharides was achieved. These data confirm that the beneficial effects of nanofiltration include both the concentration and purification of XO; the latter effect being achieved through the preferential removal of both monosaccharides and non-saccharide compounds in permeate (Vegas et al., 2005). In the processing of rice husk autohydrolysis liquors, low molecular weight cut-off ultrafiltration and nanofiltration ceramic membranes are especially interesting for the processing of XO-containing liquors, as they allow higher flux values than polymeric membranes (Vegas et al., 2008a). Operation with a ceramic membrane of 50 kDa cut-off was successful for retaining fractions with DP > 7, and provided a method for removing both high-DP XO and suspended solids (present in the autohydrolysis liquors or formed during membrane processing) (Gullón et al., 2008). Finally, low cut-off membranes have been employed for concentrating XO-containing media by reverse osmosis (Izumi et al., 2004).

As stated before, obtaining commercial-grade XO involves multistage processing of liquors from the hydrolytic breakdown of the xylan-containing substrate. *Figure 14.2* presents simplified diagrams proposed for the manufacture of refined XO concentrates.

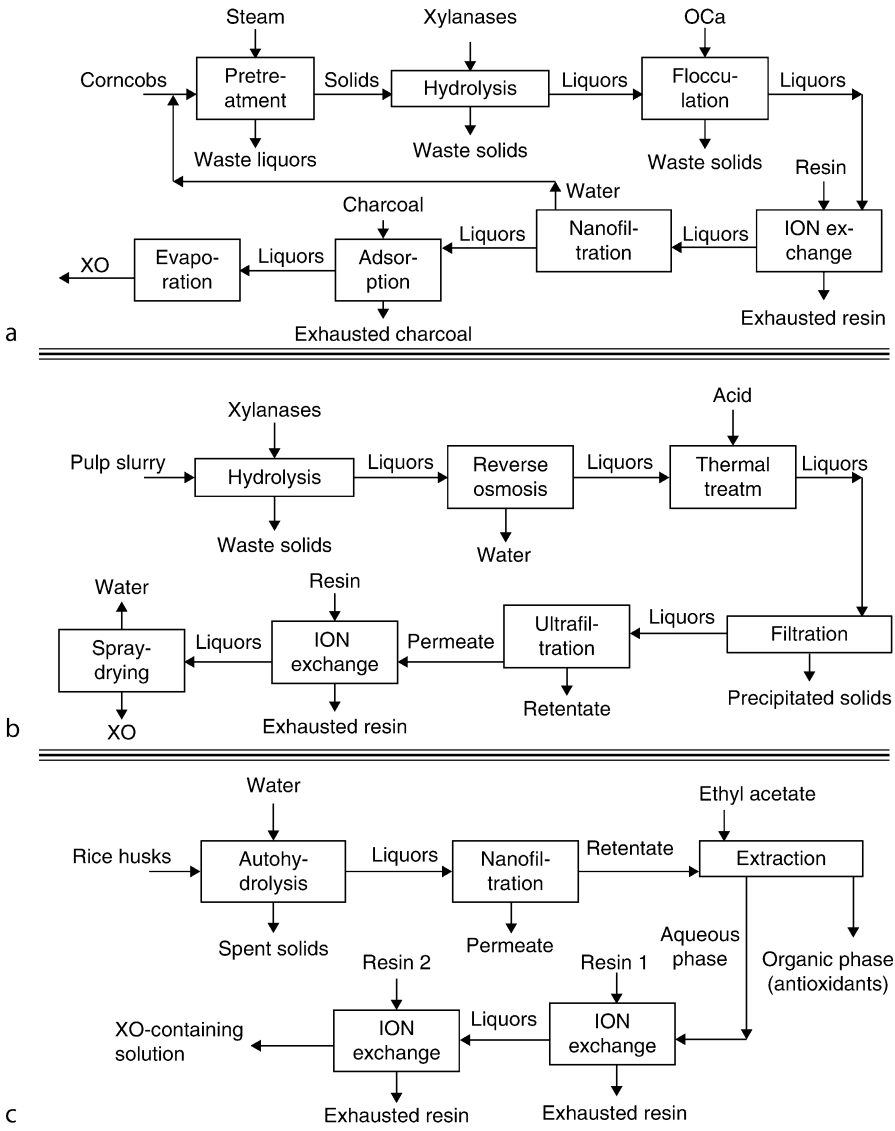


Figure 14.2
Processes for XO manufacture and refining reported by: (a) Yuan et al., 2004; (b) Izumi et al., 2004; (c) Vegas et al., 2006.

14.2.4 DP Tailoring and Structure of Xylooligosaccharides

The biological activity of xylan-derived products depends on the molecular weight distribution. Even if low-DP oligosaccharides (in the range 2–4) have been preferred for food applications, it must be highlighted that compounds with higher molecular weights can also present valuable properties. In this field, experiments in rats have proved that long-chain xylooligosaccharides (DP in the range 5–20, with an average value of 12.3) can improve the intestinal function and present hypolipemic activities (Izumi and Kojo, 2003).

The DP distribution of xylan-hydrolysis products depends on the operational conditions employed in the overall processing. In the case of hydrothermal treatments, where the polymeric xylan contained in a given feedstock is converted into soluble products (which undergo further hydrolytic degradation in the reaction media), the DP distribution is strongly affected by the severity of the operational conditions: longer reaction time and/or higher temperatures lead to reaction products of decreased molecular weight. To assess this point, the influence of the reaction conditions on the DP distribution of the reaction products obtained by autohydrolysis of rice husks was studied. Rice husk samples were treated at 172 or 188°C for selected reaction times, in order to cover the severity range (measured in terms of the severity factor R_0 defined in Section 2.2) of practical interest. The DP distribution was measured by High Performance Size Exclusion Chromatograph (HPSEC) under reported conditions (Kabel et al., 2002a), and the chromatograms were integrated on the basis of the elution times determined for standard compounds, in order to estimate the relative proportions of soluble xylan-derived products having DP $>$ 25, DP in the range 9–25 and DP $<$ 9. The corresponding results are shown in *Figure 14.3*, which presents the relative abundance of the considered fractions as a function of the logarithm of the severity factor. The severity dependence of the proportions of low, medium and high molecular weight fractions presented a similar variation pattern for the two temperatures considered, confirming the validity of the R_0 parameter for assessing the modifications in molecular weight distribution. Operating at any of the considered temperatures, the proportions of compounds with DP $>$ 25 accounted for a limited part of the total xylan-derived products even in treatments at low severities, and decreased slightly with severity. Under the severest conditions assayed, the high-molecular weight fractions accounted for about 2% of total xylan-derived products, no matter of the temperature considered. The low molecular weight fraction (DP $<$ 9) was the predominant one, with proportions that increased slightly with severity owing to the increased hydrolysis. The relative amounts of medium molecular weight compounds

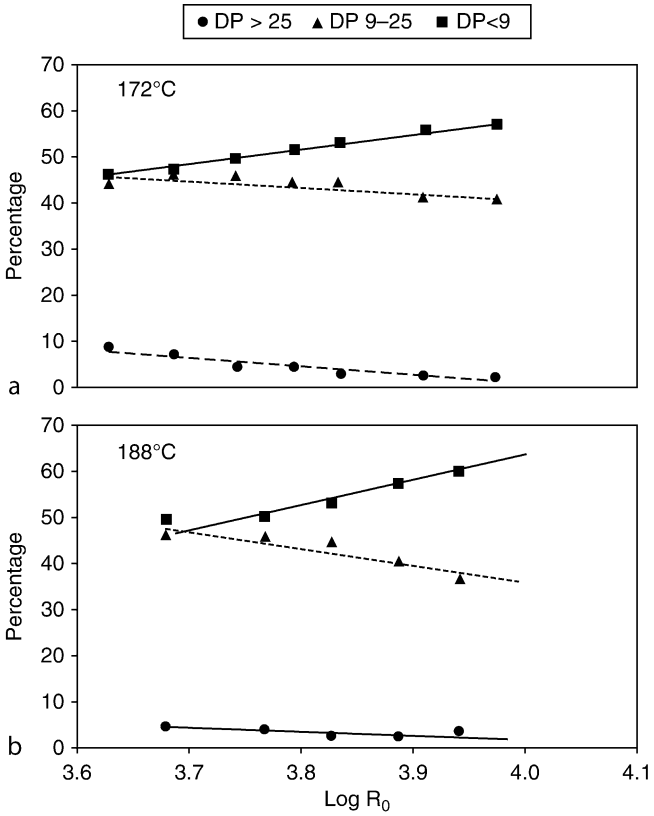


Figure 14.3
Relative proportions of XO with different DP range obtained in hydrothermal treatments of rice husks carried out at different temperatures and severities.

decreased continuously with severity (owing to the higher participation of depolymerization reactions compared to the ones causing the hydrolysis of high molecular weight fractions), to reach values around 40% under the severest conditions assayed. The main conclusion from this study was that 90–94% of the total rice husk xylan-derived products corresponded to oligosaccharides with DP $<$ 25 operating at low severities, and that operating under harsh conditions (R_0 in the vicinity of 10^4 min), about 60% of the total xylan-derived compounds corresponded to fractions with DP $<$ 9.

When low-DP XO are the target products, it must be taken into account that direct autohydrolysis under harsh conditions can be not suitable for operation: according to the kinetic models available, this operational strategy could result in

increased generation of undesired compounds (particularly, monosaccharides and monosaccharide-degradation products), which should be removed in order to obtain purified XO, and could limit the yield in XO.

Alternatively, low DP-XO can be obtained by enzymatic DP reduction of autohydrolyis reaction products using endo-1,4-β-xylanases (also named 1,4-β-xylan xylanhydrolases, E. C. 3.2.1.8). This strategy allows the production of low DP-XO at high yields.

The effects of xylanases depend on the structures of both substrate and enzymes. Based on hydrophobic cluster analysis and similarities in amino acid sequences, xylanases have been classified into families 5, 8, 10, 11 and 43 of glycoside hydrolases. Most xylanases belong to the glycosyl hydrolase families 10 and 11 (denoted GH-10 and GH-11), which present different physicochemical properties (including structure, molecular weight, pI and thermal stability), and exert different catalytic activity in the presence of substituted xylopyranosil units. GH-10 xylanases are characterized by high molecular weights and low pI values, while the low molecular mass endoxylanases with high pI values are classified as glycanase family 11.

In order to avoid the production of xylose from XO, the enzymes selected for DP reduction should be free from xylobiase activity. For this reason, cloned endo-1, 4-β-xylanases are preferable to raw xylanase preparations from wild strains of xylanolytic microorganisms. Recent results dealing with the DP reduction of xylan-derived compounds without causing the degradation of xylobiose and low-molecular weight oligomers to xylose have been reported for commercial endo-xylanase preparations from cloned microorganisms. Shearzyme 2x (a GH-10 xylanase produced using recombinant DNA techniques from a strain of *Aspergillus oryzae* carrying the gene coding for the enzyme from *Aspergillus aculeatus*), Pentopan Mono BG (a GH-11 xylanase, obtained by heterologously expressing the *Thermomyces lanuginosus* in *Aspergillus oryzae*), and Pulpzyme HC (a GH-11 xylanase produced by submerged fermentation of a genetically modified *Bacillus* microorganism) have been employed for DP tailoring of xylan-derived products (Vegas et al., 2008b). The differences in structure and mode of action of enzymes resulted in different DP distribution of the hydrolysis products. In experiments with raw autohydrolysis liquors, Shearzyme 2x led to the highest proportion of oligomers with DP in the range 2–3, and to slightly higher amounts of compounds with DP > 7 than the GH-11 enzymes. Pentopan Mono BG and Pulpzyme HC gave oligomers with DP in the range 4–5 or 6–7 as major reaction products. Experiments with refined autohydrolysis liquors confirmed the superior ability of Shearzyme for obtaining compounds with DP in the range 2–3, and the

comparative advantage of Pulpzyme for hydrolyzing XO chains to compounds with DP < 7.

Depending on the biomass feedstock, a variety of technologies have been employed for manufacturing refined, low-DP oligosaccharides. For example, an opposite philosophy to the one described above (enzymatic hydrolysis and physicochemical processing, instead of autohydrolysis and xylanase hydrolysis) has been suggested. The operational mode selected (xylanase processing of bleached kraft pulp, and further heating under acidic conditions) enabled the production of a mixture of xylose and XO having xylotriose, xylotetraose and xylopentaose as major components (Izumi et al., 2000).

The composition of XO depends on the nature of the native-xylan containing feedstocks employed as a raw material, on the possible pretreatments suffered by the raw material before performing the hydrolytic breakdown of xylan, and on the operational conditions employed in this latter stage. For example, when using hardwoods as feedstocks, preliminary processing under alkaline conditions to isolate xylan results in the saponification of acetyl groups, and the subsequent xylan hydrolysis results in non-acetylated XO. Oppositely, when native raw materials are subjected to autohydrolysis for XO production, the reaction products keep the major structural properties of the xylan present in the feedstock, presenting a rich substitution pattern.

Experimental studies have been carried out to assess the structure of XO from a variety of sources, including wheat bran, brewery's spent grains, corncobs and *Eucalyptus* wood (Kabel et al., 2002a). Under autohydrolysis conditions leading to the maximum XO yield, the xylose-degradation reactions were negligible, whereas arabinose was preferentially splitted off from the xylose-containing chains, and partially degraded to furfural and levulinic acid. Again, the relative amounts of arabinose and xylose in oligomers from autohydrolysis treatments depend on the feedstock considered: under intermediate severity conditions, XO from wheat bran autohydrolysis are essentially free from arabinose, whereas the production of XO branched with arabinose (with an arabinose to xylose ratio of 0.3) has been reported for the autohydrolysis products of brewery's spent grains. For corncobs and *Eucalyptus* wood hydrolysis products, the reported ratios arabinose/xylose were 0.04 and 0, respectively; and uronic substituents were bound in both cases. Acetylated XO containing uronic substituents are abundant in *Eucalyptus globulus* wood autohydrolyis products (Kabel et al., 2002a).

14.2.5 Biological Properties of Xylooligosaccharides

As cited before, XO cause health effects when ingested as part of the diet (for example, as active ingredients of functional foods) through the modulation of colonic microbiota. From a nutritional point of view, XO behave as nondigestible oligosaccharides (NDO's), which are not degraded by the low-pH gastric fluid and digestive enzymes, but are metabolized in the large bowel, and show prebiotic effects (discussed in detail elsewhere in this volume).

A recent review (Moure et al., 2006) identified (among others) the following potential XO biological effects:

- Antioxidant activity (with DPPH-radical scavenging and inhibition of erithrocyte hemolysis activities)
- Protection against low-density lipid peroxidation
- Prevention of atherosclerosis
- Prevention and treatment of oxidative stress, anemia and arteriosclerosis
- Treatment of vaginal and urogenital infections
- Low-glycemic index carbohydrate substitute, with applications in the prevention of type II diabetes
- Cosmetics and skin-related pharmaceuticals (for example, in the treatment of atopic dermatitis), collagen production enhancer, active components of moisturizing preparations, and treatment of epithelial covering tissue)
- Antihyperlipidemic agents, with activity against cholesterol, phospholipid and triglycerides
- Antihyperlipidemic activity
- Hair growth stimulant
- Inhibition of melanin and inhibition of melanoma cell proliferation
- Active agents against inflammation
- Therapeutic agents for osteoporosis treatment
- Hyaluronic acid-formation promoters
- Histamine-release inhibitors
- Enhancers of metal ion absorption
- Cytotoxic, antimicrobial and bacteriostatic agents
- Cancer cell apoptosis inducers
- Antiallergy agents
- Prevention and treatment of immune disorders

14.2.6 Technological Properties, Commercial Applications and Complementary Aspects

Considered as ingredients for functional foods, XO present remarkable technological properties. The sweetness of xylobiose is equivalent to 30% that of sucrose; the sweetness of other XO is moderate and possess no off-taste. XO are stable over a wide range of pH (particularly in the acidic range, even at the relatively low pH value of the gastric juice) and temperature (up to 100°C). Water activity of xylobiose is higher than that of xylose, but almost the same as glucose. Antifreezing activity of xylobiose on water at temperatures higher than −10°C is the same as that of xylose, but greater than that of glucose, sucrose and maltose. Considered as food ingredients, XO have a good fragrance, and their non-cariogenic and low calorie characteristics allow their utilization in anti-obesity diets. In food processing, XO show advantages over inulin in terms of resistance to both acids and heat, allowing their utilization in low-pH juices and carbonated drinks. Moreover, the antioxidant activity of xylan-derived fractions (sometimes called "antioxidant fiber") is well known. It is usually ascribed to the presence of phenolic groups esterified to the polysaccharide chains and/or antioxidant fractions with different chemical nature in XO concentrates (for example, melanoidins coming from Maillard reactions or free low molecular weight phenolics coming from acid-soluble lignin). Recently, the ability of XO with acidic substituents for reducing iron ions has been reported, suggesting new application fields.

XO show potential in pharmaceutical and feed formulations, as well as in agricultural uses, but their most important market seems to correspond to food applications. In this field, XO present advantages compared to other oligosaccarides in terms of healthy effects and concentration thresholds, but their comparatively high production costs are hindering a wider and faster market development. To accomplish this goal, further improvements in processing technology would be necessary.

The Japanese market pioneered the incorporation of XO into foods, and today they are used in a wide variety of commercial products, including combinations with soya milk, soft drinks, tea or cocoa drinks, nutritive preparations, dairy products (milk, milk powder and yoghurts), candies, cakes, biscuits, pastries, puddings, jellies, jam and honey products, and special preparations for health food for elder people and children. Other application areas of XO include the synbiotic foods (made up of a prebiotic and a probiotic). The first synbiotic of this type was "Bikkle" from Suntory, successfully marketed since 1993, which contains bifidobacteria, XO, whey minerals, and Oolong tea extract. Additionally,

the potential role of XO in the reduction of lifestyle-related diseases as well as the maintenance and improvement of human health is also a potential development factor. With the increasing health consciousness among consumers and the rapid progress of physiologically active functional foods, the future profile of products containing oligosaccharides with biological activities seems to be greatly promising (Nakakuki, 2005).

The bright future of XO for food applications is demonstrated by the dramatic increase in demand observed since 1994. For example, the production of Suntory increased from 70 tones in 1994 to 300 tones in 1996, and more recent data (Taniguchi, 2004) estimate the XO consumption in 650 ton of XO per year in Japan, which accounts for about one-half of the world market. Interestingly, the selling price reported for XO was the highest one among 13 different types of oligosaccharides (Taniguchi, 2004).

14.3 Mannans and Mannan-Derived Products

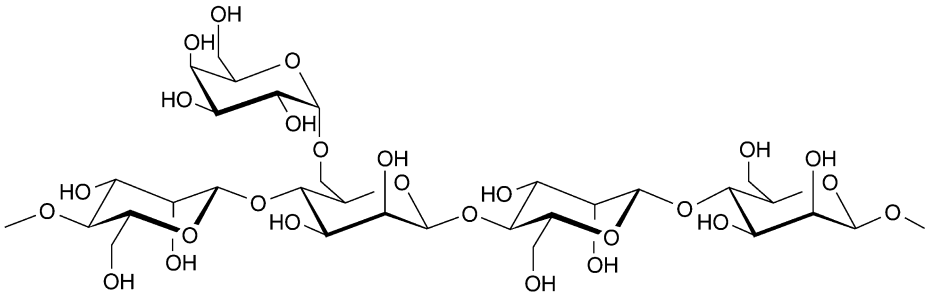
14.3.1 Structure, Occurrence, Hydrolytic Degradation and Processing

Mannans are linear or branched polymers made up of mannose, galactose, and glucose as structural monomer components. Mannan-type polysaccharides include galactomannans, glucomannans and galactoglucomannans. *Figure 14.4* shows representative mannan structures. Whereas the backbone of galactomannan is made up exclusively of β-(1→4)-linked D-mannopyranose residues in linear chains, glucomannan has both β-(1→4)-linked D-mannopyranose and β-(1→4)-linked D-glucopyranose- residues in the main chain (Ebringerova and Heinze, 2005).

Mannans are present in softwoods, hardwoods, ramie, bulbs, tubers, seeds, roots, and leaves of some non-gramineous monocotyl plants as structural, hemicellulosic polymers, as well as in gums. Galactomannans from different sources show differences in the distribution of D-galactosyl units along the polymer structure. Some of the structural units of mannans can be substituted by *O*-acetyl groups.

Water soluble galactomannans heavily substituted with galactopyranosyl residues (30–96%) are abundant in the cell walls of storage tissues (endosperm, cotyledons, perisperm) of seeds. Those from the endosperm of some leguminous seeds, such as guar (*Cyanopsis tetragonoloba*), locust bean or carob (*Ceratonia siliqua*), and tara gum (*Caesalpinia spinosa*), are widely used commercially.

General structure of galactomannan



General structure of glucomannan

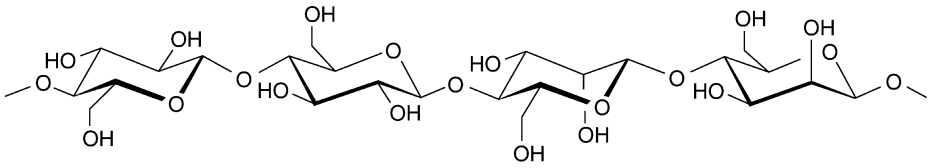


Figure 14.4
Structure of representative mannans.

The most commonly used galactomannans in the food industry are guar and locust bean gums. Guar gum is composed of a mannose backbone with galactose side chains, and is used primarily as a thickening and water binding. Locust bean gum has a reduced number of galactose residues attached to the mannose backbone compared to guar gum, and is well known for its interaction with other gums such as carrageenan and xanthan gum, giving gels with particular texture characteristics.

Details on the structure and the pseudoplastic rheological behavior of locust bean galactomannans can be found in the article by Lazaridou et al. (2001). The water-soluble galactomannan present in the seeds of the Chinese traditional medicine plant fenugreek (*Trigonella foenum-graecum*) contain mannopyranose and galactopyranose residues with a molar ratio of 1.2:1.0, where the major chain is made up of β-(1,4)-linked D-mannopyranose residues, and 83.3% of the corresponding structural units are substituted at C-6 with a single residue of α-(1,6)-D-galactopyranose. Fenugreek mannan is a highly efficient water-thickening agent with applications in the food industry.

The tubers of konjac (*Amorphophallus konjac*, a potato-like plant belonging to the *Araceae* genus employed in Asia as gelling agent, thickener, emulsifier and rheological modifier) contain glucomannan of high molecular weight, which

consists of sequences of mannose separated by glucose units in the backbone. The glucose to mannose ratio is 1 to 1.6, with one acetyl group per six glucose residues (Ebringerova and Heinze, 2005).

The chemical methods of mannan hydrolysis are based in the same concepts already described for XO. Both solubilization and DP reduction of the mannan-containing hemicellulosic polymers present in spruce wood have been achieved in a single step by steam processing and microwave irradiation (Palm and Zacchi, 2003). Steam treatments at 200°C led to products with a mean molecular weight of 3,400 g/mol and a maximum molecular weight of 12,000 g/mol. Operation under harsher conditions led to products of lower average molecular weight.

Manooligosaccharides have been prepared by thermal hydrolysis of spent coffee grounds (Asano et al., 2003) and by high-temperature hydrolysis of coffee bean and waste coffee (Nakamura et al., 2002). In the latter case, the main products were oligosaccharides containing up to 10 monosaccharide units.

Aqueous processing (with or without acidification by CO_2) has been employed for the manufacture of mannan oligosaccharides with molecular weights in the range 2,000–6,000 g/mol, starting from natural mannan polysaccharides (Murota and Yamanoi, 2003), whereas acid hydrolysis of coffee bean dregs and lees resulted in oligosaccharides containing up to 10 mannose residues (Fujii et al., 2001).

Partial hydrolysis of konjac glucomannan in media catalyzed by acids has been assayed for manufacturing edible films and for obtaining low-molecular weight compounds for which new application areas are expected. Except for the molar mass, no structural differences have been found between the polymeric substrate and its hydrolysis products.

As an alternative to acid or aqueous chemical treatments, the hydrolytic depolymerization and debranching of mannan can be carried out by enzymatic hydrolysis. For this purpose, both endohydrolases and exohydrolases are necessary. The main-chain mannan-degrading enzymes include β-mannanase, β-glucosidase, and β-mannosidase, but additional enzymes (such as acetyl mannan esterase and α-galactosidase) are required to remove side-chain substituents that are attached at various points on mannan.

Enzymatic hydrolysis of coffee mannan (isolated from green defatted *Arabica* beans by delignification, acid washing and subsequent alkali extraction) with *Sclerotium rolfsii* mannanases resulted in products with lower viscosity and enhanced processability. Upon enzymatic processing, various mannooligosaccharides (including mannotetraose, mannotriose, and mannobiose) were released (Sachslehner et al., 2000).

Guar gum galactomannan has been depolymerized by combined or sequential treatments with α-galactosidase and β-mannanase. Hydrolysis of guar and locust bean gums with β-mannanases from *Penicillium oxalicum* resulted in the production of low molecular-weight oligomers, which accounted for 92% of the total released saccharides. The DP range of oligomers was 2–7 for guar gum and 2–6 for locust bean gum (Kurakake et al., 2006). Enzymatic processing of konjac glucomannan with a purified β-mannanase from *Trichoderma* spp. resulted in the formation of oligosaccharides identified as M-M, G-M, M-G-M, M-G-M-M, and M-G-G-M, where G- and M- represent β-1,4-D-glucopyranosidic and β-1,4-D-mannopyranosidic linkages, respectively (Park, 2006).

Mannans can also be hydrolyzed by enzymes having pectinases and cellulases as major activities. Pectinases from *Aspergillus niger* with polygalacturonase activity have been employed for debranching and depolymerization of guar galactomannan (Shobha et al., 2005), leading to products of commercial interest as functional food ingredients. In the same way, processing of konjac with commercial cellulase preparations has been proposed for the manufacture of glucomannan hydrolysates (Al-Ghazzewi et al., 2007).

Multistage processes have been reported for obtaining either refined mannan-containing materials suitable as hydrolysis substrates or for mannooligosaccharide refining. Nunes et al. (2006) purified soluble galactomannans from roasted coffee infusions by precipitation with 50% ethanol, followed by anion exchange chromatograhy and further separation in Sepharose; whereas Wu and He (2003) started from mannan-containing gum, which was solvent-extracted at pH < 5.5, processed for solvent removal, diluted with water, treated with mannanases at controlled pH, heated, filtered and spray-dried.

14.3.2 Biological Properties of Mannans and Mannan-Derived Products

A number of health effects have been reported for polymeric and oligomeric mannans. Concerning prebiotic potential and other related effects, reported studies deal with:

- The ability of konjac mannan enzymatic hydrolysates to stimulate the growth of single strains of lactobacilli and bifidobacteria (*Lactobacillus acidophilus*, *Lactobacillus casei* or *Bifidobacterium adolescentis*) in single or mixed cultures. The growth of lactobacilli inhibited the growth of pathogens, Escherichia coli and Listeria monocytogenes.

Based on this finding it has been claimed that the hydrolysate can be used as a potential prebiotic and applied to a wide range of foods, feeds and pharmaceutical products has been claimed (Al-Ghazzewi et al., 2007).

- The beneficial health effects achieved by konjac glucomannan on the colon and bowel habits, including relief of constipation (which has been ascribed to increased stool bulk and improved colonic ecology). Konjac glucomannan is degraded almost 100% by the simultaneous action of enzymes and intestinal anaerobic bacteria present in human feces, producing formic acid, acetic acid, propionic acid, and 1-butyric acid. The proportions of these fatty acids were different among test subjects, and their total amounts ranged from 17.1 to 48.8% of the initial konjac glucomannan (Matsuura, 1998). The mechanisms by means of which konjac glucomannan modulates the bowel habit in healthy adults have been considered by Chen et al. (2006), who assessed the effects of konjac glucomannan ingestion at a dose of 4.5 g/day on the colon health. The beneficial effects observed were enhanced bowel movement, improved stool bulk and improved colonic ecology. Glucomannan supplementation increased significantly the concentrations of fecal lactobacilli, total bacteria, and daily fecal output of bifidobacteria, lactobacilli, and total bacteria. In addition, it increased the proportions of fecal bifidobacteria and lactobacilli with respect to the total fecal bacteria, whereas they decreased the relative proportions of clostridia compared to the placebo group. The suppression of clostridia growth in the human colon was achieved indirectly (through the action of bifidobacteria) or directly (through the physicochemical characteristics of glucomannan). Specific increases in the colonic bifidobacteria and lactobacilli caused by glucomannan might promote the bowel movement. The anti-constipation effects of glucomannan are well established. Treating patients with chronic idiopathic constipation with 1 g glucomannan/day resulted in statistically significant modification of the mouth-to-cecum transit time, compared with control groups, and a return to the normal range after the 10-day treatment, suggesting that chronic idiopathic constipation is a disease that involves the whole gut. Modifications of the intestinal habit and stool characteristics caused by glucomannan ingestion were assessed in a random, parallel, double blind, cross over trial study versus placebo involving 60 patients treated at two doses (3 and 4 g glucomannan/day), which led to the conclusion that the 4 g/day treatment resulted in the highest improvement of the assessed parameters. Studies in children showed that glucomannan is also suitable for pediatric applications.
- The suitability of mannan oligomers (β-1,4-D-mannobiose, β-1,4-D-mannotriose, β-1,4-D-mannotetraose, and β-1,4-D-mannopentose), manufactured from coffee mannan by enzymatic hydrolysis followed by purification by active carbon chromatography, in supporting the growth of enterobacterial strains. All mannooligosaccharides

were utilised by *B. adolescentis*, *Lactobacillus acidophilus*, and *Lactobacillus gasseri*. Oppositely, detrimental bacteria such as *Clostridium perfringens* and *Escherichia coli* could not use mannooligosaccharides, affirming the prebiotic properties of the studied compounds (Asano et al., 2001). Mannooligosaccharides with DP up to 10 prepared from coffee bean dregs and lees by acid hydrolysis have been claimed as growth promoters for probiotic bacteria (Fujii et al., 2001). Mannooligosaccharides from thermally hydrolyzed spent coffee grounds, administered to volunteers at 1.0 g/day or 3.0 g/day, resulted in significantly increased contents of *Bifidobacterium* (suggesting that the ingestion of mannooligosaccharides caused *Bifidobacterium* to be the predominant bacteria in the intestine) and in improved defecation and defecating conditions. The resistance of mannooligosaccharides obtained from thermal hydrolysis of spent coffee grounds to human salivary α-amylase, artificial gastric juice, porcine pancreatic enzymes and rat intestinal mucous enzymes, together with their fermentability by human fecal bacteria leading to the formation of SCFA (mainly acetic, propionic and n-butyric acids), confirmed that mannooligosaccharides are indigestible saccharides and are potential agents for improving the large intestinal environment (Asano et al., 2003).

- The ability of hydrolyzed guar gum in promoting colonic health through the production of short-chain fatty acids at comparatively high concentrations with respect to alternative oligosaccharides and commercial dietary fiber.
- The activity of partially hydrolyzed guar gum (together with a recommended oral rehydratation solution) as an active agent for treating acute diarrhea in children, through the action of short-chain fatty acids released during its fermentation by colonic bacteria. Partially hydrolyzed guar gum has been reported to have a different *in vitro* fermentation pattern than native guar gum, whereas the concentration profiles of the acids formed were dependent on the molecular weight. The molar ratios of acetate increased with the molecular weight, while molar ratios of propionate decreased. The molecular weight of substrates leading to optimal production of short-chain fatty acids has been characterized on the basis of experimental data.
- The ability of partially hydrolyzed guar gum to regulate the bowel habit, with an increase in defecating frequency and softer stools in people with constipation, but also significantly improvement of diarrhea in patients with gastrointestinal intolerance. These effects are in relation with the improvement of the intestinal microbiota balance, which also resulted in prevention from infection and colonization by *Salmonella enteritidis*. Additionally, therapeutic benefits derived from the utilization of partially hydrolyzed guar gum have been observed in the treatment of the irritable bowel syndrome, with decrease in constipation-predominant and diarrhea-predominant forms of the disease and decreased abdominal pain. The health

effects have been ascribed to the higher colonic contents of short-chain fatty acids, and higher lactobacilli, and bifidobacteria concentrations resulting from the selective fermentation of hydrolysates. The guar gum hydrolysate has been shown to enhance the fecal lactobacilli and bifidobacteria concentrations in healthy men. In the treatment of the irritable bowel syndrome, partially hydrolyzed guar gum was well tolerated and preferred over wheat bran by patients, limiting the probability of patients abandoning the prescribed regimen, and suggesting that these products are a valid option for high-fiber diet supplementation. Based on a number of technical properties and health effects (suitability for being used in enteral products and beverages, reduction of laxative dependence in a nursing home population, reduction of the incidence of diarrhea in septic patients receiving total enteral nutrition, reduction in symptoms of irritable bowel syndrome, increased presence of *Bifidobacterium* spp. in the gut), partially hydrolyzed guar gum has been recommended for clinical nutrition applications.

- The suitability of mannans for low-calorie diets, based on their indigestible nature (Fujii et al., 2001). Obesity is a well-established risk factor for cardiovascular disease, diabetes, hyperlipidemia, hypertension, osteoarthritis, and stroke. With 50% of Europeans and 62% of Americans classed as overweight, the food industry is waking up to the potential of products for weight loss and management. Konjac glucomannan is highly effective in the treatment of obesity due to its ability to induce a satiety feeling, and daily doses in the range 2–3 g/day have been recommended as a part of anti-obesity diets. In a study of obese women observing a hypocaloric diet, administration of galactomannan preparations resulted in accelerated weight loss, decreased desire to eat, and hunger sensations before meals. A galactomannan containing, high fiber diet caused beneficial health effects in obese patients, as it induced satiety and had beneficial effects on some cardiovascular risk factors, principally decrease in plasma LDL-cholesterol concentrations. Mannooligosaccharides obtained from coffee administered to humans resulted in abdominal fat reduction. A dietary supplement containing two sources of mannan (among other components) has been reported to be suitable for increasing body weight loss, reducing the percentage of body fat and absolute fat mass, and reducing the circumferences of upper abdomen, waist and hip. Konjac glucomannan was well-tolerated at doses of 2–4 g/day, and resulted in significant weight loss in overweight and obese individuals, a fact ascribed to its beneficial effects of promoting satiety and fecal energy loss. A comparative analysis of three fiber supplements (glucomannan, guar gum and alginate) plus a balanced 1,200 kcal diet showed a comparative advantage of glucomannan for achieving weight reduction in healthy overweight subjects.

- The cardioprotective effects of mannans, for which hypocholesterolemic effects derived from interactions with the bile acids have been reported, probably in relation with their ability to form gels. The bile acids interact with the gel, loosing their ability to carry cholesterol to the mucose surface, and allowing the excretion of cholesterol in feces. On the other hand, the hepatic cells compensate the loss of bile acids by synthesis from cholesterol, decreasing cholesterolemia also by this way. Similarly, fenugreek galactomannan has been reported to inhibit bile acid absorption, leading to lower cholesterol levels. Protective effects have been observed for coffee mannan-derived oligosaccharides. The administration of a coffee drink containing mannooligosaccharides (3.0 g/day) to a group of healthy adults increased in the amount of excreted fat, and decreased the fat utilization. Ingestion of partially hydrolyzed guar gum lowered the serum cholesterol level in humans by improving lipid metabolism without reduction of protein utilization. Guar gum has been successfully used for the treatment of hypercholesterolemia, even though clinical application has not become widespread because a large amount of guar gum is unpalatable. Partially hydrolyzed guar gum ingested in a yoghurt drink (which also contained sunflower oil and egg yolk) has shown ability to lower the intestinal uptake of fat and cholesterol, reducing the risk of vascular disease. The results indicated that the hydrolyzed guar gum decreased the bioaccessibility of both fat and cholesterol in a dose-dependent manner, through a depletion-flocculation mechanism, which antagonized the emulsification by bile salts and resulted in decreased lipolytic activity, and so in lower bioaccessibility of fat and cholesterol. The effects of diet supplementation with glucomannan combined with total-body exercise program has been studied in overweight (body mass index >25 kg/m^2) sedentary men and women. The combination of resistance and endurance exercise training program with glucomannan diet regimen improved both HDL-cholesterol and total cholesterol/HDL-cholesterol ratio. The hypocholesterolemic effects obtained by supplementing the diet of normocholesterolemic subjects with 2.4 g of chitosan and glucomannan/day were confirmed by the observed significant decreases of serum total, HDL- and LDL-cholesterol concentrations, these effects being likely mediated by increased fecal steroid excretion. Administration of konjac mannan fiber to hyperlipidemic, hypertensive type 2, high-risk type 2 diabetic patients, improved blood lipid profile and systolic blood pressure, possibly enhancing the effectiveness of conventional treatment. Glucomannan has been recommended as a rationale adjunct to diet therapy in primary prevention in high risk hypercholesterolemic children, whereas a combination of glucomannan and plant sterols substantially improved plasma LDL-cholesterol concentrations.

- The effects on glycemia observed in studies with different types of mannans and mannan-containing dietary supplements. Ingestion of fenugreek with water was shown to lower postprandial blood glucose levels in type II diabetic subjects. Administration slows gastric emptying and delays the absorption of glucose in intestines, decreasing the rise in blood sugar following a meal. Delayed nutrient absorption, particularly of glucose, can reduce the glycemic index of the consumed food. Another favorable property is the ability of galactomannan to form a gel layer on the surface of stomach cells that protects against damaging compounds. High-molecular weight galactomannans are more resistant to enzymatic degradation, suggesting an ability for exerting a more sustained water binding capacity. Supplementing a high-carbohydrate diet with konjac glucomannan improved the glycemic metabolic control and the lipid profile of subjects with the Insulin Resistance Syndrome, suggesting a potential for therapeutic treatment. Partially hydrolyzed guar gum significantly reduced the level of plasma glucose, improving the acute postprandial response and the insulin response. Several clinical trials with healthy subjects and diabetic patients showed that guar gum can reduce postprandial glucose excursions. In healthy subjects, guar gum reduced the maximal rise in blood glucose by 60%, and the area under the blood glucose curve by 68%. Guar gum therapy has favorable long-term effects on glycemic control and lipid levels in non-insulin dependent diabetes mellitus subjects, and could reduce the postprandial glucose in insulin-dependent diabetes mellitus patients. The ability of glucomannan for lowering postprandial insulin levels has been confirmed in trials with healthy subjects, and its utilization has been recommended for treating patients with previous gastric surgery suffering from postprandial hypoglycaemia. In a test performed with healthy nondiabetic subjects, a novel low-viscosity beverage containing guar gum was suitable for reducing the postprandial glycemic response to an oral glucose challenge, providing a means to stabilize blood glucose levels by reducing the early phase excursion, and then by appropriately maintaining the later phase excursion in healthy nondiabetic humans.
- The antioxidative capacity of the products resulting from the *in vitro* fermentation of unhydrolyzed and two types of hydrolyzed konjac mannan with different DP. The experimental determination of antioxidant activities by a variety of experimental methods, proved that the fermentation of unhydrolyzed mannan by selected strains of bifidobacteria and lactobacilli produced antioxidative capacity mainly by preventing the initiation of ferrous ion-induced peroxidation, whereas the fermentation of mannooligosaccharides increased principally the radical-scavenging ability and hindered lipid peroxide formation.

- The mineral absorption-promoting activity reported for partially hydrolyzed guar gum.
- The immunostimulatory properties reported for mannans, including acetylated mannan from *Aloe* (acemannan) and fenugreek galactomannan. Activation of immune responses by acemannan resulted in antiviral and antitumoral activities, and its action has been partially ascribed to the ability to promote differentiation of immature dendritic cells. The immunostimulatory properties of mannan-derived saccharides have been claimed in a recent patent, whereas in experiments with human immune cells, the production of reactive oxidizing species was decreased by mannan-oligosaccharides with DP in the range 6–7.
- The suitability of low molecular-weight products obtained by enzymatic hydrolysis of natural products for ameliorating sepsis, inflammatory diseases (such as rheumatoid arthritis) and allergies. These effects have been claimed in recent patents.
- The applications of mannan and galactomannan saccharides (including gums and their partially hydrolyzed reaction products) as active agents for reducing colonization of the oral cavity by plaque and disease-causing microorganisms.

14.4 Arabinogalactans

14.4.1 Structure, Occurrence, and Technological Properties of Arabinogalactans

The term arabinogalactan refers to a kind of water-soluble polysaccharide, long, densely branched, made up of galactose and arabinose moieties (Hori et al., 2007). Arabinogalactans are widely spread throughout the plant kingdom, and appear in edible and inedible plants, including leek seeds, carrots, radishes, black gram beans, pears, maize, wheat, red wine, italian ryegrass, tomatoes, ragweed, sorghum, bamboo grass and coconut meat and milk.

Arabinogalactans occur in two structurally different forms described as type I and type II:

- Type I arabinogalactans have a linear (1→4)-β-D-Galp backbone, bearing 20–40% of α-l-Araf residues (1→5)-linked in short chains, in general at position 3. It is commonly found in pectins from citrus, apple and potato.
- Type II arabinogalactans, known as arabino-3,6-galactan, have a (1→3)-*β*-D-Gal*p* backbone, heavily substituted at position 6 by mono- and oligosaccharide side

chains composed of arabinosyl and galactosyl units, and occur in the cell walls of dicots and cereals often linked to proteins (known as arabinogalactan proteins). The acidic type II arabinogalactan has uronic acid residues incorporated in its side chains and belongs to the groups of gum exudates (Ebringerova et al., 2005). Type II arabinogalactan is most abundant in the heartwood of the genus *Larix* (Western larch/*Larix occidentalis* or Mongolian larch/*Larix dahurica*) and occurs as minor, water-soluble component in softwoods. Other sources of type II arabinogalactan are coffee beans and arabic gum.

Most commercially-available arabinogalactan is produced from larch trees. Certain tree parts of western larch were reported to contain up to 35% arabinogalactan, whereas high-grade products from larch are composed of more than 90% arabinogalactan. All arabinogalactans isolated from *Larix* spp. are water-soluble, nitrogen-free polysaccharides of the 3,6-β-D-galactan type. The side chains consist of combinations of single galactose sugars, as well as longer side-chains containing galactose and arabinose residues. The galactose and arabinose units (consisting of β-galactopyranose, β-arabinofuranose, and β-arabinopyranose) are in a molar ratio of approximately 6:1, and comprise more than 99% of the total glycosyl content. A trace amount of glucuronic acid is generally also found (Odonmazig et al., 1994). *Figure 14.5* shows a tentative structure proposed for larch arabinogalactan. A curdlan-like triple helical structure, based on preliminary X-ray fiber diffraction data, has been reported for this polymer (Chandrasekaran and Janaswamy, 2002). The composition of arabinogalactan from siberian larch wood and the influence of the purification conditions on the properties of the final product have been reported recently.

Alkaline treatments of coffee bean cell walls followed by cellulase digestion allowed the identification of four different structures. The outer part of cell walls presented arabinogalactan protein-rich layers. The arabinogalactan-protein

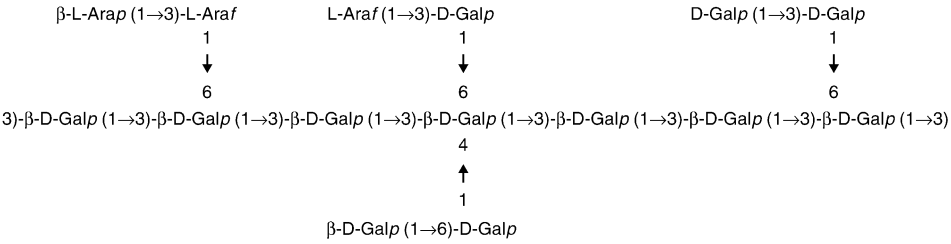


Figure 14.5
Tentative structure for larch arabinogalactan (Kelly, 1999).

(AGP) fraction of green coffee beans accounts for about 15% of the dry bean. Type II arabinogalactans are present in coffee brews, and their amount and composition depend on factors such as type of coffee, roasting and grinding degree, and brewing.

Acacia gum (gum arabic) is a natural dietary fiber produced by *Acacia* trees, principally composed of polysaccharides and proteoglycans, the latter being AGP. Gum arabic also contains complex, ramified arabinogalactans, as well as some lipids (Yadav et al., 2007). Acacia tree exudates obtained without any chemical or enzymic processing contained more than 90% soluble fiber on dry weight (Fremont, 2007). Studies on the composition of crude *Acacia* gums revealed the presence of arabinogalactan, AGP and glyco-proteins. Details on the main chemical and physical features of *Acacia* gum and its fractions isolated by hydrophobic interaction chromatography (arabinogalactan-peptide, AGP and glycoprotein fractions), have been reported.

Some technological properties of selected arabinogalactans are:

- Larch arabinogalactan is a dry, free-flowing powder, with a very slight pine-like odor and sweetish taste. Because of its excellent solubility, low viscosity, mild taste and easy dissolution in water and juices, it is easily administered. Other favorable technological properties are high water solubility and narrow molar mass distribution. According to the Generally Recognized as Safe (GRAS) Notice No. GRN 000047 (FDA, Center for Food Safety & Applied Nutrition, Office of Premarket Approval), the properties of larch arabinogalactan permit its use as a film-former, foam adhesive, additive, thickener, bulking agent, emulsifier, and as a therapeutic agent. Based on its food grade status and numerous studies supporting the safety of larch arabinogalactan, it is considered to be extremely safe with minimum to no toxicity. Larch arabinogalactan's properties make it a relatively easy therapeutic tool to employ in pediatric populations.
- Arabic gum has a unique combination of excellent emulsifying properties, low viscosity when dissolved, and is free from taste and odor, making it suitable for use in the food industry, as it can be incorporated in large amounts to foodstuffs without disturbance of their organoleptic properties. *Acacia* gums show unusual rheological properties, which have been ascribed to their surface properties, and especially to its ability to form elastic films at air/water or oil/water interfaces.
- Coffee AGP shows interesting rheological features, which suggest that coffee beans could be used as an alternative source of surface-active polymers for many commercial applications.

Reported processes for manufacturing arabinogalactan polysaccharides from different sources (including *Portulaca oleracea* and ground green or roasted coffee beans) are based on sequential stages of processing, including mechanical treatments, chemical reaction (by enzymatic hydrolysis), extraction and refining of extracts (Curti et al., 2005). The resulting products have been proposed for applications such as manufacture of functional foods, cosmetic compositions with physiological activity, formulation of glassy matrices, and beverages. *Acacia* gum commercial preparations have been proposed for the development of innovative functional foods with favorable nutritional and health claims (Meance, 2004).

14.4.2 Prebiotic Properties of Arabinogalactans

The prebiotic effects of arabinogalactans are based on their resistance to human digestive enzymes and to their fermentability by human intestinal bacteria (Crociani et al., 1994; Vince et al., 1990). Intestinal fermentation of arabinogalactan leads to the stimulation of the endogenous microbiota and to the production of short-chain fatty acids (Englyst et al., 1987; Vince et al., 1990), as well as to increased growth of beneficial anaerobes, such as bifidobacteria and lactobacilli, using culture-based methods (Vince et al., 1990).

During the last decade, larch arabinogalactan, known as larch gum in food applications, has produced emerging commercial and scientific interest, which follows closely upon recent reports related to the beneficial physiological effects of commercial arabinogalactan and its immunomodulatory properties (Ebringerova et al., 2005). As a dietary fiber supplement, larch arabinogalactan has several beneficial properties, including the abilities to promote the growth of friendly bacteria (specifically, increasing anaerobes such as bifidobacteria and lactobacilli), and to increase the production of SCFA, as well as to decrease ammonia generation. Larch arabinogalactan administration to human subjects increased the levels of beneficial intestinal anaerobes, particularly *Bifidobacterium longum*, via their fermentation specificity for arabinogalactan compared to other complex carbohydrates (Crociani et al., 1994). Arabinogalactan is fermented by mixed populations of human fecal bacteria more slowly than starch and pectin, and the fermentation occurred without accumulation of free sugars or oligosaccharides. Time-course measurements of the polysaccharide remaining in the fermentation media showed that the arabinose side chains of arabinogalactan were co-utilized with the backbone sugars, and that the polysaccharide-degrading

activity was mainly cell-associated, but extracellular polysaccharidase activity increased as the fermentations progressed. In experiments, multicomponent substrate utilization occurred, leading to molar ratios of acetate, propionate, and butyrate of 50:42:8 (Englyst et al., 1987).

Prebiotic activity has also been reported for *Acacia* gums, based on human studies that showed high fermentability with no side effects even at high supplementation level. *Acacia* gum stimulates the development of beneficial bacteria and the production of SCFA, helping to regulate different functions such as gut transit or lipids metabolism (Fremont, 2007). However, thanks to its high molecular weight and to its complicated molecular structure, *Acacia* gum does not exhibit undesired nutritional side-effects such as laxative effect and/or flatulence. Its fermentation pattern is specific, leading to the preferent formation of propionate and butyrate through a progressive fermentation that could permit these beneficial end-products to reach the distal part of the colon (Meance, 2004). Ingestion of 10 or 15 g/day of a commercial *Acacia* gum concentrate for 10 days increased the stool weight and the total lactic acid-producing bacteria and bifidobacteria counts in stools, without affecting total anaerobe and aerobe counts. The magnitude of this selective effect was greater in subjects with a low initial fecal concentration of bifidobacteria. Fecal digestibility was around 95%, and its caloric value was 5.5–7.7 kJ/g. The concentrate showed good digestive tolerance, even if daily ingestions above 30 g caused excessive flatulence. Based on these data, the commercial product has been considered as a very well tolerated dietary fiber with bifidogenic properties believed to benefit intestinal health.

Most studies dealing with arabinogalactan have been conducted *in vitro*. When considering the reported results, it is important to take into account that the human colon is a complex environment, and that *in vitro* studies may not accurately represent bacterial activities within the human colon. Daily diet supplementation with 15 or 30 g arabinogalactan resulted in significant increases of total fecal anaerobes and *Lactobacillus* spp., this latter being believed to maintain and restore normal intestinal balance; but no significant changes in parameters such as fecal enzyme activity, transit time, frequency, fecal weight, fecal pH and short-chain fatty acids were observed (Robinson et al., 2001). These results have been ascribed to the fact that SCFA are believed to be quickly absorbed following their production; therefore, it is difficult to determine the total amount produced in human subjects. Additionally, it is noteworthy that arabinogalactans have been claimed as active principles of fluid diets for preventing or curing enteropathy.

14.4.3 Other Biological Properties of Arabinogalactans

Larch arabinogalactan (AG) has been reported to show immunomodulating properties (Ebringerova et al., 2005). AG from larch wood possesses a wide spectrum of physiological activities and due to that it can be applied as a medical product and biologically active food additive. Cholesterol-lowering and immunomodulating properties have been reported for arabinogalactan from several plant sources. Many herbs with well established immune-enhancing properties, such as *Echinacea purpurea*, *Baptisia tinctoria*, *Thuja occidentalis*, *Angelica acutiloba* and *Curcuma longa* also contain significant amounts of arabinogalactans. A purified, highly branched arabinogalactan polymer induced the proliferation of mouse lymphocytes in a dose-dependent manner. Response of mice to arabinogalactan from coffee beans showed proliferation of macrophage and splenocyte, evidencing that arabinogalactan can stimulate immunocytes and enhance immune responses. *In vitro* and animal studies suggest that the effects of larch arabinogalactan on the immune system could be related to the activation of "natural killer cells," and perhaps other white blood cells as well, and also possibly alter levels of immune-related substances such as interleukins, interferon, and properdin. Thus, arabinogalactan could be suitable for treating a number of chronic diseases characterized by decreased natural killer cell activity, including chronic fatigue syndrome, viral hepatitis, HIV/AIDS, and autoimmune diseases, such as multiple sclerosis. Stimulation of natural killer cell activity by larch arabinogalactan has been associated with recovery in certain cases of chronic fatigue syndrome. By virtue of its immune-stimulating properties, larch arabinogalactan has been shown to affect a slight increase in CD4 cell counts, in addition to a decreasing susceptibility to opportunistic pathogens. On the other hand, the enhancement of the immune response may decrease the incidence of bacterial infections, particularly those caused by Gram negative microorganisms, such as *Escherichia coli* and *Klebsiella* spp. Prophylactic applications of larch arabinogalactan might include use as an immune-building agent for individuals with a propensity to ear infections and other upper respiratory infections. Arabinogalactans have been reported to act as activators of lymphocytes and macrophage, a valuable property that could be useful in the immunoprevention of cancer.

Experiments with animals showed several physiological properties of *Acacia* gum, including increases in glucose and bile acid absorption. *Acacia* gum has been proposed as a hypoglycemic agent, based on the postprandial glucose response of type II diabetic subjects to the administration of a commercial

concentrate. Other effects observed include the decrease the glycemic index of food products, making them healthier and more suitable for diabetics. However, the human response to diet supplementation with larch or tamarack arabinogalactan resulted in poor long-term results in terms of serum lipids and glucose concentrations; and administration of a commercial arabinogalactan from western larch at doses of 15 or 30 g did not result in significant changes in blood lipids or blood insulin (Robinson et al., 2001). However, significant decreases in fat consumption were observed when subjects consumed the 30 g dose of AG, a fact that could be related to increased reports of bloatedness. Thus, the sensation of fullness may have led subjects to avoid high fat foods.

The studies of Robinson et al. (2001) and Vince et al. (1990) coincided in the finding that fecal ammonia levels decreased significantly upon ingestion of arabinogalactan. This finding may be related to with the significant increases in total anaerobes, as some anaerobic colonic bacteria prefer to utilize ammonia as a nitrogen source rather than amino acids or peptides when fermenting carbohydrates. High colonic ammonia levels may have detrimental health implications, including cytopathic effects on colonic epithelial cells, effects on the intermediary metabolism and DNA synthesis of mucosal cells, and toxicity against epithelial cells. Based on these finding, arabinogalactan may be of clinical value in the treatment of portal-systemic encephalopathy, a disease characterized by ammonia build-up in the liver (Vince et al., 1990).

Additionally, a variety of health benefits, including mitogenic, antimutagenic, gastroprotective, and antimicrobial effects have been reported for arabinogalactans, which also show ability to block metastasis of tumor cells to the liver. Its utilization as a cancer protocol adjunct causing potential therapeutic benefits has been proposed.

The whole set of biological properties of arabinogalactan suggest an array of clinical uses, both in preventive medicine and in clinical medicine, as a therapeutic agent in conditions associated with different pathologies.

14.5 Pectins and Pectin-Derived Products

14.5.1 Occurrence, Structure and Applications of Pectins

Pectins are complex, acidic heteropolysaccharides with gelling, thickening and emulsifying properties, which are recognized to promote human health as dietary fiber (Gulfi et al., 2007), and are widely used in the food and pharmaceu-

tical industries for their technological properties and health effects. The food applications of pectins, mainly derived from its ability to form gels in the presence of Ca^{2+} ions or a solute at low pH, including their utilization in jams and jellies, fruit products, dairy products, desserts, soft drinks, frozen foods, and more recently in low-calorie foods as a fat and/or sugar replacer. The food applications of pectins have been revised exhaustively by Voragen et al. (1995). In the pharmaceutical industry, they are used to achieve a number of health effects (including decrease of blood cholesterol levels and treatment of gastrointestinal disorders, as explained below). Other applications of pectin include use in edible films, paper substitute, foams and plasticizers.

Almost all higher plants contain pectins, which have a lubricating and cementing function. Pectins are major components of the primary wall and the middle lamella of plant cells, where they represent around 40 wt.% (on dry matter basis) of the cell walls of fruits and vegetables. In citrus fruits, they are present at a cellular level (membranes, juice, vesicules and core) in different quantities, depending on the fruit variety and maturity stage. Several byproducts of the food industries are used for pectin extraction, including citrus peels, apple pomace, sugar beet pulp and (in a minor extent) potato fibers, sunflower heads and onions (May, 1990).

The structure of pectins is complex. Polymers are made up of different monomers, galacturonic acid being the predominant one. Two types of "regions" have been identified in pectins: the "smooth" regions, made up of galacturonic acid, and the ramified "hairy" regions, where most of neutral sugars are located.

Pectins are believed to have structural features that are common to a variety of fruit and vegetable tissues, even if the structural elements may vary depending on the source. The following pectin structural elements can be identified:

- Homogalacturonan, composed of a α-(l→4)-linked D-galacturonic acid (GalA) backbone which forms the "smooth region," accounts usually for about 60% of the total pectin, and has a length chain of 72–100 GalA residues. The structural units can be esterified at C-6, and/or *O*-acetylated at *O*-2 or *O*-3. The type and degree of substitution determine the properties of pectins.
- Xylogalacturonan, composed of homogalacturonan substituted with xylosyl moieties. The degree of xylosidation is between 25 and 75% (Schols et al., 1995).
- Rhamnogalacturonan I, whose backbone is composed of repeated subunits of α-(1→2) -L-rhamnose-α-(l→4) -D-galacturonic acid. The rhamnosyl residues of rhamnogalacturonan I can be substituted at *O*-4 with neutral sugar side chains,

mainly composed of galactosyl and/or arabinosyl residues. These side chains are substituted by either both GalA or polymeric chains, such as arabinogalactan I or arabinan. The proportion of branched rhamnosyl residues varies from about 20 up to 80% depending on the polysaccharide source.

- Rhamnogalacturonan II, which forms a distinct region within homogalacturonan. It contains clusters of four different side chains with a variety of sugar residues.
- Arabinan, consisting of α-(1→5)-linked arabinosyl backbone, which usually is substituted with α-(l→2) or α-(l→3) linked arabinosyl side chains.
- Arabinogalactan I and arabinogalactan II, whose structures have been described above.

Figure 14.6 shows the structural units of pectins. The relative proportions of the various structural elements may vary significantly among different plant tissues (Voragen et al., 1995). Recent studies have reported data on pectins' structure and discussed their macromolecular structure, as well as additional ideas on how pectins are integrated into the plant cell wall.

Pectins are usually extracted from suitable substrates by mild acidic treatments (typically in media with pH in the range 1–3, obtained by externally added mineral acids, operating at 50–90°C for 3–12 h), which lead to products essentially constituted of homogalacturonans, with limited amounts of neutral sugars (Guillotin et al., 2005; Kravchenko et al., 1992). Pectins can be further precipitated by adding alcohol (isopropanol, methanol or ethanol), and the gelatinous mass is pressed, washed, dried and ground. Depending on the source, GalA residues in homogalacturonans can be present as free carboxyl groups, methyl-esterified, or acetylated; further alkaline or acid processing allows the reduction of the ester group content. Acidic processing of pectin-containing native substrates may also result in the solubilization of phenolics with antioxidant activity, which can be recovered from the liquors for further utilization (Berardini et al., 2005).

Commercial pectins are mainly classified as a function of their methyl-esterification degree (measured as the amount of moles of methanol per 100 moles of GalA), since this is the main parameter influencing the physical properties. The commercial products can be classified as:

- High methyl-esterified pectins, which are extracted from pomace or peels of apples under acidic conditions, and precipitated with alcohol to yield products in which 50% or more of the GalA moities are methyl-esterified. This kind of pectins can be classified according to their setting time in ultra rapid set, rapid set, medium rapid set and slow set pectins (May, 1990).

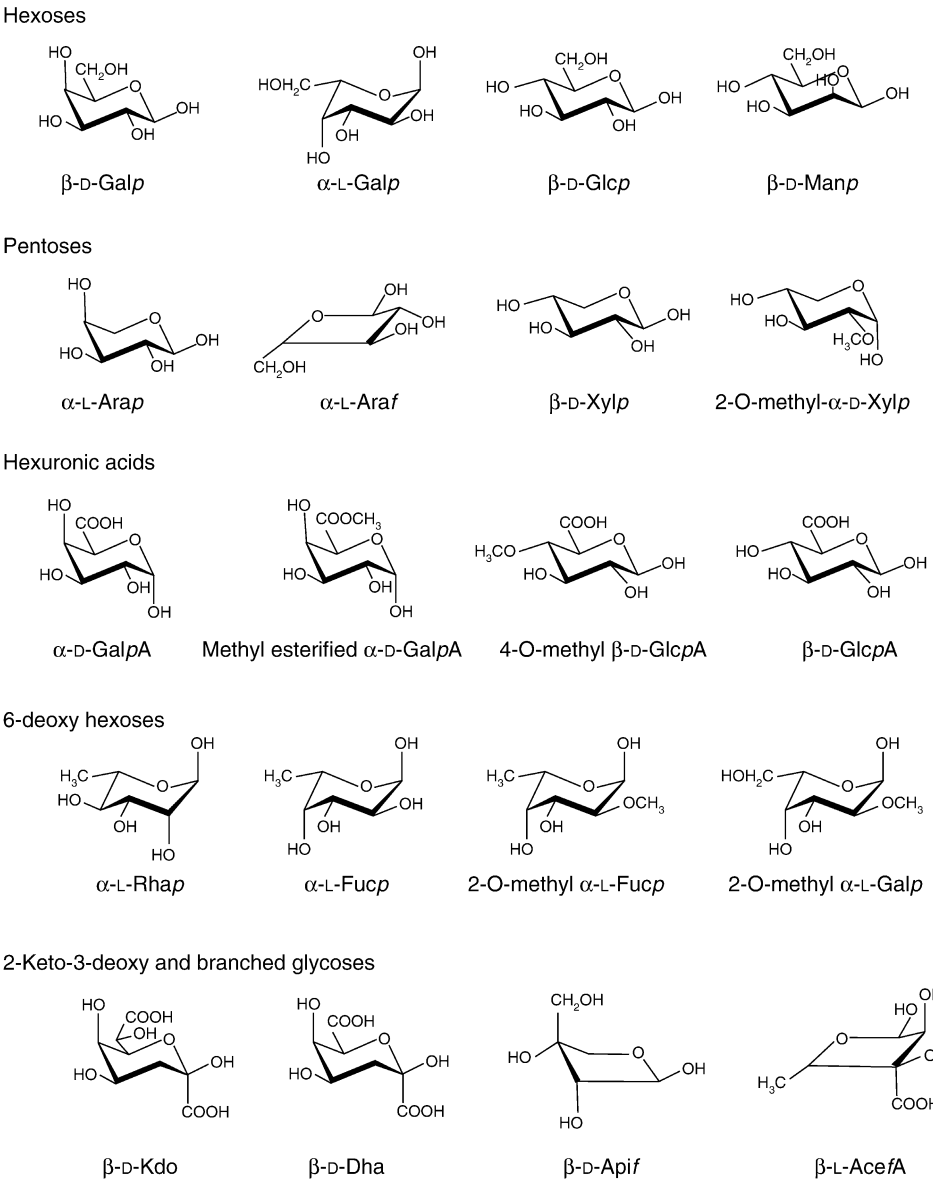


Figure 14.6
Structural elements of pectins.

- Low methyl-esterified, non-amidated pectins, which are obtained by controlled de-esterification of high methyl-esterified pectins under acidic or alkaline conditions. Processing under alkaline conditions has to be performed under low-severity conditions to avoid polymer degradation.

- Low methyl-esterified, amidated pectins, which are obtained by chemical reaction of the methyl-ester groups in the presence of ammonia and alcohol, resulting in the presence of amide groups at the C-6 position of the GalA residue. Amidation results in improved gelling properties. Only 25% substitution of GalA by amide groups is permitted in food products.

Commercial pectins also include the "acetylated pectins," which are extracted from sugar beet residues as a low-quality material in terms of gelation due to their limited molecular weight and low proportion of GalA (derived from a higher content of neutral sugars). Acetylated pectins are used for their emulsifying properties.

Depolymerization of native pectins is carried out during the extraction process, which is usually catalyzed by chemical, enzymatic or combined methods. The final DP distribution depends on the nature of the substrate and on the operational conditions used for processing.

Partial acid hydrolysis of pectins leads to depolymerization, but also to structural modification due to the different susceptibilities of the various glycosidic bonds towards hydrolysis. Mild acid hydrolysis of galacturonans (in media containing 0.2–2 M sulfuric acid for 72 h at 80°C) resulted in the cleavage of the galacturonic acid chains into oligomeric forms without any degradation (Garna et al., 2006). Pectin fractionation may take place by β-elimination (which splits specifically the glycosidic linkages next to methoxylated galacturonic acid units without steric limitation, and is the predominant mechanism under neutral or mild acidic conditions), a reaction competitive with de-esterification. This latter reaction is promoted by cold, alkaline conditions, and results in the removal of acetyl groups and methyl esters (Kravchenko et al., 1992).

Reactions of pectins with water or steam at high temperatures and pressures have been proposed as alternative methods for chemical modification. Mitchell and Mitchell (1996) claimed the production of oligomers with DP in the range 1–20, at 25% yield, by depolymerization of polyuronic acid in aqueous media, whereas Miyazawa et al. (2008) reported a severity analysis on the hydrothermal processing of pectin using a combination of semi-batch and plug-flow reactors, in which the production of oligomers, monomers, and degradation products was assessed. In a related study, galacturonic acid and its oligomers were produced by hydrolysis of polygalacturonic acid by hydrothermal treatments with hot, compressed water at 453–533 K, which led to the formation of monomeric and oligomeric reaction products with DP < 11 at yields in the range 22–33.4% of the polymeric material. Further enzymatic hydrolysis with pectinases resulted in

increased formation of monogalacturonic acid and oligomers with DP 2 and 3 (Miyazawa and Funazukuri, 2004).

Fast extraction of pectin from orange albedo was accomplished with steam processing at 15 psi for 2–6 min ("flash extraction"). The molecular weights of pectin isolates obtained under selected conditions were higher than the ones of commercial citrus pectin. Flash extraction of pectin from orange and lime peel led to premium pectin together with an oligosaccharide fraction with prebiotic activity (Hotchkiss et al., 2005). The effects of the more influential variables (processing pressure, moisture content of the feedstock treatment and processing time) on pectin extraction from orange peels have been assessed by response-surface methodology, whereas steam treatments have been coupled with hydrogen peroxide treatments for refining purposes.

Other technologies employed for the physicochemical extraction of pectins include microwave heating of orange peels in acidic media, whereas depolymerization has been carried out by ultrasound processing, extrusion at alkaline pH, and gamma ray irradiation.

Enzymes can be used for processing both pectin-containing raw materials and purified pectins (sometimes in combination with chemical processing) to accomplish a variety of objectives, including extraction, depolymerization, purification, removal of selected substituents or side chain and structural elucidation. Pectic enzymes are classified according to the mode of attack on their specific structural element of the pectin molecule. The most important enzymes employed to reduce the DP and/or to modify the structure of polymeric pectin fragments are:

- Endo-polygalacturonase (EC 3.2.1.15), which cleaves the α-(1→4)-D galacturonan linkages in homogalaturonan segments randomly, leading to the formation of oligosaccharides.
- Exopolygalacturonase (EC 3.2.1.67 and EC 3.2.1.82), which attack homogalacturonan chains from the non-reducing end, splitting GalA units.
- Rhamnogalacturonan hydrolase (EC 3.2.1.-), which hydrolyses the α-D-(1→4)-Gal*p*A-α-L-(1→2)-Rha*p* linkage in the rhamnogalacturonan I backbone, leaving Rha*p* at the non-reducing side.
- Rhamnogalacturonan lyase (EC 4.2.2.-), which enables the eliminative cleavage of α-L-(1→2)-Rha*p*-α-D-(1→4)-Gal*p*A backbone of rhamnogalacturonan I, leaving a 4-deoxy-β-L-threo-hex-4-enepyranosyluronic acid (unsaturated GalA) group at the non-reducing end.
- Endoxylogalacturonan hydrolase (EC 3.2.1.-), which hydrolyses the α(1→4)-D linkages of xylose-substituted galacturonan moieties in xylogalacturonan.

- Rhamnogalacturonan rhamnohydrolase, an exo-acting pectinase, which possesses a specificity to release terminal rhamnosyl residues (1→4)-linked to α-galacturonosyl groups.
- Rhamnogalacturonan galacturonohydrolase, which is able to release a GalA moiety connected to a rhamnose residue from the non-reducing side of rhamnogalacturonan I chains, but is unable to liberate GalA from homogalacturonan.

Additional effects (for example, removal of methylester or acetyl groups, and arabinan side chains) can be achieved by enzymes. Almost total solubilization ("liquefaction") of pectin-containing raw material can be accomplished by using enzyme cocktails containing cellulases, hemicellulases and pectic esterases. For example, liquefaction of apple cell walls may result in complete pectin removal, leading to a high-molecular weight pectic fraction, termed "modified hairy region," made up of highly branched rhamnogalacturonan. Liquefaction has been applied to other feedstocks, such as carrots, potato fiber and tissues from red beets, pear, carrot, leek, and onion. In the case of red beets, the poor enzymic conversion by pectolytic and cellulolytic enzymes was ascribed to the high degree of acetylation of pectins.

Partial enzymatic hydrolysis leads to the formation of oligosaccharides, an objective that can be accomplished by using free or immobilized enzymes. The latter method allowed the production of dimers to pentamers from citrus pectin using pectinases immobilized on affinity gel. Enzymatic processing may enable the recovery of valuable byproducts: for example, processing of bergamot peel with pectinolytic and cellulolytic enzymes led to the simultaneous solubilization of carbohydrates (with the formation of monosaccharides and oligosaccharides in relative amounts dependent on the reaction time) and to low molecular weight flavonoids (Mandalari et al., 2006).

As an example of mixed chemical-enzymatic treatments, the sequence of steps reported for the structural elucidation of pectins from sugar beet pulp includes extraction with water, oxalate, hot acid, and cold alkali, together with base-catalyzed β-elimination, de-esterification, and treatments with four different enzymes; the procedure allowed the identification of sequences of galacturonic acid residues with relatively little neutral sugar attached ("smooth" fragments), and "hairy" fragments containing feruloyl groups. For structural determination, the hairy- and alkali soluble- fractions of beet pectins have been subjected to the action of four different enzymes prior to mild hydrolysis by 0.05 M trifluoroacetic acid. Also, in structural studies, citrus peel pectins have been solubilized with oxalate or dilute hydrochloric acid, and the isolated

products have been treated with selected enzymes. Alternatively, enzymatic processing can be employed for modifying the structure of pectins obtained by chemical methods (for example, to cause demethoxylation of fractions obtained by acid hydrolysis of citrus peels, in order to obtain products with improved emulsifying properties); or for modifying the properties of the resulting solid residues. For the latter purpose, treatments with 0.06–0.5% dilute sulfuric acid at 100–140°C had a positive effect on the rate of subsequent enzymic hydrolysis of orange peel by a mixture of cellulolytic and pectinolytic enzymes.

As previously explained in the case of xylooligosaccharides, membrane technologies are playing an increasing role in the production and refining of pectin oligosaccharides. The sequential processing of enzymically hydrolyzed pectate by ultrafiltration and nanofiltration membranes enabled the separation of di- to pentamers, whereas ultrafiltration followed by diafiltration has been proposed for making a pectin concentrate from beet pulp. The same combination has been studied as an alternative purification method to alcohol precipitation, whereas enzymatic membrane reactors have been employed for the generation of pectin oligosaccharides from orange peel albedo (Hotchkiss et al., 2003), and from methylated pectins.

14.5.2 Prebiotic Potential of Pectins and Pectin-Derived Products

The prebiotic potential of pectins and pectin-derived oligosaccharides depend on the physicochemical characteristics of the considered substrates, and particularly on their molecular weight and degree of esterification.

Concerning the comparative *in vitro* degradability of pectins by human fecal flora, their relative fermentability (as measured by total production of acetate, propionate and butyrate after 24 h of fermentation) was lower than the ones of starches, locust bean gum, arabic gum and guar gum. The whole degradation process has been reported to occur in two sequential stages: in the first one, the polymeric substrates are fragmented into unsaturated oligogalacturonans (which behave as intermediate reaction products) by the action of bacterial enzymes; and in the second one, the oligogalacturonans disappear as a result of their further fermentation by the gastrointestinal microbiota, leading to the formation of short-chain fatty acids. Low-esterified pectins were depolymerized and fermented *in vitro* by human fecal flora faster than the highly esterified one.

Fermentation with individual or mixed cultures of selected intestinal bacteria (*Bacteroides thetaiotaomicron* and *Escherichia coli*) was slower than the one carried out with a complete fecal flora, owing to the lower pectin-degrading activity. No pectin-degrading activity was found in pure cultures of *Escherichia coli*, whereas the disappearance of oligogalacturonic acids in the later stages of fermentation by intestinal bacteria resulted in an increased formation of short-chain fatty acids. *In vitro* fermentation of media derived from the enzymatic liquefaction of apple parenchyma with fecal inocula resulted in the formation of a spectrum of short-chain fatty acids, suggesting their possible application as dietary fiber.

Prebiotic effects have been suggested for highly branched, arabinan containing "hairy" regions of pectins with complex molecular structure, extracted under alkaline conditions, by means of *in vitro* fermentation assays with human fecal bacteria. Comparatively, higher amounts of short-chain fatty acids were produced from the pectin hairy regions than from commercial pectins, with particularly remarkable differences in propionate concentrations. Concomitantly, pH decreased to a larger extent in the fermentation of hairy substrates. These findings are of interest owing to their beneficial influence into colon health, suggesting that hairy regions of pectins might be an interesting dietary fiber with enhanced prebiotic properties compared to commercial pectins (Gulfi et al., 2007).

Intestinal fermentation of pectic substrates depends on the molecular weight. Pectic oligosaccharides, which can be produced from waste biomass or low-cost byproducts (such as apple pomace, orange peels or sugar beet pulp) have been considered as novel prebiotics, which may have better functionality than those currently established on the market, enabling a selective composition shift in the gut microbiota by enhancing the growth of beneficial, health-promoting bacteria. The prebiotic potential of pectic oligosaccharides has been reported to be higher than that of the pectins they are derived from, owing to the more selective fermentation. This behavior has been observed, for example, in pectic oligosaccharides prepared by enzymic hydrolysis of commercial citrus pectin and apple pectin, which enabled higher growth rates of bifidobacteria compared to the original pectins. Oligosaccharides derived from pectins by either enzymic hydrolysis or by flash extraction displayed potential prebiotic properties, as they selectively increased the populations of beneficial bacteria such as bifidobacteria and lactobacilli and decreased undesirable bacteria such as clostridia (Rastall et al., 2005). In contrast, oligosaccharides derived from high-methoxy citrus pectin, low-methoxy apple pectin and orange peel enhanced the

growth of bifidobacteria and lactobacilli while limiting the growth of pathogens in mixed fecal batch cultures (Hotchkiss et al., 2004). *In vitro* fermentation of pectin oligosaccharides derived from Valencia oranges, mainly consisting of arabinogalactan pectic side chains and xyloglucan, showed bifidogenic effects, as revealed by the increases in acetate, butyrate and propionate concentrations upon fermentation (Hotchkiss et al., 2007). However, fermentation of oligosaccharides derived from orange peel pectin in mixed fecal bacterial cultures (containing glucose and pectic oligosaccharides from rhamnogalacturonan and xylogalacturonan as potential carbon sources) were suitable for increasing the concentrations of bifidobacteria and *Eubacterium rectale*, as well as the production of butyrate, but no prebiotic effect was found. The growth rates of bifidobacteria depend on the structural characteristics of pectin oligosaccharides (and particularly, on their degree of methylation). *Bifidobacterium angulatum*, *B. infantis* and *B. adolescentis* had higher growth rates on pectic oligosaccharides derived from pectins than on the original, polymeric substrates. In general, greater fermentation selectivity was obtained with substrates of lower degrees of methylation.

Based on the above ideas, it can be inferred that non-selectively fermented polysaccharides like pectin can have their bifidogenic properties improved by partial hydrolysis, enabling a selective increase in the populations of beneficial bacteria, such as bifidobacteria and lactobacilli, as well as a decrease in undesirable bacteria, such as clostridia. These effects are based on the more selective fermentation of oligomeric compounds in comparison with the parent polysaccharides (Rastall et al., 2005).

Looking for enhanced prebiotic activity and high tolerance, pectin oligosaccharides have been used in combination with neutral prebiotics as components of infant fourmulae. Administration of mixtures of pectin and neutral oligosaccharides to infants resulted in increased concentrations of bifidobacteria and lactobacilli in stools, softer stool consistency, and decreased fecal pH. In a study dealing with the evaluation of mixtures of galacto-, fructo- and pectin oligosaccharides carried out on a group of healthy, full-term, partially breast-fed children, ingestion of prebiotics resulted in an increase of the *Bifidobacterium* counts and a decrease in the proportions of the *Bacteroides* and *Clostridium coccoides* groups, suggesting that the proposed combination was clinically safe and effective on infant microbiota, as it minimized the alteration of fecal microbiota after cessation of breast-feeding and enhanced the proportions of bifidobacteria (Magne et al., 2008).

14.5.3 Other Biological Effects of Pectins and Pectin-Derived Products

Beyond prebiotic characteristics, pectins and pectin oligosaccharides have been reported to cause a number of healthy effects, including regulation of lipid and glucose metabolism, decreased glycemic response and blood cholesterol levels, anti-cancer and immunological properties, reduction of damage by heavy metals, anti-obesity effects, dermatological applications, anti-toxic, anti-infection, antibacterial and antioxidant properties. Owing to their ability for exerting multiple health-promoting effects (see below), pectic oligosaccharides have been proposed as a new class of prebiotics (Hotchkiss et al., 2004) which however have not been yet evaluated as such in humans.

Experimental evidence suggests that the application of pectin and pectin-derived products on health care seems to be dependent on the fine carbohydrate structures, bringing many possibilities of benefits for human being. Even if in some cases natural pectin had no activity, chemical and enzymic modifications may provide useful health care products. Selected health effects of pectins and pectin-derived products are discussed in the following paragraphs.

Epidemiological evidence exists that a diet high in water-soluble fiber is inversely associated with the risk of cardiovascular diseases. One of the ways of preventing the cardiovascular disease is to lower serum LDL cholesterol levels. This objective can be reached by pectin ingestion without concomitant effects on HDL cholesterol or triacylglycerol concentrations (Theuwissen and Mensink, 2008). Hypocholesterolemic action has been reported for a variety of pectin-containing feedstocks, whereas blood cholesterol-lowering activity has been also claimed for pectin hydrolysates, and low-molecular weight citrus pectin (prepared by hydrolysis with a crude enzyme preparation from *Kluyveromyces fragilis*) showed a repressive effect on liver lipid accumulation. Recent studies have been devoted to assess the mechanisms of pectin action on cholesterol, particularly through experiments with animals. Feeding rats with pectin-containing products from the enzymic liquefaction of apples resulted in physiological benefits derived from an increased excretion of bile acids and neutral sterols. Upon pectin ingestion, hepatic conversion of cholesterol into bile acids increases, which will ultimately lead to increased LDL uptake by the liver (Theuwissen and Mensink, 2008). Pectins with high methoxyl content show ability to bind lipids and bile acids. Experiments using rats fed with hydrophobic amidated pectins showed significantly altered cholesterol homeostasis, and the results suggested that this substrate might be considered as a clinically effective

hypocholesterolemic agent. Several hypotheses have been proposed to explain the hypocholesterolemic effects, including binding of bile acids by fiber, interference of lipid absorption and reduced hepatic cholesterol synthesis by propionate. Their major hypocholesterolemic effects have been reported to be related with bile acids. Increased fecal excretion of bile acids and neutral sterols has been reported in the experimental assessment of various pectic substrates, whereas ingestion of dietary fibers (including pectin) has been correlated with decreased plasma cholesterol concentrations. The decrease of cholesterol levels in liver and blood serum and its increase in feces could explain the beneficial effects of these dietary fibers in disease prevention. The interference with bile acid absorption, and hence, reduction of cholesterol absorption, seems to be the major mechanism for the observed effects (Ikeda and Sugano, 2005). Pectins, by enhancing fecal bile acid excretion, may cause increased hepatic synthesis of bile acids and liver depletion of cholesterol, resulting in a higher rate of cholesterol synthesis and reduced serum cholesterol concentrations. Increased fecal excretion of bile acids has been reported to be negatively correlated with the serum cholesterol level. Hypolipidemic activity has been ascribed to both the lower rate of absorption and higher rate of degradation and elimination of lipids. On the other hand, the hypolypidemic action of pectins depends on the raw material and on the processing conditions: some isolates presented highly significant hypolipidemic activity, while others were less significant, or even insignificant, in their action. Hypolipidemic activity has been reported for high-methoxyl pectins through experiments with rats: pectins of increased methylation degrees enabled higher concentrations of free and secondary bile acids in the cecum and colon of conventional rats. With increasing degree of methylation, more bile acids were transported into lower parts of the intestinal tract and excreted, whereas the proportion of secondary bile acids decreased. In a related study, rats fed with dietary highly methylated apple pectin resulted in decreased body weight gains and total cholesterol and triglyceride levels compared to specimens fed with an standard diet, suggesting that high methoxyl fractions from apple could be used as a functional ingredient to decrease cardiometabolic risk factors. The structure of pectins has been found significant for the physiological effects caused in hamsters fed with either lemon pectin or "smooth regions" of lemon pectin made up of polygalacturonic acid regions; the latter fraction showed comparatively better cholesterol-lowering properties. Lemon peels and lemon peel wastes have been reported to be as effective in lowering plasma and liver cholesterol in hamsters as the pectin extracted from the peels.

Concerning the effects of pectins on glucose metabolism, these compounds (alone or in combination with other dietary fibers), have been reported to play a

constructive role by releasing sugars and absorbing sugars slowly in the intestinal tract; this would be of interest, for example, for reducing the severity of diabetes mellitus (Butt et al., 2007). Pioneering studies emphasized the ability of pectins for decreasing the post-prandial hyperglycaemia, thus improving the control of blood glucose concentration. The ability of pectins to reduce the blood glucose levels (Butt et al., 2007) and the postprandial rise in blood glucose and serum insulin in patients with type-II diabetes, have been claimed in more recent studies. Pectins have been reported to be beneficial for fish oil-treated diabetic patients, whereas oligogalacturonic acids (with DP up to 20, obtained by enzymatic processing of pectin and further fractionation), and/or their physiologically acceptable salts, have been claimed as hyperglycaemia inhibitors, suitable for diabetes treatment. However, other studies did not found significant effects of pectin administration on the postprandial glucose response of rats and humans, and their potentially valuable effects could be more in relation with satiety feeling associated with sustained late blood glucose levels.

Pectins and pectin oligosaccharides may provide protection against some types of cancer. Oligosaccharides derived from pectins were able to stimulate apoptosis in these cells (Hotchkiss et al., 2004; Rastall et al., 2005), and cause the growth inhibition of colon cancer cells; this suggests that there is a possibility for developing dietary protection against colon cancer (Hotchkiss et al., 2007). The ability for inhibiting the growth of cancer cells has also been reported for citrus pectin subjected to irradiation, as well as for the dialyzed products from irradiated samples (with molecular weight <10 kDa). Some commercial pectins have also been shown to have anti-cancer activity in rats and mice: fractionated pectin significantly induced apoptosis in both androgen-responsive and androgen-independent prostate cancer cells. Lower molecular weight compounds obtained by endopolygalacturonase treatment retained the apoptosis activity, which was lost when the polymeric substrate was subjected to mild base treatment. However, pectin did not inhibit intestinal carcinogenesis in mutated mice with predisposition for intestinal tumorigenesis.

Immunomodulatory effects have been reported for pectins, which have sometimes been attributed to microbiota-dependent effects. This topic has been covered in a recent review (Vos et al., 2007) for various non-digestible-carbohydrates, including pectins, and the immunological properties of structurally characterized pectic polymers from defined sources have been assessed recently.

The ability of some pectins to bind heavy metals make them potentially suitable for treatment of poisoning by heavy metals. Study of the effects of low-esterified pectin in toxic damage to the liver caused to animals by enteral

treatment with lead acetate showed that pectin treatment promoted the decrease in lead content in the liver, and the recovery of parameters of lipid metabolism; a study on the toxic injury induced in rat liver by enteral administration of lead acetate showed that pectin administration enabled a fast reduction of lead concentration in liver, resulting in decreased lipid peroxidation, as well as the normalization of total cholesterol and triglyceride levels in blood serum and liver.

Pectin oligosaccharides have been reported to provide protection against *Escherichia coli* toxins (Hotchkiss et al., 2007; Rastall et al., 2005).

Oligo-galacturonides show ability for influencing cell-cell and cell-matrix interactions, displaying activity against the adhesion of pathogenic bacteria such as enteropathogenic and verotoxigenic strains of *E. coli* (Rastall et al., 2005).

Antibacterial activity has been reported for enzymic hydrolysates of cold water-extracted pectin from *Crataegus sanguinea*, consisting of oligomers with DP $>$ 5–6. Antibacterial activity against *Escherichia coli* has been reported for lemon pectin hydrolysates (obtained using H_2SO_4 or pectinase), with increased effects for the latter type, these were attributed to the differences in free undissociated carboxyl groups, methoxyl groups, and DP of oligogalacturonides.

Finally, it can be cited that pectins or pectin oligomers have been employed for other applications, including prevention of infection, prevention or treatment of skin inflammation, cosmetic or dermatological compositions, anti-obesity formulae, antioxidant activity, and as active principles of mineral absorption-promoter preparations.

14.6 Summary

- When ingested as a part of the diet, some biomass polysaccharides and/or their oligomeric hydrolysis products are fermented in the colon, potentially producing prebiotic effects. Xylans, mannans, arabinogalactans and pectins are suitable substrates for this purpose.
- Hydrolysis of the polysaccharides cited above can be achieved by chemical processing, enzymatic reaction, or combined chemical-enzymatic processes, resulting in products of lower DP. In many cases, refining of the reaction media is needed to obtain food-grade products, whose biological properties depend on their composition and DP distribution.
- Refining of hydrolysis media can be achieved by a number of separation methods, but multistage processing is frequently required to achieve purities in the desired range.

- Products of tailored DP (usually of oligomeric nature) may present advantages with respect to the polysaccharides they are derived from, particularly in terms of biological activity.
- The potentially prebiotic properties of biomass-derived prebiotics are related to their non-digestible nature and to their ability for being utilized by lactobacilli and bifidobacteria as specific substrates, leading to formation of SCFA (which are believed to contribute positively to colon health).
- Besides potentially prebiotic properties, the biomass-derived products considered in this study may cause a variety of favorable health effects (for example, limiting the cardiovascular risk, affecting the glucose metabolism, causing apoptosis of cancer cells or exerting immunomodulatory effects). Because of this, they can be considered as multipurpose, healthy food ingredients.
- Based on the consumers' awareness for healthy foods, the future of biomass-based prebiotics seems promisings. However, the future commercial developments must be based on sound scientific evidences of their biological properties, which need further assessment.

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List of Abbreviations

AG	Arabinogalactan
AGP	Arabinogalactan-Protein
AIDS	Acquired Immune Deficiency Syndrome
DP	Degree of Polymerization
DPPH	1,1-diphenyl-2-picrylhydrazyl
FISH	Fluorescent *in situ* Hybridization
GalA	Galacturonic Acid

G	β-1,4-D-glucopyranosil
GC-MS	Gas Chromatography-Mass Spectrometry
GH	Glycosyl Hydrolase
GRAS	Generally Recognized as Safe
HIV	Human Immunodeficiency Virus
HPSEC	High Performance Size Exclusión Cromatography
M	β-1,4-D-mannopyranosil
NDO's	Non Digestible Oligosaccharides
SCFA	Short Chain Fatty Acids
XO	Xylooligosaccharides
X2	Xylobiose

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15 Taxonomy of Probiotic Microorganisms

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15.1 Introduction

When referring to probiotics, one refers to probiotic strains, i.e., the microbial individuals, sub-cultures of billion of almost identical cells ideally derived from the same mother cell. Therefore, beneficial effects attributed to probiotics are ascribed in fact to specific strains. However, these strains have to be, by law, clearly identified at the species level (Pineiro and Stanton, 2007). In fact, probiotics have to be safe for consumption, and the evaluation of QPS – qualified presumption of safety – status by the European Food Safety Authority (EFSA) (Opinion, 2007) is discussed for species, not for single strains.

Also, corrected names have to be reported on products labels: failure of identification of the declared species is a commercial fraud and a consumer misleading, besides being an indication of unreliability of the product.

These two examples should clarify how important is the correct taxonomic identification of probiotic strains in the assessment of their reliability and efficacy.

The aim of the present contribution is to clarify which procedures, rules and scientific knowledge stand behind microbial names, as results of taxonomic analysis. Probiotic strains described to date fall in two different groups of microorganisms, namely bacteria and yeasts, which will be the focus of this treatment.

15.2 What is Taxonomy?

Taxonomy, from the Greek meaning categorization, can be viewed as a quest for order in nature. In other words, it is the analysis of the existing biodiversity in a systematic way, with the aim of arranging it in an ordered hierarchical scheme. This hierarchy is (should be) the result of the genealogical relationships between organisms (the *taxa*). The information used for these purposes has to be as complete as possible, encompassing morphology, physiology, ecology and genetics.

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A taxonomic procedure can be viewed as an iterative process, in which (i) organisms are clustered into groups on the basis of similarity; then (ii) groups are given formal names, indicated in italics, composed by a genus name and a specific identifier (e.g., *Homo sapiens*); and finally, other *taxa* are analyzed and can be either assigned to any already existing (i) and named (ii) group, or newly identified and described as novel groups. These three steps are indicated as *classification*, *nomenclature*, and *identification*, respectively (Staley and Krieg, 1989). The taxonomic hierarchy is based on the unit called species (see below). Then, Species are grouped in a Genus, Genera in a Family, Families in an Order, Orders in a Class, and Classes in a Domain. Three domains of life have been described comprising all the living organisms, two for the prokaryotes, Archaea and Bacteria, and one for eukaryotes. In the Domain Eukarya, four kingdoms are also recognized, i.e., Protista, Fungi, Animalia, and Plantae, to account for the diversity of eukaryotes, while no Kingdom category has been proposed above Classes in the Domains Archaea and Bacteria (Woese et al., 1990).

It is clear that a species in the bacterial domain must be, as a category, equivalent to a eukaryotic species, otherwise the "scheme of life" looses its meaning. However, clearly, biologically, species such as *Lactobacillus casei* (a bacterium) and ourselves as *Homo sapiens* are not comparable. Thus, it is clear that taxonomy is a necessary but conventional way of describing diversity. However, taxonomy has the important practical aim of making biodiversity accessible through cataloguing, therefore the species is considered to be equivalent as a category in the three domains, but it is circumscribed differently when analyzing different organisms.

15.2.1 Concept, Delineation and Naming of Species

The basic unit of the taxonomic scheme is the species. The species problem is one of the most debated topics in science, and will not be reviewed here: probiotic microorganisms are actually strains, not species, therefore it seems much more interesting here to focus on the relationship between species and strain, and explain how a species is circumscribed, i.e., which are the criteria used to determine if an isolate belongs to a known species or deserves a novel name.

Excellent reviews on the species concept and species circumscription are available for the two domains which include the probiotic strains known to date, i.e., Bacteria and Eukarya (Kingdom Fungi), (Giraud et al., 2008;

Rosselló-Mora and Amann, 2001). To date no archaeal strains with probiotic properties have been described, therefore that domain will not be reviewed here.

Considering species delineation, the practical criteria for the description of novel species will be reminded here. As for bacteria, the gene sequence for 16S rRNA gene is determined and analyzed to obtain the phylogenetic placement for the microorganism, then the closest neighbors are considered in a comparative study: DNA-DNA hybridization technique is then applied to assess the overall genomic similarity of the strains; if it is above 70% the strains are considered to belong to the same species, otherwise they represent different *taxa*. Also, relevant phenotypic characteristics of the strains are determined, e.g., patterns of fermentation of carbohydrates, in order to define a diagnostic trait which makes the novel species recognizable from its neighbors. More recently, other techniques have been suggested which should be useful in a more precise species delineation, such as Multi Locus Sequence Typing (Stackebrandt et al., 2002).

For fungi, and yeasts in particular, the procedure is less straightforward, and a novel species is described when genetic and/or morphological and/or reproductive differences are found, but these criteria can vary depending on the organisms under study.

This makes it clear that there are no rules for classification, as only experts can evaluate if a group of strains deserves a species status, and the standard procedure for species delineation of bacteria is pragmatic and useful, but does not have to be applied strictly.

On the contrary, rules do exist in nomenclature: once a group of strains is believed to represent a novel species, it deserves a name, which has to be given following precise regulation. Nomenclature of bacteria follows the International Code of Nomenclature of Bacteria (Lapage et al., 1990), and names are listed in the "Approved List 1980" and listed as Approved Lists 1980. Names published after 1980 or changes in names, to be valid, have to be published on the International Journal of Systematic and Evolutionary Microbiology – IJSEM – (known as International Journal of Systematic Bacteriology – IJSB – before 2000). They can also be published elsewhere, but, to be officially recognized, they have to be announced in the Validation Lists on IJSEM.

Nomenclature of yeasts follows, as for fungi, the International Code of Botanical Nomenclature, the recent version of which is the so-called Vienna Code, adopted by the Seventeenth International Botanical Congress, Vienna, Austria, July 2005 (McNeill et al., 2006). Species descriptions of unicellular eukaryotes are published on IJSEM, but also on other journals, and names are listed in the Index fungorum (see below for details).

As taxonomy is always linked to technical progress, taxonomic re-examinations often results in changes in nomenclature. Rules in nomenclature are necessary to avoid ambiguities and misunderstandings when taxonomic ree-valuations are performed and changes in names occur. The availability of lists of names, with rules of priority, also allows associating to new names characteristics linked to older ones, avoiding loss of information due to changes in names.

One of the most important points of the rules of nomenclature is that, when a novel species is described, a type strain (i.e., a strain chosen as a reference point among those grouped in the new species) has to be indicated, which has to be deposited in international culture collections, and be available to the scientific community for study and comparisons. Finally, it has to be pointed out that for bacteria one single correct name exists for a described species, while for yeasts there are two names, indicating the two different living forms, i.e., the teleomorph – the sexual reproductive stage, or the anamorph, the asexual reproductive stage.

15.2.3 Useful Links for Taxonomic Information

As anticipated, nomenclature is clearly regulated, and electronic versions of the Codes are available: the Bacteriological Code (Lapage et al., 1992) at http://www.ncbi.nlm.nih.gov/books/bv.fcgi?rid = icnb.TOC&depth = 2, and the Vienna Code (McNeill et al., 2006) at http://ibot.sav.sk/icbn/main.htm.

Also, lists of up-to-date names can be checked online, on the DSMZ (German collection of microorganisms and cell cultures, http://www.dsmz.de/bactnom/bactname.htm) or on the internet pages complied by J. P. Euzéby and updated after each issue of IJSEM (http://www.bacterio.cict.fr/). For yeasts the reference is the Index Fungorum, http://www.indexfungorum.org/.

For both bacteria and unicellular microorganisms, the journal publishing novel species descriptions is the International Journal of Systematic and Evolutionary Microbiology (www.sgmjournals.org).

Considering classification, the reference book for bacteria is the Bergey's Manual of Systematic Bacteriology, now at its second edition (2001), while, for yeasts, useful indications can be found in The Yeasts: a Taxonomic Study, fourth edition (1999) (Edited by Kurtzman and Fell), and in Yeasts: Characteristics and Identification, third edition (2000) (Cambridge: Cambridge University Press). These books provide information on morphologic, metabolic and genetic

characteristics of all known taxa and keys for their identification. Concerning prokaryotes, the taxonomic outline of the last version of Bergey's Manual underlines the phylogenetic placement of genera and taxonomic level above (Garrity et al., 2007a, http://www.taxonomicoutline.org/); a comprehensive picture of the phylogenetic diversity of prokaryotic microorganisms can be visualized at http://www.arb-silva.de/fileadmin/silva_databases/living_tree/LTP_tree_s93.pdf, which can also be used to analyze specific subtrees including the species of interest (Yarza et al., 2008).

Novel species descriptions require the deposit of the type strain in at least two recognized culture collections, the only international organizations devoted to storage and preservation of biodiversity. Well known collections are, among others, ATCC (www.atcc.org), LMG (www.bespo.be/bccm), DSMZ (www.dsmz.de) and JCM (http://www.jcm.riken.jp/), while CBS (http://www.cbs.knaw.nl/), DBVPG (http://www.agr.unipg.it/dbvpg/), and NRRL (http://nrrl.ncaur.usda.gov/) are mostly devoted to yeasts. Other information can be found on the web pages of the World Federation for Culture Collections (http://www.wfcc.info/datacenter.html), for other culture collections and abbreviations.

Finally, genome sequence data are expected to significantly contribute to taxonomy, therefore an interesting genomic database is GOLD (www.genomesonline.org), where sequencing projects are identified with a label: "Gc number" for complete genomes (http://genomesonline.org/gold.cgi?want = Published + Complete + Genomes), and a "Gi number" for incomplete sequences (http://www.genomesonline.org/gold.cgi?want = Bacterial + Ongoing + Genomes and http://www.genomesonline.org/gold.cgi?want=Eukaryotic+Ongoing+Genomes, for Eukaryotes, respectively), which will be referred to in the text.

15.3 Taxonomic Placement of Probiotic Microorganisms

Members of the Domain Bacteria are unicellular prokaryotic microorganisms, characterized by different cellular shapes (cocci, rods, spiral etc.) and cell size of few micrometers. To date, more than 8,000 species of prokaryotes (Garrity et al., 2007a) are described, grouping organisms isolated from almost every habitat on earth.

Concerning the taxonomy of the Domain, it is subdivided into 24 Phyla. Gram-positive probiotic strains known to date belong to the Phyla *Firmicutes* and

Actinobacteria, with low or high genome guanine-cytosine base (GC) content, respectively. Also, probiotic properties have been attributed to *Escherichia coli*, a Gram-negative bacterium belonging to the Phylum *Proteobacteria*.

Data will be presented as follows: after some general indications concerning the bacterial and yeast genera involved, detailed information will be given on species comprising probiotic strains. Also, an updated list of species included in the respective genera will be presented, with the double aim of providing an immediate check list for correct names of strains and a reference to the original description of the species themselves. Species belonging to different genera will be treated separately, even if closely related (e.g., *Lactobacillus* and *Pediococcus*), to facilitate the consultation of data for single species and genera. Finally, all genus names will be reported *in extenso* throughout the text, to avoid confusion between names of different genera with the same capital letter (e.g., *B.* could stand for *Bifidobacterium, Bacillus* and *Brevibacillus*).

15.3.1 Lactic Acid Bacteria (LAB)

This group of bacteria is named after the ability of fermenting carbohydrate to lactic acid. Taxonomically, it comprises diverse genera of bacteria, which appear to be also phylogenetically unrelated. The two most important genera in the probiotic field are *Lactobacillus* and *Bifidobacterium,* but some others contain species of interest, e.g., *Pediococcus, Enterococcus*, and *Lactococcus*.

15.3.1.1 Genus *Lactobacillus*

Lactobacilli are Gram-positive bacteria, unable to sporulate, occurring as rods or cocco-bacilli, with a GC composition of the genome usually below 50% (low GC bacteria). They are fastidious microorganisms, requiring rich media to grow, and microaerophilic. They are catalase negative, even if pseudocatalase activity can sometimes be present in some strains and in presence of a heme group.

They are almost ubiquitous and can be found in almost all the environments where carbohydrates are available, such as food (dairy products, fermented meat, sourdoughs, vegetables, fruits, beverages), respiratory, gastro-intestinal (GI) and genital tracts of humans and animals, sewage and plant material.

The genus *Lactobacillus* belongs to the Phylum *Firmicutes,* Class *Bacilli,* Order *Lactobacillales*, Family *Lactobacillaceae* and its closest relatives, being

grouped within the same Family, are the genera *Paralactobacillus* and *Pediococcus* (Garrity et al., 2007b).

At the time of writing (September 2008), the genus includes 116 species with valid names, and several others are in course of publication (Table 15.1).

The taxonomy of the genus is largely unsatisfactory, as the description of many novel species in the recent years has made it clear that the genus is heterogeneous and it is phylogenetically intermixed with the other two genera of the Family, i.e., *Pediococcus* and *Paralactobacillus* (Figure 15.1). Moreover, metabolic differences, in terms of type and quantity of metabolites of carbohydrate fermentation, do not match with phylogenetic groupings and are, therefore, unreliable markers for their classification. Two species of the genus, namely *Lactobacillus catenaformis* and *Lactobacillus vitulinus* are only poorly related to the other species and their taxonomic status requires attention (Hammes and Vogel, 1995; Pot et al., 1994); for this reason they were omitted in Figure 15.1.

Phylogenetically, the species form a number of stable groups, at least seven, based on the analysis of 16S rRNA gene sequences, but several species form distinct lines of descent (Felis and Dellaglio, 2007) as can be noted in the Figure 15.1, depicting the updated structure of the taxonomic Family.

Lactobacillus Acidophilus

The etymology of the name, meaning acid-loving, indicates the preference of this species for acid medium for growth. Strains of this species usually display a rod shape with rounded ends and occur singly, in pairs and in short chains.

The original description was based on strains isolated from the intestinal tract of man and animals, human mouth and vagina, but are also easily found in milk and dairy products. At the time of first description, it was a heterogeneous species, as it included strains later reclassified as novel species, e.g., *Lactobacillus johnsonii*. Its metabolism is homofermentative, converting hexoses almost completely to lactic acid (both isomers D and L are produced) (Hammes and Vogel, 1995). Phylogenetically, it is placed in the *Lactobacillus delbrueckii* group (Felis and Dellaglio, 2007). The type strain is ATCC 4356^T (=LMG 9433^T = DSM 20079^T), of human origin, active for acidophilus milk when used with yeast extract. The GC content of the genome is 34–37%.

Genome sequence data are available for the non-type probiotic strain NCFM (strain Gc00252), and another sequencing project is ongoing (Gi02787), but there is no accession number indicating which particular strain is being sequenced.

Table 15.1

List of valid names in the genus *Lactobacillus* (updated at September 2008) (*Cont'd* p. 599)

	Species of the genus *Lactobacillus*
1	*Lactobacillus acetotolerans*
2	*Lactobacillus acidifarinae*
3	*Lactobacillus acidipiscis*
4	***Lactobacillus acidophilus***
5	*Lactobacillus agilis*
6	*Lactobacillus algidus*
7	*Lactobacillus alimentarius*
8	*Lactobacillus amylolyticus*
9	*Lactobacillus amylophilus*
10	*Lactobacillus amylotrophicus*
11	*Lactobacillus amylovorus*
12	*Lactobacillus animalis*
13	*Lactobacillus antri*
14	*Lactobacillus apodemi*
15	*Lactobacillus aviarius*
	Lactobacillus aviarius subsp. *araffinosus*
	Lactobacillus aviarius subsp. *aviarius*
16	*Lactobacillus bifermentans*
17	*Lactobacillus brevis*
18	*Lactobacillus buchneri*
19	*Lactobacillus camelliae*
20	***Lactobacillus casei***
21	*Lactobacillus catenaformis*
22	*Lactobacillus ceti*
23	*Lactobacillus coleohominis*
24	*Lactobacillus collinoides*
25	*Lactobacillus composti*
26	*Lactobacillus concavus*
27	*Lactobacillus coryniformis*
	Lactobacillus coryniformis subsp. *coryniformis*
	Lactobacillus coryniformis subsp. *torquens*
28	***Lactobacillus crispatus***
29	*Lactobacillus crustorum*
30	*Lactobacillus curvatus*
31	***Lactobacillus delbrueckii***

Table 15.1 (*Cont'd* p. 600)

	Species of the genus *Lactobacillus*
	Lactobacillus delbrueckii subsp. *bulgaricus*
	Lactobacillus delbrueckii subsp. *delbrueckii*
	Lactobacillus delbrueckii subsp. *indicus*
	Lactobacillus delbrueckii subsp. *lactis*
32	*Lactobacillus diolivorans*
33	*Lactobacillus equi*
34	*Lactobacillus equigenerosi*
35	*Lactobacillus farciminis*
36	*Lactobacillus farraginis*
37	*Lactobacillus fermentum*
38	*Lactobacillus fornicalis*
39	*Lactobacillus fructivorans*
40	*Lactobacillus frumenti*
41	*Lactobacillus fuchuensis*
42	*Lactobacillus gallinarum*
43	*Lactobacillus gasseri*
44	*Lactobacillus gastricus*
45	*Lactobacillus ghanensis*
46	*Lactobacillus graminis*
47	*Lactobacillus hammesii*
48	*Lactobacillus hamsteri*
49	*Lactobacillus harbinensis*
50	*Lactobacillus hayakitensis*
51	*Lactobacillus helveticus*
52	*Lactobacillus hilgardii*
53	*Lactobacillus homohiochii*
54	*Lactobacillus iners*
55	*Lactobacillus ingluviei*
56	*Lactobacillus intestinalis*
57	*Lactobacillus jensenii*
58	***Lactobacillus johnsonii***
59	*Lactobacillus kalixensis*
60	*Lactobacillus kefiranofaciens*
	Lactobacillus kefiranofaciens subsp. *kefiranofaciens*
	Lactobacillus kefiranofaciens subsp. *kefirgranum*
61	*Lactobacillus kefiri*
62	*Lactobacillus kimchii*

Table 15.1 (*Cont'd* p. 601)

	Species of the genus *Lactobacillus*
63	*Lactobacillus kitasatonis*
64	*Lactobacillus kunkeei*
65	*Lactobacillus lindneri*
66	*Lactobacillus malefermentans*
67	*Lactobacillus mali*
68	*Lactobacillus manihotivorans*
69	*Lactobacillus mindensis*
70	*Lactobacillus mucosae*
71	*Lactobacillus murinus*
72	*Lactobacillus nagelii*
73	*Lactobacillus namurensis*
74	*Lactobacillus nantensis*
75	*Lactobacillus oligofermentans*
76	*Lactobacillus oris*
77	*Lactobacillus panis*
78	*Lactobacillus pantheris*
79	*Lactobacillus parabrevis*
80	*Lactobacillus parabuchneri*
81	***Lactobacillus paracasei***
	Lactobacillus paracasei subsp. *paracasei*
	Lactobacillus paracasei subsp. *tolerans*
82	*Lactobacillus paracollinoides*
83	*Lactobacillus parafarraginis*
84	*Lactobacillus parakefiri*
85	*Lactobacillus paralimentarius*
86	*Lactobacillus paraplantarum*
87	*Lactobacillus pentosus*
88	*Lactobacillus perolens*
89	***Lactobacillus plantarum***
	Lactobacillus plantarum subsp. *argentoratensis*
	Lactobacillus plantarum subsp. *plantarum*
90	*Lactobacillus pontis*
91	*Lactobacillus psittaci*
92	*Lactobacillus rennini*
93	***Lactobacillus reuteri***
94	***Lactobacillus rhamnosus***

Table 15.1

	Species of the genus *Lactobacillus*
95	*Lactobacillus rogosae*
96	*Lactobacillus rossiae*
97	*Lactobacillus ruminis*
98	*Lactobacillus saerimneri*
99	*Lactobacillus sakei*
	Lactobacillus sakei subsp. *carnosus*
	Lactobacillus sakei subsp. *sakei*
100	***Lactobacillus salivarius***
101	*Lactobacillus sanfranciscensis*
102	*Lactobacillus satsumensis*
103	*Lactobacillus secaliphilus*
104	*Lactobacillus senmaizukei*
105	*Lactobacillus sharpeae*
106	*Lactobacillus siliginis*
107	*Lactobacillus spicheri*
108	*Lactobacillus suebicus*
109	*Lactobacillus thailandensis*
110	*Lactobacillus ultunensis*
111	*Lactobacillus vaccinostercus*
112	*Lactobacillus vaginalis*
113	*Lactobacillus versmoldensis*
114	*Lactobacillus vini*
115	*Lactobacillus vitulinus*
116	*Lactobacillus zymae*

Species with QPS status are underlined, principal species including probiotic strains are in bold and are described in the text

Lactobacillus Casei – Lactobacillus Paracasei

The two species *Lactobacillus casei* and *Lactobacillus paracasei* are presented together because they are closely related and their nomenclatural status has been discussed for a long time and only recently solved (Judicial Commission, 2008).

The name *casei* indicate the origin of the species in cheese and dairy products in general, but it has been isolated also from sourdough, silage, human GI tract (GIT), vagina, sewage etc. The name *paracasei* indicate the close resemblance of this *taxon* to *Lactobacillus casei.*

Figure 15.1

Phylogenetic tree showing the relationships among the species of the Family *Lactobacillaceae*, including genera *Lactobacillus* (abbreviated with "*L.*" in the tree), *Paralactobacillus* and *Pediococcus* (abbreviated with "*P.*" in the tree). The tree was calculated with a maximum-likelihood-derived distance method and clustering was performed with neighbor joining method. The bar indicates number of substitutions per site.

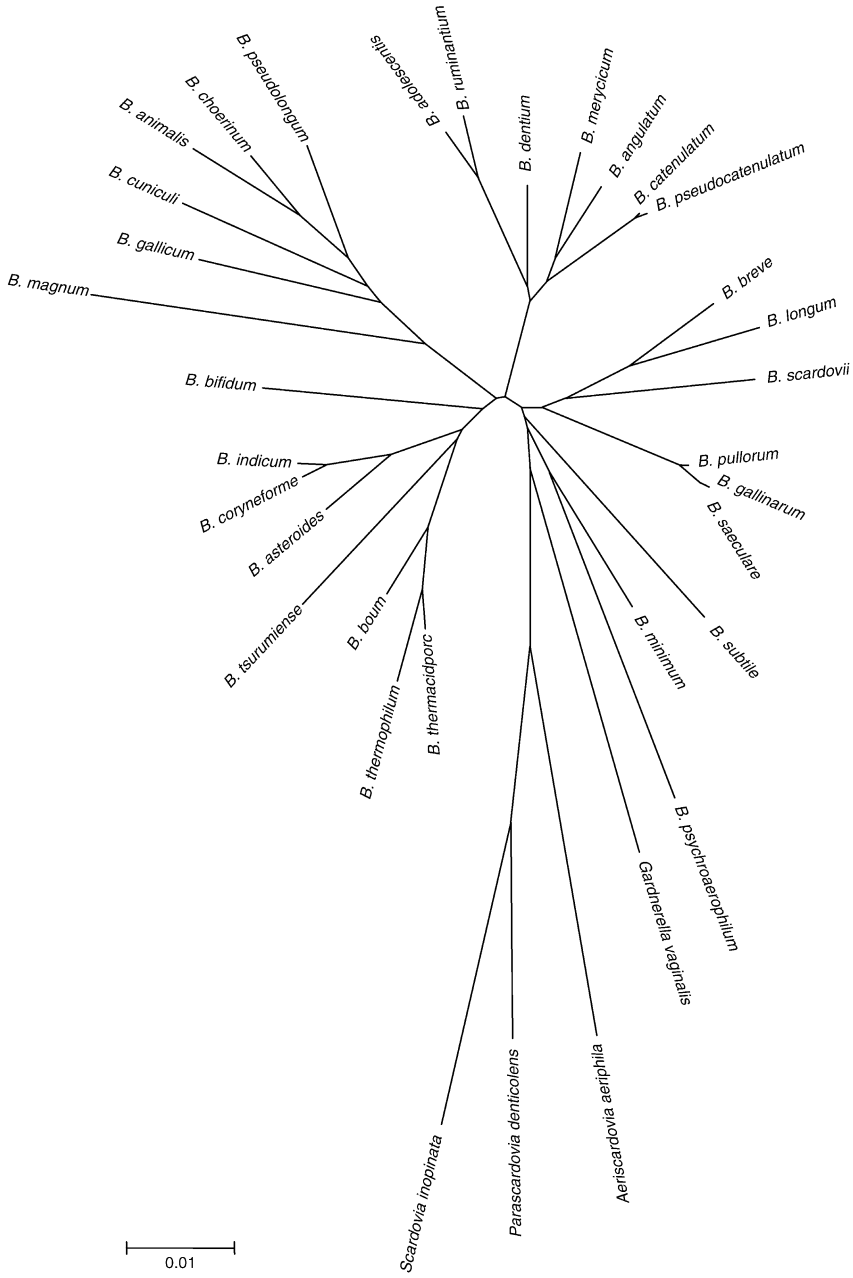

Figure 15.2

Phylogenetic tree depicting relationships among the species of the genus *Bifidobacterium* (abbreviated as "*B.*" in the tree) and related genera. Tree was calculated with the same procedure reported for *Figure 15.1*.

The first description of these rods with square ends, often occurring in chain, was based on few phenotypic traits, and the choice of strain ATCC 393 as the type has been considered inadequate for a long time. This has lead to the description of *Lactobacillus paracasei*, phenotypically very similar but genomically distinct. The details of the taxonomic controversy will not be explained here, but it is important to point out that the debate has been definitively concluded as follows: both species names are valid, with ATCC 393^T as the type strain of *Lactobacillus casei* and NCFB 151^T (=ATCC 25302^T) as type strain of *Lactobacillus paracasei* (Judicial Commission, 2008). This is expected to have important consequences also in marketing of probiotic strains: many of the cultures on the market called *Lactobacillus casei* are actually more similar to the type strain of *Lactobacillus paracasei* and therefore should be given that name.

As for metabolism, both species are facultatively heterofermentative microorganisms, producing lactic acid (L or both isomers, depending on the strains) from hexoses, but are also able to ferment pentoses (Hammes and Vogel, 1995). Phylogenetically, the two species together with *Lactobacillus rhamnosus* form a distinct clade in the genus *Lactobacillus* (Felis and Dellaglio, 2007), and the genome GC content for both species ranges between 45 and 47%.

Genome data have been obtained or are in progress for several strains in the two species (Gc00438, Gc00822, Gi02995, Gi03110, Gi02219, and Gi02799), including the type strain of *Lactobacillus casei* ATCC 393^T. However, the identification of the strains is not always clear, e.g., ATCC 334 (Gc00438) is indicated as *Lactobacillus casei* while it is a *Lactobacillus paracasei* strain (Judicial Commission, 2008).

Lactobacillus Crispatus

The curled morphology of the cells in liquid medium gives the name to the species, of straight or slightly curved rods, occurring singly or in short chains. Phylogenetically related to *Lactobacillus acidophilus* and *Lactobacillus delbrueckii,* its neighbor species appear to be *Lactobacillus kefiranofaciens.* Historically, the species was described based on strains previously assigned to the species *Lactobacillus acidophilus*, which showed a similar homofermentative metabolism, and also similar genome GC content (35–38% for *Lactobacillus crispatus*). Strains have been isolated from saliva, feces and urogenital tract of man and chicken, and also found in patients with different infections, but not in a causative role. The type strain is ATCC 33820^T (=LMG 9479^T = DSM 20584^T), isolated from human specimen (eye). Genome projects involving six non-type strains are ongoing (Gi02791, Gi02792, Gi03435, Gi03436, Gi03437, and Gi03438).

Lactobacillus Delbrueckii subsp. Bulgaricus

Lactobacillus delbrueckii is the type species of the genus *Lactobacillus*, and it currently includes four subspecies (Table 15.1), three of which, i.e., subsp. *delbrueckii*, *bulgaricus*, and *lactis*, were first described as separate species. The unification under the same name then was performed, the name being *Lactobacillus delbrueckii*, as a rule of priority as it was described before the others. The species name is to honor Delbrück, a German bacteriologist, while *bulgaricus* indicate the geographical origin of the first isolated strains, Bulgaria. The evolution of strains of the subsp. *bulgaricus* has probably been driven by mankind: it has been used for centuries in the production of yogurt and of other dairy products; therefore its genetic make up is devoted to fermentation in milk at high temperature (Germond et al., 2003). This could also explain the unusually high genome GC content for the species, 49–51%, and the scarce ability to ferment sugars other than lactose. Also, genome reduction has been observed (van de Guchte et al., 2006), probably motivated by the proto-cooperative interaction with *Streptococcus thermophilus,* the partner for yogurt production.

Phylogenetically, the species *Lactobacillus delbrueckii* belongs to the homonymous clade in the genus *Lactobacillus*, which includes, besides the already mentioned *Lactobacillus acidophilus* and *Lactobacillus crispatus, Lactobacillus acetotolerans*, *Lactobacillus amylolyticus*, *Lactobacillus amylophilus*, *Lactobacillus amylotrophicus*, *Lactobacillus amylovorus*, *Lactobacillus fornicalis*, *Lactobacillus gallinarum*, *Lactobacillus gasseri*, *Lactobacillus hamsteri*, *Lactobacillus helveticus*, *Lactobacillus iners*, *Lactobacillus intestinalis*, *Lactobacillus jensenii*, *Lactobacillus johnsonii*, *Lactobacillus kalixensis*, *Lactobacillus kefiranofaciens*, *Lactobacillus kitasatonis*, *Lactobacillus psittaci*, and *Lactobacillus ultunensis.*

The type strain of the species *Lactobacillus delbrueckii* corresponds to the type strain of the homonymous subspecies and is ATCC 9649^T (=DSM 20074^T = LMG 6412^T), isolated from distillery sour grain mash incubated at 45°C, while the type strain of subsp. *bulgaricus* is ATCC 11842^T (=DSM 20081^T = LMG 6901^T), isolated from Bulgarian yogurt. Genome data are available for three strains of the species (Gc00443, Gc00394, and Gi02793), including also the type strain (Gc00394).

Lactobacillus Johnsonii

Another bacteriologist, Johnson, gives his name to the species *Lactobacillus johnsonii,* which shares with *Lactobacillus delbrueckii* not only the origin of the name, but also the phylogenetic placement and the homofermentative metabolism. Strains of this species were initially identified as *Lactobacillus acidophilus*

and *Lactobacillus crispatus*: with those two species *Lactobacillus johnsonii* shares different traits, such as similar GC content (33–35% in this case), the isomer of lactic acid produced (DL) and the homofermentative metabolism. Strains of *Lactobacillus johnsonii* have been isolated from human specimens but also from feces of animals. Type strain is ATCC 33200^T (=DSM 10533^T = LMG9436^T) and genome data available at the moment are for a probiotic strain NCC533 (Gc00171), while another is in progress, but no indication of the strain is available to date (Gi02798).

Lactobacillus Plantarum

The species *Lactobacillus plantarum* is one of the first taxa described in the genus. As the name indicates, it is commonly found in plant material, but it is also commonly found in a variety of environments, i.e., dairy products, human specimens, sewage etc. This flexibility determines also the genetic and phenotypic heterogeneity of the species, in which a novel subspecies has been recently delineated (*Table 15.1*). Anyway, in general, the morphology of cells is rod-like with rounded ends, metabolism is facultatively heterofermentative, genome GC content is in the range of 44–46% and both isomers of lactic acid are produced from carbohydrates. From a phylogenetic standpoint, *Lactobacillus plantarum* closest relatives are *Lactobacillus pentosus* and *Lactobacillus paraplantarum*, and the three species form a distinct line of descent in the genus *Lactobacillus*.

The type strain is ATCC 14917^T (=DSM 20174^T = LMG 6907^T), isolated from pickled cabbage, and genome sequencing has been performed on a non-type strain with probiotic properties, WCFS1 (Gc00122), the first *Lactobacillus* strain sequenced (Kleerebezem et al., 2003), which is already becoming the model organism for the genus.

Lactobacillus Reuteri

The bent rods with rounded ends belonging to this species owe their name to the German microbiologist Reuter, who was the first to isolate and study these heterofermentative bacteria. Strains of *Lactobacillus reuteri* are isolated from intestine and feces of humans and animals, sourdough and meat. Often an antimicrobial compound, reuterin, is produced. *Lactobacillus reuteri* strains produce both isomers of lactic acid and have a genome GC content between 40 and 42%. Its closest phylogenetic relatives are *Lactobacillus antri*, *Lactobacillus coleohominis*, *Lactobacillus fermentum*, *Lactobacillus frumenti*, *Lactobacillus gastricus*, *Lactobacillus ingluviei*, *Lactobacillus mucosae*, *Lactobacillus*

oris, *Lactobacillus panis*, *Lactobacillus pontis*, *Lactobacillus secaliphilus*, and *Lactobacillus vaginalis*, which constitute the *Lactobacillus reuteri* subclade in the genus *Lactobacillus* (Felis and Dellaglio, 2007).

The type strain of the species, isolated from human intestine is ATCC 23272^T (=DSM 20016^T = LMG 9213^T), which genome has also been sequenced by two groups (Gc00573 and Gc00786), together with other four strains (Gi01862, Gi01863, Gi00762, and Gi02800).

Lactobacillus Rhamnosus

The ability to ferment rhamnose is one of the peculiarities of these rod-shaped bacteria, phylogenetically related to *Lactobacillus casei,* which can be isolated from dairy products, sewage, and human specimens. Strains of *Lactobacillus rhamnosus* have a facultatively heterofermentative metabolism, and are, in general very similar to *Lactobacillus paracasei* strains. Also, strains of this species are among the few in the genus *Lactobacillus* being assigned to Risk group 2 and are subjected to restricted distribution (http://www.dsmz.de/microorganisms/html/bacteria.genus/lactobacillus.html) as some strains have been isolated from endocarditis and other opportunistic infections. Nevertheless, the species has been included in list for QPS status and some strains are used as probiotics. Type strain of the species is ATCC 7469^T (=DSM 20021^T = LMG 6400^T) and three genome sequencing projects are ongoing (Gi02218, Gi00316, and Gi02801).

Lactobacillus Salivarius

As the name indicates, strains of *Lactobacillus salivarius* are common in human saliva, as well as in mouth and intestinal tract of man and animals. Historically considered a homofermentative species, after a recent taxonomic reevaluation (Lee et al., 2006), it is regarded as a facultatively heterofermentative species, able to ferment both hexoses and pentoses. Many recently described species have been found to be phylogenetically related to *Lactobacillus salivarius*, which clade is at present constituted by *Lactobacillus acidipiscis*, *Lactobacillus agilis*, *Lactobacillus animalis*, *Lactobacillus apodemi*, *Lactobacillus aviarius*, *Lactobacillus ceti*, *Lactobacillus equi*, *Lactobacillus ghanensis*, *Lactobacillus hayakitensis*, *Lactobacillus mali*, *Lactobacillus murinus*, *Lactobacillus nagelii*, *Lactobacillus ruminis*, *Lactobacillus saerimneri*, *Lactobacillus satsumensis*, and *Lactobacillus vini*.

Strains of this species produce L-lactic acid, and have a genome GC content of 34–36%. Type strain is ATCCC 11742^T (=DSM 20555^T = LMG 9477^T), isolated from human saliva, while the genome sequence has been obtained for a non-type strain with probiotic properties, UCC118 (Gc00362).

Interestingly, labels of some commercial products refer to the name "*Lactobacillus sporogenes*": this name is invalid, does not indicate any *Lactobacillus* species, but the spore-forming *Bacillus coagulans* (Sanders et al., 2003) (see below).

15.3.1.2 Genus *Bifidobacterium*

The genus *Bifidobacterium*, even if traditionally listed among LAB, is only poorly phylogenetically related to genuine LAB: it belongs to the Phylum *Actinobacteria*, Class "*Actinobacteria*," Order *Bifidobacteriales*, Family *Bifidobacteriaceae*, its neighbor genera being *Aeriscardovia, Gardnerella, Parascardovia,* and *Scardovia* (Garrity et al., 2007c). Species of these genera use a metabolic pathway for the degradation of hexoses, the so-called "bifid shunt," different from that of *Lactobacillus* and related genera (Sgorbati et al., 1995; Ventura et al., 2004). The key enzyme, considered a taxonomic character for the identification of this group of bacteria, is fructose-6-phosphoketolase (EC 4.1.2.2). The genus includes, at present, 30 species (› *Table 15.2*).

Bifidobacteria are Gram-positive rods, which can sometimes be branched, a characteristic which gives the name to the genus. Bifidobacteria do not form spores, are nonmotile, and anaerobic. Their genome GC content varies from 42 to 67 mol% and in fact they belong to the high GC Gram-positive bacteria. Five phylogenetic groups have been observed in the genus with also species constituting single lines of descent (Felis and Dellaglio, 2007; Matsuki et al., 2003).

Bifidobacterial strains exhibiting probiotic properties belong to the species *Bifidobacterium adolescentis, Bifidobacterium animalis, Bifidobacterium bifidum, Bifidobacterium breve*, and *Bifidobacterium longum*, which are not related from a phylogenetic standpoint.

Bifidobacterium Adolescentis

Bacteria belonging to this species have been isolated from feces of human adult, bovine rumen and sewage. They are phylogenetically related with *Bifidobacterium angulatum*, *Bifidobacterium catenulatum*, *Bifidobacterium dentium*, *Bifidobacterium merycicum*, *Bifidobacterium pseudocatenulatum* and *Bifidobacterium ruminantium*, and their phenotypic differentiation from *Bifidobacterium dentium* is sometimes difficult. It is characterized by genome GC content of about 58%, while the cell-wall amino acids consist of Lys (Orn) – D-Asp. The type strain, isolated from human intestine is ATCC 15703^T (=DSM 20083^T = LMG 10502^T),

Table 15.2

List of valid names in the genus *Bifidobacterium* (updated at September 2008) (*Cont'd* p. 610)

	Species of the genus *Bifidobacterium*
1	***Bifidobacterium adolescentis***
2	*Bifidobacterium angulatum*
3	***Bifidobacterium animalis***
	Bifidobacterium animalis subsp. *animalis*
	Bifidobacterium animalis subsp. *Lactis*
4	*Bifidobacterium asteroids*
5	***Bifidobacterium bifidum***
6	*Bifidobacterium boum*
7	***Bifidobacterium breve***
8	*Bifidobacterium catenulatum*
9	*Bifidobacterium choerinum*
10	*Bifidobacterium coryneforme*
11	*Bifidobacterium cuniculi*
12	*Bifidobacterium dentium*
13	*Bifidobacterium gallicum*
14	*Bifidobacterium gallinarum*
15	*Bifidobacterium indicum*
16	***Bifidobacterium longum***
	Bifidobacterium longum subsp. *infantis*
	Bifidobacterium longum subsp. *longum*
	Bifidobacterium longum subsp. *Suis*
17	*Bifidobacterium magnum*
18	*Bifidobacterium merycicum*
19	*Bifidobacterium minimum*
20	*Bifidobacterium pseudocatenulatum*
21	*Bifidobacterium pseudolongum*
	Bifidobacterium pseudolongum subsp. *globosum*
	Bifidobacterium pseudolongum subsp. *Pseudolongum*
22	*Bifidobacterium psychraerophilum*
23	*Bifidobacterium pullorum*
24	*Bifidobacterium ruminantium*
25	*Bifidobacterium saeculare*
26	*Bifidobacterium scardovii*
27	*Bifidobacterium subtile*

Table 15.2

	Species of the genus *Bifidobacterium*
28	*Bifidobacterium thermacidophilum*
	Bifidobacterium thermacidophilum subsp. *Porcinum*
	Bifidobacterium thermacidophilum subsp. *thermacidophilum*
29	*Bifidobacterium thermophilum*
30	*Bifidobacterium tsurumiense*

Species with QPS status are underlined, principal species including probiotic strains are in bold and are described in the text

which genome sequence is also available (Gc00470); a second one is ongoing (Gi01706).

Bifidobacterium Animalis

The first description of this species was based on strains isolated from feces of different animals, but different data revealed that strains described as *Bifidobacterium lactis*, isolated from dairy products, belong to the same species, but to different subspecies (Masco et al., 2004). Strains of the subsp. *lactis* are more oxygen tolerant than those of the subsp. *animalis*, and this trait is very useful for probiotic application, as it allows them to survive in high number in the non-anaerobic conditions of commercial products; another difference between the two subspecies is the ability to grow in milk. *Bifidobacterium animalis* belongs to the *Bifidobacterium pseudolongum* phylogenetic group, which includes *Bifidobacterium choerinum*, *Bifidobacterium cuniculi*, *Bifidobacterium gallicum*, and, obviously, *Bifidobacterium pseudolongum*. The DNA G + C content of the species is 61%. The type strain of *Bifidobacterium animalis* subsp. *animalis* is ATCC 25527^T (=LMG 10508^T = DSM 20104^T), isolated from rat feces, type strain of *B. animalis* subsp. *lactis* is LMG 18314^T (=DSM 10140^T), isolated from fermented milk. Genome data for three strains will be available in the future for three non-type strains of also probiotic interest (Gi01876, Gi01988, and Gi03035).

Bifidobacterium Bifidum

Bifidobacterium bifidum is the type species of the genus, strictly anaerobic. It is among the most recognizable species in the genus on the basis of the fermentation pattern, and is also clearly different from other bifidobacteria phylogenetically. Cells have, in particular conditions, a peculiar "amphora-like" shape, and often appear also as branched rods (from which the name *bifidum*). Strains of this

species have been isolated from feces of humans, both adults and infants, human vagina and animal feces, but are readily used for production of fermented dairy products for probiotic purposes. The type strain is ATCC 29521^T (=DSM 20239^T = LMG 10645^T), isolated from feces of a breast-fed infant; the genome sequence for three strains, including two different subcultures of the type strain, are in progress (Gi02210, Gi02653, Gi02657).

Bifidobacterium Breve

Strains of this species are characterized by the short size, with or without bifurcation, with lysine and glycine as aminoacid in the murein, and have a genome GC content of about 58%. Strains of this species have been isolated from intestine of infants, human vagina, and sewage, and are phylogenetically related to *Bifidobacterium longum*. The type strain is ATCC 15700^T (=DSM 20213^T = LMG 11042^T) and three genome sequencing programs are ongoing, two for the different subcultures of the type strain and one for a non-type with probiotic properties (Gi00078, Gi02209, and Gi02654).

Bifidobacterium Longum

Differently from *Bifidobacterium breve*, cells of strains in this species show a very elongated cell shape with rare branching. It is anaerobic and considered the most common species of bifidobacteria, and is isolated from feces of infants and adults, human vagina, but also animal feces. Its closest phylogenetic relative is *Bifidobacterium breve*, and the two species form a couple quite unrelated to the other species in the bifidobacterial phylogenetic tree. Other relevant characteristic for strains in the species are the genome GC content of about 60%, and the presence of many plasmids, a unique feature among fecal bifidobacteria.

Two taxa formerly described as distinct species have been included in *B. longum*, namely *Bifidobacterium infantis* and *Bifidobacterium suis*. These bacteria, first recognized as biotypes of the species, have very recently been recognized as novel subspecies. The three subgroups have been isolated from slightly different niches, such as the GIT of human adults (subsp. *longum*), the GIT of infants (subsp. *infantis*), and pig's feces (subsp. *suis*).

The type strain of subsp. *longum* (and therefore of the species) is ATCC 15707^T (=DSM 20219^T = LMG 13197^T), isolated from feces of a human adult, for subsp. *infantis* is ATCC 15697 ^{T} (=DSM 20088^T = LMG 8811^T), isolated from feces of a human infant, and for subsp. *suis* is ATCC 27533^T (=DSM 20211^T = LMG 21814^T), isolated from feces of a piglet (Mattarelli et al., 2008).

Sequence data are already or will be soon available for six strains, including the type strain (Gc00811, Gc00108, Gi02659, Gi01539, and Gi02485).

15.3.1.3 Other LAB: Genera *Streptococcus*, *Lactococcus*, *Enterococcus*, and *Pediococcus*

Other species belonging to the true LAB group play an important role in food microbiology and nutrition and are, in some cases, also considered probiotics. Two taxa having an outstanding role are *Streptococcus thermophilus* and *Lactococcus lactis*. Strains of the former species are, in combination with *Lactobacillus delbrueckii* subsp. *bulgaricus*, the actors of yogurt production, while the latter is largely used for production of dairy products and, in recent years, has become the model organism for lactic acid bacteria. Other genera, which include strains attributed with probiotic properties are *Enterococcus* and *Pediococcus* and will be briefly treated below.

15.3.1.4 Genera *Streptococcus* and *Lactococcus*

The genera *Lactococcus* and *Streptococcus* form the Family *Streptococcaceae* of the Order *Lactobacillales* in the Class *Bacilli* of the Phylum *Firmicutes*.

The genus *Streptococcus* contains about 67 species of coccoid Gram positive bacteria, mostly known for the pathogenicity of some species. The species *Streptococcus thermophilus*, on the contrary, is known for its GRAS (generally recognized as safe status), due to the long history of use in food production. Also, genomic data have demonstrated that *Streptococcus thermophilus* has lost or inactivated the virulence-related genes characterized in pathogenic streptococci, during the adaptation to milk, confirming its safety (Delorme, 2008).

The genus *Lactococcus* groups coccoid bacteria formerly referred to as mesophilic lactic streptococci. It is constituted by six species and the most important for dairy fermentation is *Lactococcus lactis*, which is divided into three subspecies, namely *Lactococcus lactis* subsp. *cremoris, Lactococcus lactis* subsp. *hordniae* and *Lactococcus lactis* subsp. *lactis*, which include the biovar *diacetylactis*. The diversity within the species has recently been re-evaluated with molecular analyzes, confirming that phenotypic and genotypic diversity are not coherent (Rademaker et al., 2007).

Streptococcus Thermophilus

As the name clearly indicates, strains of this *taxon* have a preference for growth at high temperature: all of them grow at 45°C, most are able to grow up to 50°C, and some survive also after heating 60°C for 30 min. This characteristic is an indication of man-driven adaptation to yogurt production, as already explained for *Lactobacillus delbrueckii* subsp. *bulgaricus*. In the case of *Streptococcus thermophilus*, however, genome GC content is in the range 37–40%, therefore no shift towards higher percentages is evident (Hardie and Whiley, 1995). A close phylogenetic relationship with *Streptococcus salivarius* has determined some nomenclatural changes in the past, with fluctuations of *Streptococcus thermophilus* between the status of species or of subspecies ("*Streptococcus salivarius* subsp. *thermophilus*"). However at present it is fully considered in the taxonomic rank of species, although some confusion is still present in literature data.

Streptococcus thermophilus has complex nutritional requirements, in particular considering aminoacids, which are most probably the result of the adaptation to growth in a rich medium such as milk, and also a consequence of proto-cooperation with *Lactobacillus delbrueckii* subsp. *bulgaricus*. The type strain is ATCC 19258^T (=DMS 20617 = LMG 6898), isolated from milk, and genome sequence data are available or in progress for four strains (Gc00451, Gc00234, Gc00233, and Gi00621).

Lactococcus Lactis

Lactococcus lactis is the type species of the genus *Lactococcus* (Table 15.3) and, as both genus and species names suggest, it is strictly associated with milk. However,

Table 15.3

List of valid names in the genus *Lactococcus* (updated at September 2008)

	Species of the genus *Lactococcus*
1	*Lactococcus chungangensis*
2	*Lactococcus garvieae*
3	*Lactococcus lactis*
	Lactococcus lactis subsp. *cremoris*
	Lactococcus lactis subsp. *hordniae*
	Lactococcus lactis subsp. *lactis*
4	*Lactococcus piscium*
5	*Lactococcus plantarum*
6	*Lactococcus raffinolactis*

Lactococcus lactis, underlined, is the only species in the genus under consideration for QPS status

one of the three subspecies it includes has been described based on strains isolated from leafhopper (subsp. *hordniae*). Moreover, *Lactococcus lactis* is considered an "old" microorganism, originally colonizing plants, and only in recent time adapted to milk. Morphologically, cells are usually spherical or ovoid, in pairs or in chains, mesophilic with a homofermentative metabolism. Genome GC content is in the range 34–36%.

Nowadays, *Lactococcus lactis* is among the lactic acid bacterial species with the biggest economic importance, as strains are largely applied as starter cultures for a variety of products; it is the model species for the genetic and metabolic study of low GC Gram-positive bacteria, second only to *Bacillus subtilis* in importance. This is also causing some confusion in nomenclature of strains in the species: it has been anticipated above that genomic groups within the species are not phenotypically homogeneous. From a taxonomic standpoint, nomenclature of subspecies depends on the results of phenotypic tests (degradation of maltose, ribose, deamination of arginine, growth in presence of sodium chloride among others) (Teuber, 1995). However, the availability of genome data and the interest in genetic traits is prevailing over classical phenotypic studies: a clear example is that of strain MG1363 (Wegmann et al., 2007): it displays many of subsp. *lactis* phenotypic traits, but it is usually referred to as subsp. *cremoris*, due to its genetic similarity to the type strain of subsp. *cremoris*. The availability of the genome sequence and the use of the strain as an exemplary for the name *Lactococcus lactis* subsp. *cremoris*, therefore the genetic-based nomenclature will probably overcome the classical and phenotype-based correct one, generating a dichotomy in taxonomic procedure of identification of strains at the subspecies level.

Type strains of the three subspecies are ATCC 19435^T (=DSM 20481^T = LMG 6898^T) for subsp. *lactis* (and therefore for the whole species), ATCC 19257^T (=DSM 20069^T = LMG 6897^T) for subsp. *cremoris*, both isolated from milk and ATCC 29071^T (=DSM 20450^T = LMG 8520^T) for subsp. *hordniae*, isolated from the insect *Hordnia circellata*.

Three genome sequences are already available (Gc00054, Gc00450, and Gc508) and other will be available soon (Gi03379).

15.3.1.5 Genus *Enterococcus*

Related to the above described two genera, the genus *Enterococcus* is also important for the field of probiotics. The first bulk of species in the genus (*Enterococcus faecalis* and *Enterococcus faecium*) were previously described as streptococci, but

many others have followed, increasing the number of species in the genus to 35 (Table 15.4).

Potential probiotic properties have been reported for strains of *Enterococcus faecium* but bacteria in the genus *Enterococcus* are amongst the leading causes of community- and nosocomial infections. Therefore the Scientific Committee of the European Authority for Food Safety (EFSA) did not propose QPS status for any species of genus *Enterococcus*.

From a taxonomic standpoint, the genus *Enterococcus* falls into the Family *Enterococcaceae*, together with *Atopobacter, Catellicoccus, Melissococcus, Pilibacter, Tetragenococcus*, and *Vagococcus*.

Enterococcus Faecium

As the name clearly indicates, source of first isolation of *Enterococcus faecium* was fecal material, but strains of this species are frequently isolated not only from the GIT of animals (mammals, birds, and reptiles), but also from raw milk and dairy products (Devriese and Pot, 1995).

Cells are ovoid, occurring in pairs and short chains, not pigmented and not motile. Genome GC content ranges from 37 to 40%. *Enterococcus faecium* gives a name also to a phylogenetic group of closely related species in the genus, which, after description of novel species (Table 15.4), includes *Enterococcus durans, Enterococcus hirae, Enterococcus mundtii, Enterococcus ratti*, and *Enterococcus villorum*.

Type strain is ATCC 19434^T (=DSM 20477^T = LMG 11423^T), and genome sequence data are not available at the moment but are in progress for 11 strains (Gi00227, Gi00228, Gi00229, Gi02729, Gi03235, Gi03232, Gi03265, Gi03306, Gi03225, Gi02730, and Gi03362).

15.3.1.6 Genus *Pediococcus*

Another genus including coccoid bacteria, relevant for the area of probiotics, is *Pediococcus*. Its peculiarity resides in the particular type of cell division observed, in two directions of the same plain, so that cells, during division, form tetrads. Interestingly, the closest relatives to pediococci are lactobacilli, the shape and mode of division of which are different (Simpson and Taguchi, 1995).

Moreover, the description of *Pediococcus siamensis* (Table 15.5) has determined a rearrangement of the phylogenetic structure of the genus, with the recognition of two phylogenetically distinct subpopulations, one formed by

Table 15.4

List of valid names in the genus *Enterococcus* (updated at September 2008)

	Species of the genus *Enterococcus*
1	*Enterococcus aquimarinus*
2	*Enterococcus asini*
3	*Enterococcus avium*
4	*Enterococcus caccae*
5	*Enterococcus camelliae*
6	*Enterococcus canintestini*
7	*Enterococcus canis*
8	*Enterococcus casseliflavus*
9	*Enterococcus cecorum*
10	*Enterococcus columbae*
11	*Enterococcus devriesei*
12	*Enterococcus dispar*
13	*Enterococcus durans*
14	*Enterococcus faecalis*
15	***Enterococcus faecium***
16	*Enterococcus gallinarum*
17	*Enterococcus gilvus*
18	*Enterococcus haemoperoxidus*
19	*Enterococcus hermanniensis*
20	*Enterococcus hirae*
21	*Enterococcus italicus*
22	*Enterococcus malodoratus*
23	*Enterococcus moraviensis*
24	*Enterococcus mundtii*
25	*Enterococcus pallens*
26	*Enterococcus phoeniculicola*
27	*Enterococcus pseudoavium*
28	*Enterococcus raffinosus*
29	*Enterococcus ratti*
30	*Enterococcus saccharolyticus*
31	*Enterococcus silesiacus*
32	*Enterococcus sulfurous*
33	*Enterococcus termitis*
34	*Enterococcus thailandicus*
35	*Enterococcus villorum*

No species in the genus *Enterococcus* is under consideration for QPS status, principal species including probiotic strains are in bold and are described in the text

Table 15.5

List of valid names in the genus *Pediococcus* (updated at September 2008)

	Species of the genus *Pediococcus*
1	***Pediococcus acidilactici***
2	*Pediococcus cellicola*
3	*Pediococcus claussenii*
4	*Pediococcus damnosus*
5	*Pediococcus dextrinicus*
6	*Pediococcus ethanolidurans*
7	*Pediococcus inopinatus*
8	*Pediococcus parvulus*
9	***Pediococcus pentosaceus***
10	*Pediococcus siamensis*
11	*Pediococcus stilesii*

Species with QPS status are undelined, principal species including probiotic strains are in bold and are described in the text

Pediococcus claussenii, Pediococcus pentosaceus and *Pediococcus acidilactici*; the other including *Pediococcus damnosus, Pediococcus inopinatus, Pediococcus parvulus, Pediococcus ethanolidurans, Pediococcus stilesii, Pediococcus siamensis* and *Pediococcus cellicola. Pediococcus dextrinicus* is scarcely related with other species in the genus, and is more similar to lactobacilli (Felis and Dellaglio, 2007).

Pediococcus Acidilactici

The name of this first described species of the genus *Pediococcus* clearly indicates its ability to produce lactic acid. Strains of this species are mostly isolated from plant material (silage, cereal and potato mashes, barley, malt), but some strains have been isolated also from meat products. It produces both isomers of lactic acid from glucose and other carbohydrates, while it is usually unable to degrade maltose, a sugar usually associated with plant environment. Strains can also grow up to 50°C and in presence of sodium chloride at high concentration (10%). Genome GC content is in the range 38–44%.

The species has always shown high overall similarity with *Pediococcus pentosaceus* (see below), and this is confirmed also from their phylogenetic relatedness: they form a distinct clade in the phylogenetic tree of the genus, together with *Pediococcus claussenii*, and *Pediococcus stilesii.*

The type strain is DSM 20284^T (=LMG 11384^T), and no genome sequence data are available nor in progress (GOLD database).

Pediococcus Pentosaceus

As the name indicates, strains of this species are able to degrade pentoses (except strains of the formerly valid subsp. *intermedius*). It is very similar to *Pediococcus acidilactici* in terms of shape, culture conditions and physiological traits, and they are also isolated from almost the same plant niches. In principle the two species can be phenotypically distinguished on the basis of maltose degradation, and the slightly heat resistance (39–45°C but not 50°C) and genome GC content (35–39%), but reliable differentiation can only be obtained with molecular methods. Some strains are able to produce pediocin.

The type strain is ATCC 33316^T (=DSM 20336^T = LMG 11488^T), and genome data are available for a non-type strain (Gc00439).

15.3.2 Non LAB: Genera *Propionibacterium, Bacillus, Brevibacillus, Sporolactobacillus, Escherichia*

15.3.2.1 Genus *Propionibacterium*

The genus *Propionibacterium*, similarly to the genus *Bifidobacterium,* belongs to the Class *Actinobacteria* (Garrity et al., 2007c), which comprises high G + C content Gram-positive non sporeforming bacteria; its neighbor genera in the Family *Propionibacteriaceae* are *Brooklawnia, Jiangella, Luteococcus, Microlunatus, Propioniferax, Propionomicrobium*, and *Tessaracoccus.*

Propionibacteria take the name from the ability to produce propionic acid, acetic acid and carbon dioxide from carbohydrates and lactic acid; these characteristics are desirable for the production of some types of cheeses, e.g., the Swiss type, where propionibacteria are used as starter cultures. In general, morphology of propionibacteria might be very different: coccoid, bifid or even branched rods have been described, which vary also with the physiological state and culture conditions (Jan et al., 2007).

The genus comprises 12 species (*Table 15.6*) divided in two ecologically distinct groups, in terms of habitat/source of isolation: the "*acnes* group," of human origin, and the "dairy" or "classical" propionibacteria, isolated from milk and dairy products. The latter group includes the only species proposed for QPS status, i.e., *Propionibacterium freudenreichii.* Other species have been isolated from different environments (e.g., *Propionibacterium cyclohexanicum*, from spoiled orange juice), and phylogenetically assigned to different subgroups,

Table 15.6

List of valid names in the genus *Propionibacterium* (updated at September 2008)

	Species of the genus *Propionibacterium*
1	*Propionibacterium acidipropionici*
2	*Propionibacterium acnes*
3	*Propionibacterium australiense*
4	*Propionibacterium avidum*
5	*Propionibacterium cyclohexanicum*
6	***Propionibacterium freudenreichii***
	Propionibacterium freudenreichii subsp. *freudenreichii*
	Propionibacterium freudenreichii subsp. *Shermanii*
7	*Propionibacterium granulosum*
8	*Propionibacterium innocuum*
9	*Propionibacterium jensenii*
10	*Propionibacterium microaerophilum*
11	*Propionibacterium propionicum*
12	*Propionibacterium thoenii*

Species with QPS status are underlined, principal species including probiotic strains are in bold and are described in the text

demonstrating that the correlation between phylogenetic and ecological structure of the genus is not obvious.

Propionibacterium Freudenreichii

Propionibacterium freudenreichii is the type species of the genus *Propionibacterium*, and it owes its name to the microbiologist von Freudenreich, who first described, with Orla-Jensen, the "*Bacterium acidi propionici* a." It includes two subspecies, namely *Propionibacterium freudenreichii* subsp. *freudenreichii* and *Propionibacterium freudenreichii* subsp. *shermanii*. Strains of this species are commonly found in cheese, where are often used as starter cultures; to date only strains belonging to this species have probiotic properties confirmed in humans. As other propionibacteria, strains of this species can be rod-shaped or branched, can be observed as single cells or in pairs, or in groups, and grow anaerobically.

Propionibacterium freudenreichii belongs to the group of dairy species, but its closest phylogenetic relatives are two recently described species, i.e.,

Propionibacterium cyclohexanicum and *Propionibacterium australiense*, isolated from spoiled orange juice and granulomatous bovine lesions, respectively. The type strain of the subspecies is ATCC 6207^T, isolated from Swiss cheese.

Currently the genome sequencing for two strains in this species is ongoing, one for a non-type strain, not identified at the subspecies level (Gi00483), and a second one for the type strain of the subsp. *shermanii* CIP 103027^T (Gi 00772).

15.3.2.2 Spore-Forming Bacteria: Genera *Bacillus, Brevibacillus, Paenibacillus*, and *Sporolactobacillus*

The first description of genus *Bacillus* dates back to 1872 (Cohn) and it was based on two species, i.e., *Bacillus anthracis* and *Bacillus subtilis*. Nowadays, 147 species are ascribed to this genus (*Table 15.7*), and many other species have also been transferred to other genera, i.e., *Alkalibacillus, Alicyclobacillus, Aneurinibacillus, Brevibacillus, Geobacillus, Gracilibacillus, Lysinibacillus, Marinibacillus, Paenibacillus, Pullulanibacillus, Salimicrobium, Sporolactobacillus, Sporosarcina, Ureibacillus, Virgibacillus*, and *Viridibacillus* (Garrity et al., 2007b).

According to a recent re-analysis of all archaea and bacteria described to date (Yarza et al., 2008) the genus *Bacillus* is sub-divided in at least eight phylogenetic groups, intermixed with other bacterial genera (see http://www.arb-silva.de/fileadmin/silva_databases/living_tree/LTP_tree_s93.pdf), in the taxonomic lineage of *Firmicutes*.

Members of the genus *Bacillus* (and related genera) are a heterogeneous group of aerobic rod shaped bacteria, producing lactic acid and other metabolites (e.g., carbon dioxide, diacetyl, bacteriocins), with a range of mole G + C genome content from 32 to 69%, with the most striking characteristic of producing endospores, a characteristic important also for probiotic application. Their heterogeneity results in difficult identification based on phenotypic but also on genetic tests (Sanders et al., 2003).

Bacillus species are commonly associated with soil, but frequent sources of isolation are also water, dust, and air, but are also easily found in the GIT of humans, mammals, aquatic animals and invertebrates, whether as contaminant or as residing *taxa*. Industrially, bacilli are employed for production of antibiotics, industrial chemicals, and enzymes. Also, the potential for food spoilage is known for some species. Furthermore, heat resistant spores are very problematic for some specific areas such as dried milk industry, and some species are known as possible agents in biological warfare in terroristic actions. However, *B. subtilis*

Table 15.7

List of valid names in the genus *Bacillus* (updated at September 2008) (*Cont'd* p. 622)

	Species of the genus *Bacillus*
1	*Bacillus acidiceler*
2	*Bacillus acidicola*
3	*Bacillus aeolius*
4	*Bacillus aerius*
5	*Bacillus aerophilus*
6	*Bacillus agaradhaerens*
7	*Bacillus akibai*
8	*Bacillus alcalophilus*
9	*Bacillus algicola*
10	*Bacillus altitudinis*
11	*Bacillus alveayuensis*
12	*Bacillus amyloliquefaciens*
13	*Bacillus anthracis*
14	*Bacillus aquimaris*
15	*Bacillus arseniciselenatis*
16	*Bacillus arsenicus* Shivaji et al. (2005)
17	*Bacillus asahii*
18	*Bacillus atrophaeus*
19	*Bacillus aurantiacus*
20	*Bacillus azotoformans*
21	*Bacillus badius*
22	*Bacillus barbaricus*
23	*Bacillus bataviensis*
24	*Bacillus benzoevorans*
25	*Bacillus bogoriensis*
26	*Bacillus boroniphilus*
27	*Bacillus butanolivorans*
28	*Bacillus carboniphilus*
29	*Bacillus cellulosilyticus*
30	*Bacillus cereus*
31	*Bacillus chagannorensis*
32	*Bacillus cibi*
33	*Bacillus circulans*
34	*Bacillus clarkii*
35	***Bacillus clausii***
36	***Bacillus coagulans***
37	*Bacillus coahuilensis*

Table 15.7 (*Cont'd* p. 623)

	Species of the genus *Bacillus*
38	*Bacillus cohnii*
39	*Bacillus decisifrondis*
40	*Bacillus decolorationis*
41	*Bacillus drentensis*
42	*Bacillus edaphicus*
43	*Bacillus endophyticus*
44	*Bacillus farraginis*
45	*Bacillus fastidiosus*
46	*Bacillus firmus*
47	*Bacillus flexus*
48	*Bacillus foraminis*
49	*Bacillus fordii*
50	*Bacillus fortis*
51	*Bacillus fumarioli*
52	*Bacillus funiculus*
53	*Bacillus galactosidilyticus*
54	*Bacillus gelatini*
55	*Bacillus gibsonii*
56	*Bacillus ginsengihumi*
57	*Bacillus halmapalus*
58	*Bacillus halodurans*
59	*Bacillus hemicellulosilyticus*
60	*Bacillus herbersteinensis*
61	*Bacillus horikoshii*
62	*Bacillus horti*
63	*Bacillus humi*
64	*Bacillus hwajinpoensis*
65	*Bacillus idriensis*
66	*Bacillus indicus*
67	*Bacillus infantis*
68	*Bacillus infernus*
69	*Bacillus insolitus*
70	*Bacillus isabeliae*
71	*Bacillus jeotgali*
72	*Bacillus koreensis*
73	*Bacillus kribbensis*
74	*Bacillus krulwichiae*
75	*Bacillus lehensis*

Table 15.7 (*Cont'd* p. 624)

	Species of the genus *Bacillus*
76	*Bacillus lentus*
77	*Bacillus licheniformis*
78	*Bacillus litoralis*
79	*Bacillus luciferensis*
80	*Bacillus macauensis*
81	*Bacillus macyae*
82	*Bacillus mannanilyticus*
83	*Bacillus marisflavi*
84	*Bacillus massiliensis*
85	*Bacillus megaterium*
86	*Bacillus methanolicus*
87	*Bacillus mojavensis*
88	*Bacillus mucilaginosus*
89	*Bacillus muralis*
90	*Bacillus murimartini*
91	*Bacillus mycoides*
92	*Bacillus nealsonii*
93	*Bacillus niabensis*
94	*Bacillus niacini*
95	*Bacillus novalis*
96	*Bacillus odysseyi*
97	*Bacillus okhensis*
98	*Bacillus okuhidensis*
99	*Bacillus oleronius*
100	*Bacillus oshimensis*
101	*Bacillus panaciterrae*
102	*Bacillus patagoniensis*
103	*Bacillus plakortidis*
104	*Bacillus pocheonensis*
105	*Bacillus polygoni*
106	*Bacillus pseudalcaliphilus*
107	*Bacillus pseudofirmus*
108	*Bacillus pseudomycoides*
109	*Bacillus psychrodurans*
110	*Bacillus psychrosaccharolyticus*
111	*Bacillus psychrotolerans*
112	***Bacillus pumilus***
113	*Bacillus pycnus*

Table 15.7

	Species of the genus *Bacillus*
114	*Bacillus qingdaonensis*
115	*Bacillus ruris*
116	*Bacillus safensis*
117	*Bacillus salarius*
118	*Bacillus saliphilus*
119	*Bacillus schlegelii*
120	*Bacillus selenatarsenatis*
121	*Bacillus selenitireducens*
122	*Bacillus seohaeanensis*
123	*Bacillus shackletonii*
124	*Bacillus silvestris*
125	*Bacillus simplex*
126	*Bacillus siralis*
127	*Bacillus smithii*
128	*Bacillus soli*
129	*Bacillus sonorensis*
130	*Bacillus sporothermodurans*
131	*Bacillus stratosphericus*
132	*Bacillus subterraneus*
133	*Bacillus subtilis*
	Bacillus subtilis subsp. *spizizenii*
	Bacillus subtilis subsp. *subtilis*
134	*Bacillus taeanensis*
135	*Bacillus tequilensis*
136	*Bacillus thermantarcticus*
137	*Bacillus thermoamylovorans*
138	*Bacillus thermocloacae*
139	*Bacillus thioparans*
140	*Bacillus thuringiensis*
141	*Bacillus tusciae*
142	*Bacillus vallismortis*
143	*Bacillus vedderi*
144	*Bacillus vietnamensis*
145	*Bacillus vireti*
146	*Bacillus wakoensis*
147	*Bacillus weihenstephanensis*

Species with QPS status are underlined, principal species including probiotic strains are in bold and are described in the text

is traditionally used in Eastern countries to produce specific foods, which demonstrates its safe use for food application, together with other species. In fact, members of the genus *Bacillus* are used as probiotics, where one of the main advantages over classical lactobacilli is the availability of endospores, which may be stored desiccated almost indefinitely (Fritze and Claus, 1995).

Spore-forming bacteria proposed for probiotics production belong mainly to the species *Bacillus cereus, Bacillus clausii, Bacillus coagulans, Bacillus indicus, Bacillus licheniformis, Bacillus pumilus, Bacillus subtilis*, "*Bacillus laterosporus*," "*Bacillus laevolacticus*," "*Bacillus polymyxa*" and "*Bacillus polyfermenticus*." Species names reported in inverted commas are not valid either because they are not updated ("*B. laterosporus*" is now *Brevibacillus laterosporus*, "*Bacillus polymyxa*" corresponds to *Paenibacillus polymyxa*, and "*Bacillus laevolacticus*" belongs to the genus *Sporolactobacillus*, with the same species name) or they have not been formally described ("*Bacillus polyfermenticus*"). Some details on the genera *Brevibacillus* and *Sporolactobacillus* are given below, before species explanations.

The genus *Brevibacillus*, the name meaning short rod, has been described in 1996 to accommodate, on the basis of phylogenetic relatedness, ten species of Gram-positive, motile, aerobic spore-forming bacteria previously belonging to the genus *Bacillus* (Shida et al., 1996). After publication of that new genus name, other four species have been described, i.e., *Brevibacillus ginsengsoli, Brevibacillus invocatus, Brevibacillus levickii*, and *Brevibacillus limnophilus* (Table 15.8). Ecologically, *Brevibacillus* inhabit the same environments as *Bacillus*. *Brevibacillus brevis* is the type species of the genus.

Species in the genus *Paenibacillus* are facultatively anaerobic or strictly aerobic bacteria, rod shaped, generally Gram-positive but also variable, usually motile, almost all catalase positive. They have different degrading abilities, excreting diverse proteolytic and/or extracellular polysaccharide-hydrolyzing enzymes. The G + C contents range from 45 to 54 mol%. The type species is *Paenibacillus polymyxa*. The genus groups more than 90 species, none of them has been proposed for QPS status, strains considered probiotics seem to belong to *Paenibacillus polymyxa* only.

The genus *Sporolactobacillus* comprises six species of catalase-negative, facultative anaerobic or microaerophilic endosporeformers (Table 15.9). Due to the metabolic similarities with genuine lactic acid bacteria, species of this genus were originally thought to belong to the genus *Lactobacillus*, but then assigned to *Bacillus*, due to their ability to form spores, and eventually described as novel genus, in the Family *Sporolactobacillaceae* (Garrity et al., 2007b). The type species for *Sporolactobacillus* is *Sporolactobacillus inulinus*. Members of the genus have been isolated from chicken feed, soil, dairy products, and pickle.

Table 15.8

List of valid names in the genus *Brevibacillus* (updated at September 2008)

	Species of the genus *Brevibacillus*
1	*Brevibacillus agri*
2	*Brevibacillus borstelensis*
3	*Brevibacillus brevis*
4	*Brevibacillus centrosporus*
5	*Brevibacillus choshinensis*
6	*Brevibacillus formosus*
7	*Brevibacillus ginsengisoli*
8	*Brevibacillus invocatus*
9	***Brevibacillus laterosporus***
10	*Brevibacillus levickii*
11	*Brevibacillus limnophilus*
12	*Brevibacillus parabrevis*
13	*Brevibacillus reuszeri*
14	*Brevibacillus thermoruber*

No species has been proposed for QPS status. Species including proposed probiotic strains are in bold and are described in the text

Table 15.9

List of valid names in the genus *Sporolactobacillus* (updated at September 2008)

	Species of the genus *Sporolactobacillus*
1	***Sporolactobacillus inulinus***
2	*Sporolactobacillus kofuensis*
3	*Sporolactobacillus lactosus*
4	***Sporolactobacillus laevolacticus***
5	*Sporolactobacillus nakayamae*
	Sporolactobacillus nakayamae subsp. *nakayamae*
	Sporolactobacillus nakayamae subsp. *Racemicus*
6	*Sporolactobacillus terrae*

No species has been proposed for QPS status. Species including proposed probiotic strains are in bold and are described in the text

Bacillus Cereus

The species *Bacillus cereus*, the species name meaning wax-coloured, is associated with food poisoning, due to the production of toxins, however strains belonging to the "var. *vietnami*" have been indicated as probiotics (Duc et al., 2004).

The indication of a variety (the "var.") has no meaning in bacterial taxonomy. In fact this name has been assigned to one strain exhibiting physiological traits similar to *Bacillus cereus* but apparently not phylogenetically related to it. According to Hoa et al. (2000), these data could justify the description of a novel species. For the time being, this description has not appeared, therefore the name cannot be considered valid. Also, another organism attributed with probiotic properties is "*Bacillus toyoi*" or "*Bacillus cereus* var. *toyoi*," used mostly for animals (e.g., De Cupere et al., 1992; Scharek et al., 2007), but similar considerations presented for "var. *vietnami*" apply.

In general, *Bacillus cereus* is motile, hemolytic on blood agar, penicillin resistant. Its closest relatives are *Bacillus mycoides, Bacillus weihenstephanensis, Bacillus thuringensis, Bacillus anthracis* and *Bacillus pseudomycoides*. According to the tree obtained by Yarza et al. (2008) this group of bacteria seems to be more closely related with the genus *Gemella* than with other *Bacilli*.

Type strain of *Bacillus cereus* is DSM 31^T (=ATCC 14579^T) which has also been sequenced (Gc00135) together with other strains, already completed (Gc00617, Gc00215, and Gc00173) or ongoing (40 more), due to the clinical interest for the species.

Bacillus Clausii

Bacillus clausii, described in 1995 with other alkaliphilic species in the genus *Bacillus*, is named after Claus, a German microbiologist who made fundamental contribution to the study of bacilli, and it groups bacteria isolated from soil. The closest phylogenetic relatives are *Bacillus oshimensis, Bacillus lehensis, Bacillus patagoniensis, Bacillus gibsonii, Bacillus murimartini*, and *Bacillus plakortidis*. As a species, it is characterized by the production of catalase and oxidase, is able to reduce nitrate and to hydrolyze starch and gelatin, finally, it grows between 30 and 50°C and with up to 10% sodium chloride. Genome GC content is around 43 mol% (Nielsen et al., 1995). The type strain, isolated from Garden soil, is ATCC 700160^T (=DSM 8716^T). Genome sequencing projects exist for strains in the species, one complete (Gc00228), and one incomplete (Gi00061), strains chosen for sequencing are not the type strain.

Bacillus Coagulans

Bacillus coagulans was first described in 1915 isolated from spoiled canned milk, in which it had caused coagulation (from which the names) is a thermotolerant and microaerophilic species, associated to spoilage of foods such as milk

products, vegetables and fruits. Also *Bacillus coagulans* is exploited industrially, e.g., as a source of thermostable enzymes, and it is employed also as growth promoting additive in food and feed, often under the invalid name of "*Lactobacillus sporogenes*." Different morphologies of the cells, spore surfaces and sporangia have been reported, complicating the recognition of *Bacillus coagulans* as a single species. An emended description of the species based on a polyphasic approach and on 31 strains confirmed the heterogeneity of the species (De Clerck et al., 2004), the strains of which have been isolated from different environments, suggesting high flexibility. The closest phylogenetic relatives are *Bacillus oleronius, Bacillus sporothermodurans, Bacillus acidicola*, and *Bacillus ginsenghumi*, which, as a clade, appear to be quite unrelated to *Bacillus subtilis* group. The type strain is ATCC 7050^T (=LMG 6326^T = DSM 1^T) isolated from evaporated milk, but other strains have been isolated from soil. A genome project is ongoing (Gi01001) for a non-type strain.

Bacillus Licheniformis

The first description of the *taxon* called today *B. licheniformis* dates back to 1898, and it owes its name to its shape, the name meaning lichen-shaped. As other bacilli, it is Gram-positive, microaerophilic, and motile. *Bacillus licheniformis* is an industrially important strain as it produces enzymes and bacteriocins. Strains of *Bacillus licheniformis* have been isolated from soil, milk, water, but also septic wounds, and are generally considered safe. However, Salkinoja-Salonen et al. (1999) and other studies have reported on the toxigenic activity of *Bacillus licheniformis, Bacillus pumilus*, and *Bacillus subtilis*. Interestingly, these three species, proposed for probiotic application, also belong to the same phylogenetic group.

The type strain is ATCC 14580^T (=DSM 13^T = LMG 12363^T), and genome data for DSM 13^T have been obtained by two groups independently (Gc00213, and Gc00221).

Bacillus Pumilus

Strains of this species, the name of which means "small," are used for enzyme production on industrial scale, and for many other applications.

Strains in this species are aerobic and motile, positive for catalase as well as for oxidase, beta-galactosidase and amylase. Acid production is observed from glucose, arabinose, mannitol and xylose. As for pH it is a neutrophilic species, while, considering heat resistance of vegetative cells, it is mesophilic. This species is also associated to food poisoning due to toxin production. It belongs to *Bacillus*

subtilis phylogenetic group (see below). Its type strain is ATCC 7061^T (=DSM 27^T = LMG 18928^T). Genome sequences for four strains are completed or in progress, including one for the type strain (Gc00656, Gi03241, Gi01901, and Gi00674).

Bacillus Subtilis

The slender shape of these rods, less than 1 μm wide, gives the names to the *taxon*, which is the type species of the genus. As anticipated, *Bacillus subtilis* is the classical model organism for genetic research in Gram-positive bacteria, but it is also widely used in traditional and industrial fermentation processes as well as in agriculture. Phylogenetically it is very closely related to species *Bacillus altitudinis, Bacillus aerius, Bacillus aerophilus, Bacillus amyloliquefaciens, Bacillus atrophaeus, Bacillus licheniformis, Bacillus mojavensis, Bacillus pumilus, Bacillus safensis, Bacillus sonorensis, Bacillus stratosphericus, Bacillus vallismortis*, and *Bacillus velezensis* but also to *Brevibacterium halotolerans*. Its sporangia are not swollen, and spores are ellipsoidal. It grows between 15–20 and 45–55°C, with an optimum at 28–30°C. It also grows in presence of 7% sodium chloride. It is a strictly aerobic and catalase positive species, able to hydrolyse starch and casein.

Recently, a second subspecies has been described, i.e., subsp. *spizizenii,* named after American bacteriologist Spizizen, on the basis of significant sexual isolation found between two genotypically distinguishable populations within the species and DNA-DNA hybridization levels in the range of 58–68%. These values are thus just below the threshold usually applied for the delineation of species (70%) but, due to the high overall similarity of this second group of bacteria with the type strain of *Bacillus subtilis* the two populations were retained in the same species (Nakamura et al., 1999). Type strains are ATCC 6051^T (=DSM 10^T = LMG 7135^T), for subsp. *subtilis*, and NRRL B-23049^T (=LMG 19156^T), for subsp. *spizizenii*. Genome sequence data are available or in progress for 10 strains (Gc00010, Gi01245, Gi03233, Gi01244, Gi01246, Gi03234, Gi03274, Gi03229, Gi03230, and Gi03239).

Brevibacillus Laterosporus (Formerly "Bacillus laterosporus")

Brevibacillus laterosporus strains take their name from the lateral position of spores. These bacteria show low level of larvicidal activity, and have also been associated with human infections. As for temperature for growth, it ranges between 15 and 50°C, with an optimum at 30°C. The species reduces nitrate and is capable of casein and gelatin hydrolysis, but not of starch.

Phylogenetically the genus appears quite homogeneous. Type strain is ATCC 4517^T (=DSM 25^T) while no sequence data are in progress at the moment, according to lists in GOLD database.

Paenibacillus Polymyxa (Formerly "Bacillus polymyxa")

As anticipated, *Paenibacillus polymyxa* is the type strain of the genus. Its closest phylogenetic relatives are *Paenibacillus kribbensis, Paenibacillus peoriae, Paenibacillus jamilae, Paenibacillus brasilensis*, and *Paenibacillus terrae*. It is able to hydrolyze pectin and xylan and fix nitrogen, and it produces acid from various sugars. Another interesting characteristic is the production of slime, which gives the origin to the name, which literally means "much slime." Its habitat is probably soil. The type strain is ATCC 842^T, and genome sequence data are available (Gi00423) for a non-type strain.

Sporolactobacillus Inulinus

Sporolactobacilli, in general, and species *inulinus* in particular, display intermediate characteristics with respect to *Bacillus* and *Lactobacillus*. In common with the former is the ability to form spores, and the presence of diaminopimelic acid as cell wall component. Similarly to the latter, they are catalase negative, microaerophilic and produce lactic acid from glucose through homolactic fermentation. The lactic acid isomer produced is D (-). Genome GC content is 38–39%. The type strain is ATCC 15538^T (=DSM 20348^T = LMG 11481^T), and no genome sequence days appear to be in progress in GOLD database.

Sporolactobacillus Laevolacticus

The taxonomic status of this motile bacterium producing D(–) – lactic acid has been questioned for a long time, but only recently (Andersch et al., 1994; Hatayama et al., 2006) its description has been validly published, including motile bacteria with diaminopimelic acid in the cell wall. The species is facultatively anaerobic, catalase positive, and mesophilic, with 40°C as the maximum temperature for growth. It hydrolyzes starch and degrades sugars through homolactic fermentation. Strains have been isolated from rhizospheres of plants. The type strain is ATCC 23492^T (=DSM 442^T = LMG 6329^T), and no sequence data are available.

Notably, *Bacillus polyfermenticus* is not a validly published name. Therefore, even if research reports on its probiotic properties have been published, no reliable characteristics can be assigned to this name, and no strain can be indicated as reference point.

15.3.2.3 Genus *Escherichia*

The genus *Escherichia* belongs to the Phylum *Proteobacteria*, Class *Gammaproteobacteria*, Order *Enterobacteriales*, Family *Enterobacteriaceae*. Genera in the same family are: *Arsenophonus, Brenneria, Buchnera, Budvicia, Buttiauxella, Cedecea, Citrobacter, Dickeya, Edwardsiella, Enterobacter, Erwinia, Ewingella, Hafnia, Klebsiella, Kluyvera, Leclercia, Leminorella, Moellerella, Morganella, Obesumbacterium, Pantoea, Pectobacterium, Phlomobacter, Photorhabdus, Plesiomonas, Pragia, Proteus, Providencia, Rahnella, Raoultella, Saccharobacter, Salmonella, Samsonia, Serratia, Shigella, Sodalis, Thorsellia, Tatumella, Trabulsiella, Wigglesworthia, Xenorhabdus, Yersinia*, and *Yokenella* (Garrity et al., 2007d).

Escherichia includes six species of Gram-negative rods, chemo-organotrophic with both oxidative and fermentative metabolism, catalase-positive which produce acid and gas from glucose.

From a taxonomic standpoint, the genus should also include the four species of the genus *Shigella*, which are retained separate only for historical and clinical reasons (Lan and Reeves, 2002).

Escherichia coli is the best known species of the genus, and it is a widespread commensal of the lower intestinal tract of humans and other vertebrates. Clones of *Escherichia coli* cause intestinal and extra-intestinal diseases with devastating effects on the host. However, as a commensal, some *Escherichia coli* strains are also used as probiotics. The type strain of *Escherichia coli* is ATCC 11775^T (=LMG 2092^T = DSM 30083^T = JCM 1649^T). It was isolated from urine but it has not been sequenced although about 71 genome sequences are reported as completed (13) or ongoing (58) projects for the species *Escherichia coli*, not considering *Shigella*.

In a recent genomic study, Willenbrock et al. (2007) analyzed the genomic content of four probiotic strains by microarray. Results showed that the probiotic strains were most similar, in terms of gene pool, with *Escherichia coli* K-12 strains, and with H10407, which is an enterotoxigenic strain, the virulence of which is plasmid-encoded. Some virulence related genes where also detected in these isolates, indicating that both pathogenic and non-pathogenic *Escherichia coli* strains use common strategies for adaptation to their niche. Finally, genetic flexibility was witnessed by the presence of strain-specific phage genes, transposases, insertion elements and mobile-elements-related genes. These data cannot be used in devising a taxonomic scheme as they are not stable elements, but are of utmost importance for characterization at the strain level.

15.3.3 Yeasts

Yeasts are unicellular eukaryotic microorganisms. The strains used as probiotics are referred to as "*Saccharomyces boulardii*" however this species name has no meaning in taxonomy as it is a synonym for *Saccharomyces cerevisiae* (Edwards-Ingram et al., 2004; Hennequin et al., 2001; McCullough et al., 1998; Mitterdorfer et al., 2002).

According to Kurtzman and Fell (1999), the genus *Saccharomyces* includes 14 species. However more recently, Kurtzman (2003), on the basis of Multigene Sequence Analysis, proposed a new *Saccharomyces* genus that includes only seven of the previous species (*Saccharomyces cariocanus, Saccharomyces cerevisiae, Saccharomyces bayanus, Saccharomyces mikatae, Saccharomyces kudriavzevii*, *Saccharomyces paradoxus*, and *Saccharomyces pastorianus*); the rest of the previous species are in a new genus, namely *Kazachstania. Saccharomyces cerevisiae* is the type species of the genus.

Considering the taxonomic lineage, the genus *Saccharomyces* is grouped in the Domain Eukaryota, Kingdom Fungi, Phylum Ascomycota, Subdivision Saccharomycotina, Class Saccharomycetes, Order Saccharomycetales, Family Saccharomycetaceae. It can be noted that names above the genus level are not written in italics, according to the rules of the International Code of Botanical Nomenclature (McNeill et al., 2006) and differently from bacterial nomenclature.

Cells of *Saccharomyces cerevisiae* reproduce vegetatively by multilateral budding (a characteristic of the Class Saccharomycetes), and are transformed directly to asci, containing ascospores, when grown on acetate agar. Also, a characteristic of the genus *Saccharomyces* is the vigorous fermentation of sugars, which is the desired characteristic of strains of *Saccharomyces cerevisiae* (and other species) used for food production, from breadmaking to production of alcoholic beverages from a wide range of vegetable raw materials. Besides its commercial importance, *Saccharomyces cerevisiae* is a very important model system for molecular biology and genetics, as the basic cellular mechanisms of this simple eukaryote are largely conserved also in higher organisms, including mammals. The name of the anamorph for *Saccharomyces cerevisiae* is *Candida robusta*, and a large number of synonyms have been determined. The type strain is CBS 1171^T (=ATCC 18824^T = DBVPG 6173^T = NRRL Y-12632^T), isolated in a Dutch brewery (Vaughan-Martini and Martini, 1999). Genome

sequence for one strain has been determined (Gc00006) but 35 others are ongoing.

15.4 Summary

- Names are the results of taxonomic procedures, i.e., extensive and time consuming characterization of organisms under different aspects (genetic, physiology, etc) in order to define their diversity.
- Taxonomic studies of microorganisms have always been dependent on scientific and technological advancements and development of novel techniques of investigation, due to their extremely small size. Application of new techniques can highlight novel traits and therefore can modify the understanding of diversity of organisms, thus changing also names.
- Correct names of organisms are essential as they (i) allow retrieving all the updated information on *taxa*, (ii) constitute a standard for the unambiguous identification of bacteria, both for scientific purposes, for definition of lists of safe and pathogenic bacteria, and for reliable commercial information on microorganisms.
- Taxonomic traits, i.e., characteristics useful for characterization of genera, species and subspecies can be different in different organisms, as they depend also on ecology and evolution. Therefore, classification of different groups of bacteria could be slightly different and it has to be considered an "agreement among experts." On the other hand, nomenclature, i.e., the assignment of names to recognized taxa, is strictly regulated, to ensure clarity.
- Probiotic properties are strains specific and not species specific, but an accurate identification at species level is essential to have more information on the strains.

List of Abbreviations

EFSA European Food Safety Authority
GIT gastro-intestinal tract
LAB lactic acid bacteria
QPS qualified presumption of safety

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16 Ecological Interactions of Bacteria in the Human Gut

Gwen Falony · Luc De Vuyst

16.1 Introduction

The colon or large intestine is one of the most important organs of the human body (Macfarlane and Cummings, 1991). Moreover, its inhabitants, the colon microbiota, are the key elements of the human digestive ecosystem. The vast complexity of the human large-intestinal microbiota has inspired researchers to consider it as an organ itself, located inside the colon and acquired postnatally (Bäckhed et al., 2005; Zocco et al., 2007). From a physiologist's point of view, this image of the colon microbiota is relevant: like an organ, it is composed of different cell lineages that communicate with both one another and the host; it consumes, stores, and redistributes energy; it mediates physiologically important chemical transformations; and it is able to maintain and repair itself through self-replication (Bäckhed et al., 2005). As a microbial organ, the human colon community does not only broaden the digestive abilities of the host (Gill et al., 2006), but also influences body processes far beyond digestion (Roberfroid, 2005b; Turnbaugh et al., 2007).

From a microbiologist's point of view, the simplified perception of the microbial colon community as a functional entity narrows down colon ecosystem research toward a 'black box' approach, not only with respect to the identification of its inhabitants, but also concerning the numerous metabolic activities and interactions that take place in the large instestine (Pryde et al., 2002). As the exact composition of the colon microbiota remains at present largely unexplored, input/output-based studies might appear manageable tools to investigate a terrifying complexity. However, even though such studies would generate comprehensive results, they inherently neglect the sometimes subtle fluctuations in composition and metabolic activity within the microbial colon community. Such changes – with a possible impact on the host's health – can only be monitored after thorough dissection of the colon microbiota and subsequent identification

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of the dominant microbial clusters and their interactions (Flint et al., 2007). The latter will eventually lead toward a global, ecological understanding of the colon ecosystem.

During the last few years, the implementation of culture-independent molecular techniques in the field of gut research has revealed the presence of an unsuspected microbial diversity in the human colon (Eckburg et al., 2005; Frank et al., 2007; Li et al., 2008). Further exploration of this microbial wealth is a necessary step toward a better understanding of the relationship between the human host and its symbionts. However, defining a complex ecosystem, such as the human large intestine, does not end with the construction of a catalogue of its members, but also implies the determination of the habitat (ecological or environmental area inhabited) and niche (relational position within the ecosystem) of each (cluster of) inhabitant(s) and its functional role(s) (Ley et al., 2006). This functional characterization of large bacterial groups within the colon ecosystem is probably one of the greatest challenges for microbiologists in the years to come (Turnbaugh et al., 2007).

16.2 Defining the Colon Ecosystem

The human colon represents one of the most complex microbial ecosystems known to men (Zoetendal et al., 2006). The concept of an ecosystem as the basic unit in ecology was first proposed by Arthur George Tansley in 1935 (Trudgill, 2007). It was defined as a natural unit consisting of all plants, animals, and micro-organisms (biotic factors) in an area functioning together with all of the non-living physical (abiotic) factors of that environment (Evans, 1956; Tansley, 1935; Townsend et al., 2003). An ecosystem is by definition an artificial subunit of an environment: a mental construct imposed on a complex reality for study purposes solely (Tansley, 1935). Ecosystem boundaries are mental boundaries, as all ecosystems are not only part of larger ones, but also overlap, interlock, and interact with one another (Tansley, 1935; Townsend et al., 2003). The human colon ecosystem, characterized by a constant in- and outflux of nutrients, microorganisms, and end-products of colon fermentation, makes no exception to this rule.

From a broad ecological point of view, the human body can be considered as a super-organism composed of an amalgam of both microbial and human cells, depending on each other for their survival (Lederberg, 2000). The relationship between the host and the microbial populations inhabiting its colon is often described as commensal (one partner benefits while the other seems unaffected)

as opposed to mutualistic (both partners derive benefit) (Bäckhed et al., 2005; Hooper and Gordon, 2001; Ley et al., 2006). However, it has been suggested that the use of this denomination only reflects a current lack of knowledge concerning the exact nature of host-microbiota interactions (Bäckhed et al., 2005).

The activities of the colon microbiota have a major impact upon nutrition and health of the host via the supply of nutrients, conversions of metabolites, and interactions with host cells (Flint et al., 2007). Moreover, it has been argued that the mere stability of the colon ecosystem, as a result of partially host-driven selection, contributes to the health and well-being of an individual (Ley et al., 2006).

16.2.1 The Human Colon Environment

The human colon can be pictured as an unbranched tube into which nutrients enter at one side and feces are excreted at the other (Macfarlane and Cummings, 1991). It has an average length of 1.5 m and an undisected surface of approximately 1.3 m^2. The large intestine contains about 220 g of wet contents, including an estimated total bacterial mass of 90 g. Daily, it receives around one to two liters of chyme – a semi-fluid mass composed of (partially) undigested food ingredients ($\pm$ 95 g day^{-1}), body excretions, and bacterial as well as human cells – which is subsequently reduced to a final volume of approximately 0.2 l semi-solid feces as a result of absorption of fluids (Macfarlane and Cummings, 1991; Roberfroid, 2005b). Transit of contents is relatively slow, with an average transit time of 60 h in people living on a Western-type diet, whereby gut transit always slows down from the proximal colon toward the rectum (Macfarlane and Cummings, 1991). However, the constant flow of nutrients through the gut ecosystem forces colon bacteria to avoid washout by either maintaining a sufficient reproduction rate or attaching to or colonizing host tissues (Flint et al., 2007).

Roughly, the colon can be divided into three different regions with respect to nutrient availability and bacterial activity, namely the proximal or ascending colon, the transversal colon, and the distal or descending colon (Macfarlane and Cummings, 1991). Food residues and nutrients (carbohydrates, proteins, etc) not absorbed in the small intestine primarily arrive in the ascending colon, where they can be fermented by the resident microbiota. High carbohydrate availability results in high growth rates, fast bacterial turnover, and a relatively low lumenal pH of 5.4–5.9. The gradual carbohydrate and moisture depletion toward the end of the descendal colon is reflected in lower growth rates, slower bacterial turnover, and a corresponding lumenal pH of 6.6–6.9, as compared to the proximal colon.

Whereas carbohydrate fermentation occurs mainly in the proximal colon, protein degradation increases progressively toward the end of the distal part of the colon (Macfarlane et al., 1992). End-products of carbohydrate fermentation include short-chain fatty acids (SCFA), such as acetate, propionate and butyrate, and gases (see Section Dietary Carbohydrate Metabolism in the Human Colon: Metabolic Cross-Feeding), while protein fermentation also generates branched SCFA, ammonia, amines, phenols, and indols (*Figure 16.1*) (Roberfroid, 2005a). The transversal colon is characterized by intermediate conditions with lower bacterial growth rates as compared to the proximal colon and a lumenal pH at an intermediate value of approximately 6.2.

16.2.2 Spatial Heterogeneity

The human colon ecosystem represents in terms of cell density the most successful microbial ecosystem presently described (Ley et al., 2006). The human intestinal

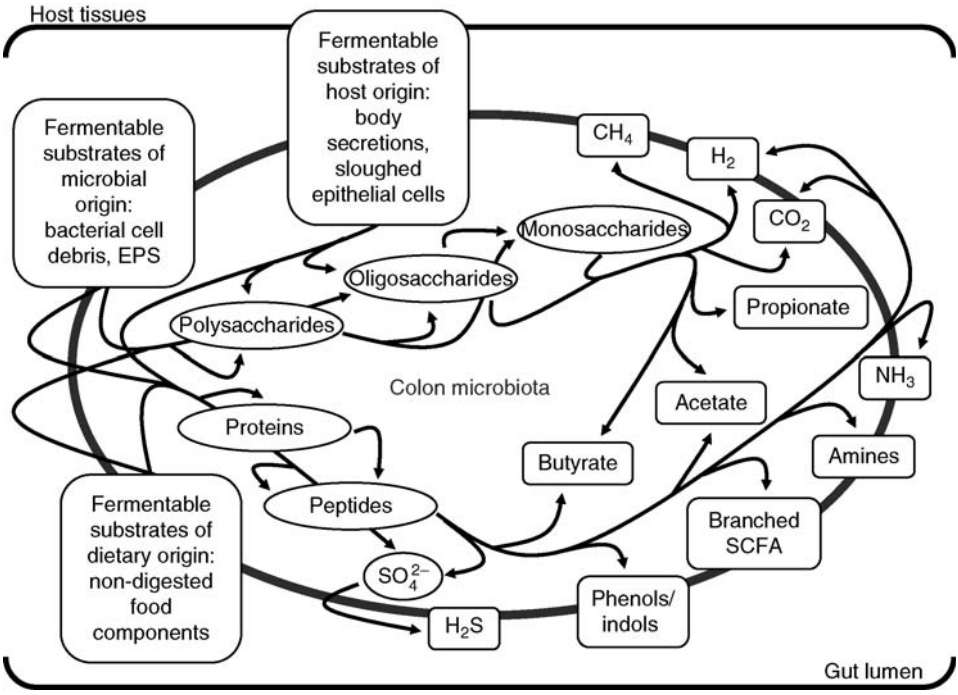

Figure 16.1
Overview of the main routes of carbohydrate and protein fermentation in the human colon.

tract is packed with up to 100 trillion microorganisms, reaching cell densities of up to 10^{12} cells ml^{-1} toward the end of the gut – the highest recorded number for any microbial habitat up to now (Whitman et al., 1998). The human microbiome (the collective genome of the microbiota) is thought to contain more than 100 times the total number of human genes, making the human being more prokaryote than eukaryote (Bäckhed et al., 2005; Gill et al., 2006). The key of the success of the human colon as a microbial habitat lies in the fact that it represents a nutrient-rich and at the same time spatially heterogeneous environment (Flint et al., 2007). While the abundance of nutrients is indispensable to comply with the carbon and energy requirements of the enormous numbers of residing microorganisms, the heterogeneity of the environment assures the availability of a multitude of ecological niches (Flint, 2006; Ley et al., 2006).

The spatial heterogeneity of the colon originates from a variety of factors including host characteristics (e.g., genotype, immune status) and diet composition (e.g., degree of consumption of meat and vegetables/fruits, level of carbohydrate and protein uptake, consumption of pre- and probiotics). Moreover, niche diversity is enhanced by the development of a complex network of often nutrition-based microbial interactions within the colon microbiota itself, with cooperation and competition as the main driving forces (Flint et al., 2007; Flint, 2006). Cooperative interspecies interactions can occur during hydrolysis of complex carbohydrates and sequential utilization of fermentation and partial breakdown products, as well as by the exchange of growth factors (Dethlefsen et al., 2006; Flint et al., 2007). Competition for resources and adhesion sites to the colon are considered the main limiting factors for microbial growth, combined with other bacterial interference mechanisms such as the production of toxic metabolites and specific antimicrobial compounds such as bacteriocins (Dethlefsen et al., 2006; Makras and De Vuyst, 2006; Makras et al., 2004). In this context, the abundant and diverse presence of phages should be mentioned as a possible interfering component, which may contribute to the diversity of the colon's microbial genetic landscape (Breitbart et al., 2003).

Besides competitive and cooperative interactions, colonization history is considered a microbial factor adding to niche diversity (Curtis and Sloan, 2004; Tilman, 2004). By colonizing a pre-existing microbial niche, microorganisms influence their direct surroundings and locally alter the colon environment. In a way, the assembly of the colon microbiota can be thought of as being partially recursive, creating and responding to its own selective pressure (Day et al., 2003). The influence of colonization history contributes to interindividual differences in microbiota composition (see Section Defining the Colon Ecosystem: Microbial

Diversity). Moreover, it enhances and extends the consequences of historical contingencies such as antibiotic interventions (Dethlefsen et al., 2006). In this respect, the colon microbiota does not necessarily represent a climax population: internal dynamics can assure the stable persistence of an out-of-equilibrium situation (Sarr et al., 2005).

As a consequence of the interplay between host and dietary factors, combined with the effects of microbial interactions, the human colon environment is populated with extensively adapted species, displaying a high level of interdependence – often translated in complex growth requirements and possible obligate interactions such as syntrophy (Flint et al., 2007; Ley et al., 2006). The latter is not only reflected by the difficulties encountered when attempting to isolate individual members of the resident microbiota (Ducluzeau, 1989; Duncan et al., 2007b), but it also complicates the prediction of the human colon community responses to external factors – even when isolated populations respond deterministically (Dethlefsen et al., 2006; Flint et al., 2007).

16.2.3 Microbial Diversity

For many decades, the use of culture-dependent microbiological investigation techniques, involving selective plating and incubation, and subsequent identification of bacterial isolates, has dominated gut diversity research (Macfarlane and Macfarlane, 2004; Tannock, 1999). Although the application of such traditional microbiological methods has contributed substantially to the current understanding of the composition of the colon ecosystem, cultivation biases gave rise to some persistent myths concerning the dominating genera. Due to the lack of total anaerobic culturing techniques and the use of cultivation media that do not meet the complex nutritional demands of many colon inhabitants, the presence of relatively easily culturable microorganisms with a high degree of oxygen tolerance has been overestimated in the past (Walter, 2008). Such microorganisms include lactobacilli, clostridia, enterococci, and, on the species level, *Escherichia coli*, as was later revealed by the introduction of culture-independent molecular techniques in the world of gut microbiology (Eckburg et al., 2005; Frank et al., 2007; Walter, 2008). At this moment, up to 80% of the bacterial species inhabiting the human colon is considered unculturable using presently available methodologies (Blaut et al., 2002; Duncan et al., 2007b; Hold et al., 2002). However, it has been suggested that this percentage mainly reflects the limited recent efforts on cultivation rather than an inherent culturability (Flint et al., 2007).

Nowadays, the most comprehensive and least biased enumerations of microbial diversity come from sequencing of 16S rRNA genes, obtained directly from DNA extracted from colon samples, using PCR primers targeted to broad phylogenetic groups (Ley et al., 2006). The main conclusion of the first attempts to implement such techniques on a large scale basis is the fact that microbial colon diversity has been largely underestimated throughout the years (Eckburg et al., 2005; Frank et al., 2007). Diversity at the strain level is overwhelming: estimations vary between 500 and 1,000 species, representing over 7,000 strains (Hooper and Gordon, 2001; Xu et al., 2007). However, despite the large progress that has been made during recent years, current knowledge of the composition of the human colon microbiota remains partial, fragmented, and undetailed.

Notwithstanding the huge diversity reported at strain level, the human colon appears to be a remarkably selective environment. Recent 16S rRNA gene sequencing-based surveys of the distal gut and fecal microbiota of adult individuals revealed that the colon ecosystem is dominated by only four of the 55 bacterial divisions (deep evolutionary lineages, sometimes referred to as 'phyla') described up to date (Bäckhed et al., 2005; Eckburg et al., 2005; Frank et al., 2007). Together, species belonging to the divisions *Firmicutes* (64% – mostly belonging to the *Clostridia* class, including the genera *Eubacterium*, *Faecalibacterium*, *Roseburia*, and *Lactobacillus* – the latter representing a minor group), *Bacteroidetes* (23% – mainly *Bacteroides* spp., with *Bacteroides thetaiotaomicron* as a common representative), *Proteobacteria* (8% – among which *Enterobacteriaceae* and sulfate–reducing colon bacteria), and *Actinobacteria* (3% – including *Bifidobacterium* spp.) make up more than 98% of the bacterial colon population (› *Table 16.1*) (Eckburg et al., 2005; Frank et al., 2007). Other bacterial divisions less abundantly encountered include *Cyanobacteria*, *Fusobacteria*, *Spirochaetes*, and *Verrucomicrobia* (Bäckhed et al., 2005; Eckburg et al., 2005; Frank et al., 2007; Ley et al., 2006). Of the 13 archaeal divisions known to date, *Methanobrevibacter smithii* seems to be the only common resident of the human large intestine (Bäckhed et al., 2005; Eckburg et al., 2005), although some studies indicate a greater diversity (Scanlan et al., 2008). Reports on the presence of fungi are scarce (Scupham et al., 2006).

The reasons for the selectivity of the colon environment are to be found in the strict requirements for the colon microbial community membership. The requisites needed include an arsenal of enzymes to utilize available nutrients; cell-surface molecular paraphernalia to attach to a suited habitat, evade bacteriophages, and appease the human immune system; a genetic flexibility toward mutation and adaptation; the ability to avoid washout through rapid proliferation; and the stress resistance needed to be able to cross a toxic, dry, and aerobic environment when

Table 16.1

Niches of some prominent microbial groups in the human colon (Dethlefsen et al., 2006; Duncan et al., 2007b; Flint, 2006; Macfarlane and Cummings, 1991)

Firmicutes – Clostridial cluster IV. Contains *Faecalibacterium prausnitzii*, an abundant fermenter of starch and inulin-type fructans with an absolute requirement for acetate. Produces butyrate and lactate. Other Cluster IV bacteria have been reported to degrade a range of carbohydrates, including starch, fucose, cellulose (*Ruminococcus* spp. – in combination with methane producers), and other complex plant cell wall materials. Metabolites reported are butyrate, lactate, and acetate.
Firmicutes – Clostridial cluster IX. Poorly characterized bacterial group. Includes species belonging to the genera *Megasphaera* and *Veillonella*, among others. Predominantly saccharolytic. Lactate consumption by *Megasphaera elsdenii* has been reported. Production of butyrate and propionate.
Firmicutes – Clostridial cluster XIVa. Broad bacterial cluster including species belonging to the genera *Anaerostipes*, *Clostridium*, *Coprococcus*, *Eubacterium*, *Roseburia*, and *Ruminococcus*. Mainly saccharolytic, growth reported on fucose, starch, xylan, and inulin-type fructans. Lactate conversion by *Anaerostipes caccae* and *Eubacterium hallii*. Butyrate production with (*Anaerostipes*, *Roseburia, Eubacterium*) or without (*Clostridium*, *Coprococcus*) acetate requirement. Other reported metabolites are ethanol, lactate, succinate, and propionate. Acetogenesis demonstrated for *Ruminococcus hydrogenotrophicus*.
Firmicutes – scattered. Predominant proteolytic and amino acid-fermenting colon bacteria, mostly belonging to the genera *Peptococcus*, *Peptostreptococcus*, and *Clostridium*. Phylogenetically scattered group.
Bacteroidetes. Mainly composed of species belonging to the genus *Bacteroides*. Degradation of xylan, mucin, inulin-type fructans, and other poly- and oligosaccharides. Periplasmic starch degradation. Includes some proteolytic bacteria belonging to the genera *Cetobacterium* and *Alistipes*. Production of acetate, propionate, and succinate.
Actinobacteria. Contains bacterial species belonging to the genera *Bifidobacterium* and *Atopobium*. The genus *Bifidobacterium* includes starch-, inulin-type fructan-, xylan-, and mucin-degrading species, producing lactate, acetate, formate, and ethanol. Atopobia mainly produce acetate, formate, and lactate.
Proteobacteria. The δ-subdivision of the *Proteobacteria* phylum includes H_2-consuming, sulphate-reducing bacteria, belonging to the genera *Desulfovibrio* and *Desulfobulbus*. Other genera consume partially reduced fermentation products, such as lactate and ethanol, while reducing sulphate to sulfide.
Archaea. *Methanobrevibacter smithii* is the predominant methanogen present in the human colon.

passed on from one host to another (Ley et al., 2006). A shallow, widely radiated, fan-like phylogenetic architecture (wide diversity at strain level but far fewer intermediate and deep lineages) is a typical feature for communities that suffer extreme selection followed by a period of détente (Ley et al., 2006). Only limited groups of bacteria are genetically armed to be able to reach the human colon alive,

but once settled, they find to their disposition a wide range of habitats and niches allowing them to proliferate and – on a microcommunity level – to differentiate.

16.2.4 Ecosystem Stability

Microbial ecosystems are generally characterized by a remarkable degree of stability (Sonnenburg et al., 2004; Walter, 2008). The human colon ecosystem makes no exception to this rule: although the colon microbiota is dynamic and able to adapt to environmental challenges, it remains relatively stable over time. It is characterized by a remarkable resistance to chaotic blooms of subpopulations and resilience after disturbances caused by, for example, antibiotic therapy, stress, or drastic changes in diet (Bäckhed et al., 2005; Ley et al., 2006; Makras et al., 2004). The latter phenomenon, commonly referred to as colonization resistance, mainly finds it origin in the highly niche-specific nature of most colon inhabitants. As every ecological niche can only support one or a conjunction of a limited number of microorganisms, it becomes extremely difficult for other bacteria to claim their piece of the environmental colon cake (Ley et al., 2006; Makras et al., 2004).

A key element in the construction process of a stable gut ecosystem is the development of oral tolerance by an individual in the first few months after birth. Oral tolerance, or the specific suppression of cellular and humoral immune responses to an antigen by prior administration of this antigen through the oral route, prevents hypersensitive reactions toward food proteins and bacterial antigens present in the gut environment (Weiner, 2000). It leads toward an individual acceptance of a particular microbiota by the human immune system – a peaceful coexistence between the human super organism and its microbial gut residents – while unaccepted elements might cause responses.

Another factor contributing to the stability of a microbial ecosystem is the diversity displayed by a bacterial community. A huge degree of diversity is usually translated into a broad repertoire of stress responses, ensuring the stability of key system processes (Ley et al., 2006). In the colon, this stability is of supreme importance, as drastic changes can severely affect human health (Bäckhed et al., 2005). Host selection for a stable colon microbiota avoids the occurrence of keystone species – defined as species with a unique and central role in a bacterial community – and favors functional redundancy among the residing microorganisms (Ley et al., 2006). As the human colon microbiota is characterized by shallow levels of microbial diversity (Eckburg et al., 2005; Frank et al., 2007), the latter selectivity for functional redundancy is reflected as habitat specialization. Within

the same bacterial genus, different ecotypes have emerged: functional redundant species coexisting by expressing different degrees of substrate specificity and degradation efficiency (Bäckhed et al., 2005). This combination of both specialization and generalization does not only ensure the stability of the ecosystem as a whole, but also provides individual strains with the ability to respond to a variety of different stress factors (Bäckhed et al., 2005).

The stability of the colon ecosystem makes it possible to differentiate between autochthonous microorganisms, true residents of a particular niche within the colon ecosystem, and allochthonous microorganisms, 'hitchhiking' through the gut (Ley et al., 2006; Walter, 2008). Autochthonous microorganisms have developed long-term associations with their particular hosts. They form stable populations in particular regions of the human colon, although the composition of those bacterial communities can differ substantially from one host to the other (Eckburg et al., 2005; Frank et al., 2007). Whereas a core composed of the dominant phyla is thought to be present in every individual, fingerprints of the colon microbiota at species/strain level are individual-dependent (Ley et al., 2006; Turnbaugh et al., 2007). The allochthonous colon population is mainly composed of microorganisms originating from ingested food, water, or other components from the environment (Ley et al., 2006). Bacteria considered autochthonous in other body ecosystems, such as the oral cavity, also contribute to the allochthonous colon community (Walter, 2008; Xu and Gordon, 2003). Although the composition of this subgroup of colon inhabitants can differ greatly in time, all members share the transient character of their large-intestinal existence.

Although the human gastro-intestinal tract is germ-free at birth, an immediate vertical transfer of microorganisms occurs between the mother and her baby. It has been shown that babies acquire their initial colon microbiota from the vagina and feces of the mother and/or from the environment (Ley et al., 2006). Due to the stability of the colon ecosystem, kinship relations remain observable over time. Through childhood, the complexity of the colon microbiota increases gradually (Dethlefsen et al., 2006). This evolution toward a higher degree of complexity persists in adulthood, as the colon ecosystem is a particularly open environment and, therefore, highly susceptible to colonization and perturbation. Moreover, while the harsh conditions of microbiota transfer at birth favor cooperation and altruism among gut-colonizing species, the period of détente that follows offers possibilities to cheaters, colon inhabitants that benefit from the residing community without contributing to it themselves. However, such cheaters remain a minority, limiting the probability that they are immediately transferred to a new-born (Ley et al., 2006).

16.2.5 Spatial Organization of Microbial Communities

Human feces are without any doubt the most accessible source of information concerning the composition and metabolic activity of the colon microbiota (Macfarlane and Macfarlane, 2004). Nevertheless, fecal microbial diversity is not considered representative for the entire colon ecosystem; at its best, it can provide relevant information regarding the composition of lumenal bacterial communities growing in the distal colon (Zoetendal et al., 2002; Zoetendal et al., 2006). Moreover, most feces-based studies focusing on the diversity of the colon microbiota still tend to start from the premise of colon bacteria occurring as independent individual cells, growing in suspension in a nutrient-rich semi-fluid environment. However, there is microscopic evidence that bacteria in the human colon commonly occur in microcolonies or disparate associations with other species on the surfaces of particulate materials such as food residues (Macfarlane and Dillon, 2007). While interindividual differences might be the main factor contributing to the diversity of the whole human colon microbiota, regional intraindividual variations make a convincing runner-up (Eckburg et al., 2005).

Formation of independent functional microbial entities or biofilms is a common phenomenon in microbial ecosystems. It allows microorganisms to develop coordinated multicellular behavior, both on an intra- and interspecies level, often through quorum sensing (bacterial cell-cell communication through the production and detection of diffusible signaling molecules, resulting in collectively controlled gene expression and synchronized group behavior) (Lazdunski et al., 2004; Salmond et al., 1995). Biofilm communities frequently possess phenotypic features unknown to the individual members, such as a highly efficient substrate metabolism, increased resistance to antibiotics, and decreased pH susceptibility. Moreover, microbial colon biofilms are thought to exhibit a greater resistance to the host's defensive immune system than their non-adherent counterparts (Macfarlane and Dillon, 2007). Although close spatial relationships between microorganisms on surfaces often limit growth by slowing down mass transfer and thus enhancing the effects of nutrient depletion, this drawback becomes negligible by the advantages offered by interbacterial metabolic communication (Macfarlane et al., 2000).

To this date, little is known concerning the formation, structure, and metabolic properties of biofilms in the human large intestine (Kleessen and Blaut, 2005; Macfarlane and Dillon, 2007). The mechanisms involved in attachment, coaggregation, and quorum sensing that might be relevant to large-intestinal biofilm formation remain largely unexplored (Macfarlane and Dillon, 2007). However, as

colon diversity studies indicate the coexistence of distinct sub-ecosystems in the human large intestine (Eckburg et al., 2005), biofilm formation might be of key importance for large-intestinal ecosystem stability and host health.

16.2.5.1 The Lumenal Sub-Ecosystem

The decrease in nutrient availability combined with the increase in pH that characterize the colon lumen environment upon food passage through the large intestine, are thought to have a determining influence on lumenal species composition (Macfarlane and Cummings, 1991). While bifidobacteria and species belonging to clostridial clusters IV and XIVa are presumably more abundant in the proximal colon, the numerical importance of bacteroides and proteolytic *Firmicutes* is thought to increase toward the distal part (Dethlefsen et al., 2006; Walker et al., 2005). However, incomplete peristalsis permitting backflow and mixing contributes to the complexity of this image (Ley et al., 2006), as does the development of lumenal biofilms (Macfarlane and Dillon, 2007).

Lumenal biofilms are composed of bacteria adhering to particulate food residues passing through the gut. They are thought to play a key role in the rapid initial colonization of particulate substances entering the human colon, hence contributing to the stability of the gut ecosystem. The particularity of such biofilms – as well as of mucosal biofilms discussed below – lies in the transient character of the substrates they attach to, which makes them clearly distinct from communities growing on inert sites such as tooth surfaces or catheters (Flint, 2006). Notwithstanding the fact that current understandings of the primary development of such lumenal biofilms are still limited, two recent studies provided valuable information concerning their nature and composition, although presenting different – almost contrasting – results (Macfarlane and Macfarlane, 2006; McWilliam Leitch et al., 2007).

A first study focused on bacteria adhering to particulate matter found in human feces (Macfarlane and Macfarlane, 2006). It revealed that approximately 5% of the bacterial cell mass in the lumen of the large intestine is strongly adherent to the surface of partly digested food particles, with a considerably higher percentage loosely attached. Surprisingly, the composition of these adhering communities was found to be phenotypically similar to that of the unattached populations, with bacteroides and bifidobacteria predominating. In this study, the nature and composition of the insoluble substrates have not been determined. Unfortunately, bacterial identification has been carried out using a culture-dependent approach,

which could explain uncommon composition of the fecal microbiota reported. Fermentation experiments avoiding this culturability bias and using a variety of complex carbohydrates as substrates for both biofilms and non-adhering communities revealed that biofilm populations excel in polysaccharide breakdown, while their free-living counterparts prefer oligosaccharides as an energy source. Acetate is the main fermentation product produced by the biofilm communities, while a higher butyrate production has been noted for the non-adherent bacterial consortium.

A second study concentrated on the selective colonization of insoluble substrates by human fecal bacteria (McWilliam Leitch et al., 2007). Fecal samples from four individuals have been used in different fermentation experiments with wheat bran, high amylose starch, and porcine gastric mucin as added substrates. As culture-independent identification techniques have been applied, the detected proportion of butyrate-producing colon bacteria belonging to clostridial clusters IV and XIVa is far greater than in the study mentioned above, both in the fecal material used as inoculum and in the microbial communities recovered from the insoluble substrates. The study revealed the determining influence of the host as well as the nature of the substrate on biofilm formation. Overall, it could be concluded that bran surface is mainly covered by clostridial cluster XIVa bacteria and *Bacteroides* spp. On mucin, the bacterial species most commonly recovered are related to *Bifidobacterium bifidum* and *Ruminococcus lactaris*. On starch, *Ruminococcus bromii*, *Bifidobacterium adolescentis*, *Bifidobacterium breve*, and *Eubacterium rectale* are most abundant. The fact that *Bacteroides* spp. have not been found to dominate the starch-attached bacterial populations is rather surprising, given the well-described arsenal of starch-degrading mechanisms that can be deployed by species such as *Ba. thetaiotaomicron* (Bjursell et al., 2006; Xu et al., 2003). The authors speculated that such species depend to a large extent on the availability of solubilized polysaccharides that are released by the activities of primary colonizing bacteria. In contrast with the results of an earlier study focusing on fermentation of soluble carbohydrates by human colon-derived microbial communities (Walker et al., 2005), pH did not influence the composition of the adherent microbiota during fermentation of insoluble substrates, a clear manifestation of a biofilm effect. Specific differences attributed to the composition of the donor microbiota could be noted throughout all fermentation experiments. Colonization of starch was, for example, dominated by *Bifidobacterium* spp. for two donors, while for the remaining two a consortium of *E. rectale* and *R. bromii* had the upper hand. This observation suggests that the interactions between the latter two species might be cooperative, whereas those

between them and *Bifidobacterium* spp. are presumably competitive (McWilliam Leitch et al., 2007).

16.2.5.2 Mucosal Biofilms

The human gastrointestinal tract is lined with a perpetually and rapidly renewing epithelium with the surface of a tennis court, shedding around 2×10^6 to 5×10^6 cells min^{-1} into the colon lumen (Croft and Levitan, 1970). The mucus gel layer overlying this gastrointestinal epithelium is the anatomical site at which the first host-colon bacteria encounters take place (Deplancke and Gaskins, 2001). Considered an integral component of the colon ecosystem, the mucus layer acts as a medium for protection, lubrication, and transport between the lumenal contents and the epithelial lining. It is composed predominantly of mucin glycoproteins that are synthesized and secreted by goblet cells. The thickness of the mucus layer increases gradually from the proximal colon up to the rectum (Deplancke and Gaskins, 2001). Mucin as well as epithelial cells carry carbohydrate chains that can act as receptors for binding of microorganisms and as potential energy sources for them (Flint, 2006). Due to the abundance of mucins as an energy source in the colon ecosystem, the human large intestine harbors some specialized mucin degraders, including *Akkermansia muciniphil*a, *Ba. bifidum*, and *Ruminococcus torques* (Derrien et al., 2004; Flint, 2006; Hoskins, 1993). As the habitat of these species remains currently unclear, it is not certain whether they are involved in mucosal biofilm development. Other species definitely appear to grow in a close relationship with the colon mucosa; for example, *Ba. thetaiotaomicron* has been shown to induce epithelial cells to augment the production of fucose, a primary component of mucin, hence meeting its own nutritional needs (Hooper and Gordon, 2001; Hooper et al., 1999).

Studies concerning the mucosa-associated colon community and, more specifically, its distribution along the human large intestine are rather scarce, mostly due to practical and ethical limitations (Macfarlane and Dillon, 2007). Moreover, biopsies and surgical material are usually obtained from diseased individuals or from patients who have received antibiotic therapy, and from those whose colons have been washed-out before endoscopy or colonoscopy (Macfarlane and Dillon, 2007; Zoetendal et al., 2006). As a consequence, microbial biofilms found attached to these tissues may not be representative for the composition of communities found in healthy subjects.

Bacterial communities growing on the colon epithelium as well as those colonizing the mucus layer are thought to be more subject to a variety of host factors than their lumenal counterparts. Besides general limitations such as nutrient availability, imposed on the whole of the large-intestinal ecosystem, mucosal colon biofilms are highly dependent on the rate of synthesis and the chemical composition of mucus, epithelial turnover rates, and disposability of adhesion sites. Moreover, bacteria growing in a close association with the human body are directly exposed to a stringent control by components of both the innate and the adaptive immune system (Macfarlane and Dillon, 2007).

Recent studies on the diversity of mucosa-associated biofilms in the human large intestine showed that the host specificity of the colon microbiota is reflected in the composition of mucosal biofilms (Eckburg et al., 2005; Frank et al., 2007). Molecular analysis of mucosal populations within an individual host demonstrated that most bacteria found on the colon epithelium belong to the same phylogenetic groups as those encountered in fecal material. *Bacteroidetes* and *Firmicutes* belonging to clostridial clusters IV and XIVa appear to be most abundant. Notwithstanding this superficial phylogenetic overlap, statistical diversity analyses reveal significant differences between the mucosal and lumenal colon microbiota. Although some patchiness and heterogeneity has been reported (Eckburg et al., 2005), colon mucosa-associated bacterial populations are uniformly distributed along the gastrointestinal tract (Zoetendal et al., 2006).

Microscopic investigation of the three-dimensional structure of mucosal biofilms has shown that bacteria are present throughout the entire colon mucus layer, although they are usually not found in healthy crypts (Macfarlane et al., 2004). Live/dead staining of the bacterial structures (microcolonies) observed indicate that bacteria are actively growing in the mucus layer and that their presence is not only a result of passive transfer of cells from fecal material in the colon lumen (Macfarlane et al., 2004).

From the proximal to the distal colon, the human large-intestinal ecosystem is characterized by a gradual depletion of fermentable carbohydrates (Macfarlane et al., 1992). This depletion is translated in a rise in pH, a slower microbial turnover, and a lower metabolic activity of the microbiota present upon colon transit (Macfarlane and Cummings, 1991). However, this gradient does not seem to affect every colon sub-ecosystem to the same degree. Mucosal biofilms appear to maintain relative similar compositions and structures throughout the entire large intestine (Lepage et al., 2005). The limited bacterial species that can survive in close juxtaposition with the large-intestinal epithelium (due to the inherent

selectivity of the labitat) are most probably spared from abrupt changes in pH by the buffering influence of the colon epithelium and the constant uptake of fermentation products by epithelial cells (Macfarlane and Cummings, 1991). Moreover, they are constantly provided with mucin as a fermentable energy source (Macfarlane et al., 2004). In a dynamic environment, the colon epithelial surface can be regarded as a beacon of stability, ensuring survival of a strictly selected resident microbiota.

16.3 Dietary Carbohydrate Metabolism in the Human Colon

Growth and metabolic activity of the colon microbiota are highly dependent on nutrient availability. In the human large intestine, carbon and energy requirements of the resident bacterial populations are met by a continuous supply of fermentable substrates from dietary, microbial, and human origin (*Figure 16.1*) (Macfarlane and Cummings, 1991). The host contribution to the intestinal energy metabolism consists mainly of body secretions such as pancreatic enzymes and mucins, but also sloughed epithelial cells become available as substrates for colon fermentations. The microbiota contributes to the colon energy cycle by acting as its own energy storage (Bäckhed et al., 2005): around 50% of the bacteria encountered in human feces are either damaged or dead, indicating that a substantial part of the intestinal microbial mass is in fact available as an energy source for bacterial recycling (Ben-Amor et al., 2005). Moreover, some members of the human colon microbiota have been shown to produce sugar polymers, which can act as a fermentable substrate for other intestinal bacteria, hence contributing to the complexity of the colon nutrient cycle (Salazar et al., 2008). However, the most abundant carbon and energy source present in the human colon consists of complex carbohydrates and, to a lesser extent, fats and proteins of food origin that have (partially) escaped digestion in the upper part of the gastrointestinal tract (Macfarlane and Cummings, 1991).

16.3.1 Substrate Availability

It has been estimated that – for a person consuming a Western diet – the amount of undigested carbohydrates that reach the colon varies between 20 and 65 g day^{-1} (Macfarlane and Cummings, 1991). Most of these carbohydrates are

from plant origin and belong to a heterogeneous group of food ingredients known as dietary fibers: poly- and oligosaccharides of plant origin that resist both hydrolysis by mammal digestive enzymes and absorption in the small intestine, but that are at least partially hydrolyzed and fermented by the colon microbiota (Roberfroid, 2005a). The dietary fiber fraction of the human diet mainly comprises plant cell-wall polysaccharides, but it also covers storage carbohydrates and substances released by plants in response to injuries. From a chemical point of view, it includes cellulose, hemicelluloses, pectins, gums and mucilages, mixed-linkage β-glucans, resistant starch, and inulin (Roberfroid, 2005a). The largest dietary fiber fraction that enters the colon is made up of resistant starch. Daily, between 8 and 40 g of physical inaccessible, granulated, or retrograded starch reaches the large intestine undigested and becomes available as a substrate for colon fermentation (Macfarlane and Cummings, 1991). Concentrations of free monosaccharides in the colon are generally presumed to be low (Flint, 2006), although it has been shown that they may play an important role as short-living substrates involved in cross-feeding interactions (Falony et al., 2006).

16.3.2 Functional Redundancy

As plant poly- and oligosaccharides make up the largest fraction of the diet-derived energy sources in the human colon, the possession of an enzymatic arsenal necessary to tackle these substrates is an important factor for the survival of large-intestinal microorganisms. Not surprisingly, glycoside hydrolases, esterases, and other enzymes required to degrade plant polysaccharides are found in a wide range of colon bacteria, including *Bacteroides* spp., *Bifidobacterium* spp., and *Firmicutes* belonging to clostridial clusters IV and XIVa (Ramsay et al., 2006; Schell et al., 2002; Xu et al., 2003). This functional redundancy persists even at the strain level: in the few genomes of colon bacteria studied up to now, multiple gene copies of proteins involved in anchoring, uptake, and degradation of carbohydrates have been detected (Bäckhed et al., 2005; Klijn et al., 2005).

Complex carbohydrates entering the human colon vary among each other in chemical composition, degree of polymerization (DP), structure, accessibility (association with other molecules), and solubility (Flint et al., 2008). Although this substrate diversity is thought to create a vast array of microbial niches, a driving force for colon microcommunity differentiation, many common gut-colonizing bacteria appear to be generalists – bacterial strains capable to degrade

a variety of substrates – with comparable growth abilities on overlapping ranges of substrates (Dethlefsen et al., 2006). The key to successful proliferation in the colon seems to lie in combining a broad range of enzymatic carbohydrate breakdown activities with adequate oligosaccharide uptake systems and, optionally, an effective anchoring mechanism (Flint et al., 2008). Niche adaptation among carbohydrate-degrading species is mainly reflected by the organization and regulation of the corresponding gene products (Flint, 2004). The presence of generalists provides the colon ecosystem with a two-leveled strategy to respond to both quantitative and qualitative changes in diet composition (Louis et al., 2007). At the strain level, bacterial metabolic regulation allows generalist microorganisms to meet alterations in substrate nature or supply rate by switching to alternative metabolic routes, often reflected by fluctuations in the bacterial fermentation product profile (Macfarlane and Macfarlane, 2003; Van der Meulen et al., 2004, 2006b). On the community level, due to the widespread functional redundancy among related, habitat-specialized species, even sustained changes in dietary input will provoke only shallow changes in bacterial composition (Bäckhed et al., 2005). Both interrelated mechanisms contribute to the stability of the colon ecosystem.

Ba. thetaiotaomicron is often presented as a perfect example of a successful, flexible, niche-adapted, human commensal with a wide carbohydrate consumption range (Bjursell et al., 2006; Comstock and Coyne, 2003; Xu et al., 2003). This species, which has only been isolated from human and rodent intestines and feces up to now, has been reported to make up 6% of the colon microbiota (Eckburg et al., 2005). A large part of its 6.3-Mb genome – sometimes referred to as its 'glycobiome' – encodes for 163 paralogs of outer-membrane, starch-binding and –importing proteins, 226 predicted glycoside hydrolases (amylases, fructofuranosidases, pullulanase, etc) and 15 polysaccharide lyases (e.g., pectate lyase) (Xu et al., 2003; Xu and Gordon, 2003). Moreover, the species has been shown to be able to redirect its carbohydrate-harvesting activities according to nutrient availability (Bjursell et al., 2006; Sonnenburg et al., 2005). This provides *Ba. thetaiotaomicron* not only with the weapons necessary to grow on a wide range of undigested polysaccharides, but also to adapt to changes in the composition of the human diet, allowing it to survive in and dominate the densely populated human intestinal ecosystem (Louis et al., 2007). A comparable, though less extensive, genetic potential for colon substrate metabolism has been shown to be present in *Bifidobacterium longum* NCC2705. This strain is equipped with more than 40 glycosyl hydrolases (amylases, fructofuranosidases, etc) that are predicted to be involved in the degradation of higher order oligosaccharides

(Klijn et al., 2005; Schell et al., 2002). Also, it possesses at least nine transport systems involved in the uptake of oligofructose (Parche et al., 2007).

16.3.3 Niche Specialization

Notwithstanding the widespread functional redundancy among colon bacteria regarding carbohydrate degradation, some niches require a higher degree of specialization. Efficient degradation of recalcitrant insoluble polysaccharides such as plant cell-wall components and starch granules, for example, is dependent on substrate colonization and formation of degradative biofilms (Macfarlane and Macfarlane, 2006). Surface adhesion, the first step of substrate colonization, is an up to now poorly understood process. It is thought to be a highly specific chain of events involving diverse substrate-binding modules located in enzymes and structural proteins, as well as the carbohydrate moieties of glycoproteins (Flint et al., 2008).

Presently, degradation of insoluble plant materials by human colon bacteria has not been characterized in detail (Flint et al., 2008). However, similar interactions as those thought to be active in the human large intestine have been described for species inhabiting the rumen ecosystem. Cellulose degradation by *Ruminococcus flavefaciens*, for example, involves the production of an elaborate cell surface-anchored cellulosome, that is thought to play a key role in the breakdown of plant cell walls (Rincon et al., 2005). Such cellulosomes are discrete, extracellular, multi-component, and multi-enzyme complexes that provide enhanced synergistic activity among the different resident enzymes to efficiently deconstruct cellulosic and hemicellulosic components of plant cell walls (Flint et al., 2008). Related cellulolytic bacteria are reported to be present in the human colon of methane-producing individuals (Robert and Bernalier-Donadille, 2003).

16.3.4 Secondary Degraders

Degradation of insoluble plant components or large polysaccharides generally provokes physical changes to the available substrates, for example regarding their solubility or physical accessibility. Moreover, such breakdown processes are often accompanied by the release of oligosaccharides in the microenvironment surrounding the degradation site, creating possibilities for opportunistic competitors (Flint et al., 2007). The availability of more readily fermentable substrates,

such as monosaccharides and short oligosaccharides, enhances biofilm maturation. Mature microbial consortia covering transient degradable substrates, such as food residues, are composed of primary degraders, growing directly on the surface of the substrates, surrounded by co-aggregating or even non-adhering secondary colonizers, feeding on partially hydrolyzed breakdown products (Flint et al., 2008; Macfarlane and Macfarlane, 2006). This mechanism of cross-feeding, i.e., the provision of breakdown products of polysaccharides as secondary substrates after partial hydrolysis by primary degraders, has been demonstrated for rumen bacteria to explain fiber digestion in ruminants (Dehority, 1991; Flint et al., 2007) and has recently been described for colon isolates growing *in vitro* on indigestible oligosaccharides (Belenguer et al., 2006; Falony et al., 2006).

To avoid opportunistic substrate competition by secondary degraders, several colon bacteria developed strategies to ensure that they remain the main beneficiaries of their hydrolytic activity. Starch degradation by *Ba. thetaiotaomicron*, for example, initiates with outer membrane binding of soluble starch molecules (Cho and Salyers, 2001; Xu et al., 2003). A second step consists of limited polysaccharide degradation using a periplasmic membrane-bound glycoside hydrolase (neopullulanase), followed by the uptake of starch debris ranging in size from maltose to maltoheptaose. Most of the glycosidic activity displayed by *Bacteroides thetaiotaomicron* is located in the periplasm, where oligosaccharides are further degraded before being transported into the cell (Salyers, 1984). The ability to take up large oligosaccharides with a DP up to eight has not only been reported for *Ba. thetaiotaomicron*, but is also thought to be present in some bifidobacteria (Schell et al., 2002). Uptake and subsequent degradation of substrates offers bacteria a substantial advantage, as intracellular breakdown is usually not accompanied by the release of large amounts of short oligosaccharides or monosaccharides in the cellular environment (Van der Meulen et al., 2006b).

16.3.5 Metabolic Cross-Feeding

The major end-products of bacterial fermentation in the large intestine are SCFA and gases (*Figure 16.2*) (Macfarlane and Cummings, 1991). The principal SCFA that result from carbohydrate fermentation are acetate, propionate, and butyrate, although formate, valerate, and caproate are also produced, albeit in lesser amounts (Macfarlane and Macfarlane, 2003). Other fermentation products, including ethanol, lactate, and succinate, do not accumulate in the colon

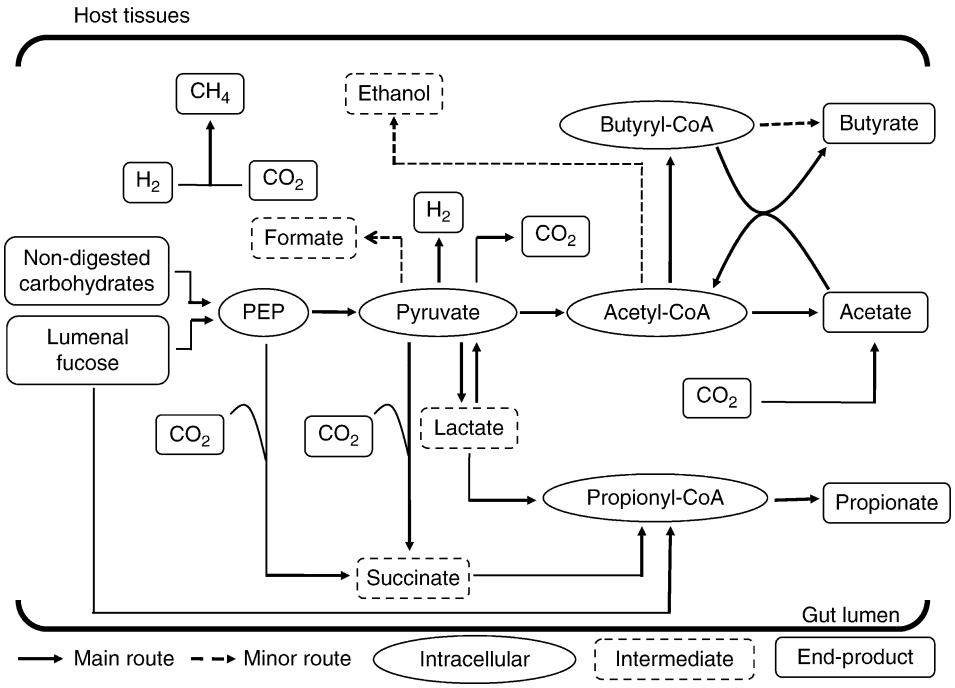

Figure 16.2
Principal substrate fluxes during colon carbohydrate fermentation.

ecosystem of healthy individuals. They serve as intermediates in metabolic cross-feeding interactions between colon bacteria and are metabolized to SCFA to varying extents. Combined total concentrations of acetate, propionate, and butyrate range between 90 and 130 mmol (kg gut contents)$^{-1}$ (Cummings et al., 1987); a total daily production of 400 mmol SCFA has been suggested (Cummings et al., 1989). Although SCFA production rates vary throughout the gut and concentrations are highest in the proximal colon, the acetate:propionate:butyrate ratio is similar in different regions of the large intestine, namely about 57:22:21 (Cummings et al., 1987). However, SCFA levels in bowel contents are not absolute; they merely reflect the balance between bacterial production and colon absorption (Macfarlane and Macfarlane, 2003).

Gas production is an integral part of colon fermentation processes. Gases generated are H_2, CO_2, and, in up to 50% of healthy individuals living on a Western diet, CH_4 (Macfarlane and Cummings, 1991). Gas generated in the colon is either excreted in breath or expelled as flatus, although CO_2 and H_2 also serve as intermediates in cross-feeding interactions between colon bacteria. The total

amount of gas produced on a daily basis is diet-dependent, with estimated values ranging between 0.5 and 4 l day^{-1}. Flatus gas has an average composition of 68% N_2, 16% H_2, 9% CO_2, 6% CH_4, and 2% O_2 (Macfarlane and Cummings, 1991).

16.3.5.1 Production and Consumption of SCFA

Acetate. With concentrations of approximately 70 mmol (kg contents)$^{-1}$ in the human cecum, lowering to 50 mmol (kg contents)$^{-1}$ in the sigmoid colon, acetate is quantitatively the most important end-product of colon fermentation processes (Cummings et al., 1987). The microbial origins of colon acetate are legion: not only is it produced by nearly all heterotrophic gut anaerobes (› *Table 16.1*), up to one-third of the colon acetate pool originates from reductive acetogenesis by, for example, *Ruminococcus hydrogenotrophicus* (Miller and Wolin, 1996). As an intermediate in cross-feeding interactions between colon bacteria, acetate plays a key role in large-intestinal butyrate production. Acetate consumption is shown to be common among butyrate-producing gut bacteria, whereas it remains rare in other functional groups of colon inhabitants (› *Table 16.1*) (Barcenilla et al., 2000). It has been demonstrated *in vitro* that acetate can contribute up to 90% of the carbon needed for butyrate production by the human fecal microbiota, depending on the primary substrate administered (Duncan et al., 2004a).

Propionate. Propionate concentrations in the human large intestine vary between 25 mmol (kg contents)$^{-1}$ in the cecum and 20 mmol (kg contents)$^{-1}$ in the sigmoid colon (Cummings et al., 1987). For many years, colon propionate production has been attributed to *Bacteroides* spp. (Macfarlane and Cummings, 1991). However, the metabolic outcome of bacteroidal substrate degradation is highly dependent on nutrient availability and CO_2 concentrations (Macy et al., 1978; Salyers, 1984). When sufficient carbohydrates are present, there is a reduced need for *Bacteroides* spp. to decarboxylate succinate to propionate, and the former metabolite tends to accumulate (Salyers, 1984; Van der Meulen et al., 2006b). It remains unclear which is the main end-product of bacteroidal metabolism in the highly competitive colon environment (Macfarlane and Macfarlane, 2003). Recently, it has been demonstrated that clostridium cluster IX bacteria, responsible for propionate production in other gastrointestinal ecosystems, such as the rumen, can make out up to 13% of the total fecal microbiota (Walker et al., 2005). The composition of this bacterial subgroup in the human colon remains at present poorly characterized. However, as it is thought to include *Megasphaera* spp.

and *Veillonella* spp., it could be involucrated in conversion of both succinate and lactate to propionate (Bourriaud et al., 2005; Flint, 2006). Propionate has been reported to have an inhibitory effect on hepatic fatty acid synthesis, making stimulation of large-intestinal propionate production a possible strategy in the struggle against obesity (Delzenne et al., 2002).

Butyrate. With cecal and sigmoid colon concentrations of 26 and 18 mmol (kg contents)$^{-1}$ respectively, butyrate is quantitatively the third major end-product of large-intestinal fermentation processes (Cummings et al., 1987). Colon butyrate producers mainly belong to the *Firmicutes* division and can be situated in the clostridial clusters I, III, IV, XI, XIVa, XV, and XVI (Pryde et al., 2002). However, more than 90% of colon butyrate-producing microorganisms are in fact cluster IV and XIVa bacteria, related to *Faecalibacterium prausnitzii* and *E. rectale* or *Roseburia* spp., respectively (Barcenilla et al., 2000; Louis et al., 2007). Together, these two particularly abundant groups have been reported to constitute between 7 and 24% of the total colon microbiota in healthy individuals (Aminov et al., 2006; Hold et al., 2003; Louis et al., 2007). The pathway for microbial butyrate production involves the condensation of two molecules of acetyl coenzyme A (acetyl-CoA) and their subsequent reduction to butyryl-CoA (Diez-Gonzalez et al., 1999; Duncan et al., 2002a). For the final step of the pathway, the actual butyrate formation, two alternative metabolic routes have been described (› *Figure 16.2*). Butyrate can be produced using a butyrate kinase, as has been demonstrated in some strains of *Butyrivibrio fibrisolvens* (Diez-Gonzalez et al., 1999; Duncan et al., 2002a). Alternatively, a butyryl-CoA:acetyl-CoA transferase can move the CoA-moiety to external acetate, leading to the production of butyrate and acetyl-CoA, as is the case for *Roseburia intestinalis* (Duncan et al., 2002a; Louis et al., 2004). In the acetate-rich colon ecosystem, butyryl-CoA:acetyl-CoA activity has been proven common among butyrate-producing strains, in contrast to butyrate kinase activity (Louis et al., 2004). This makes (external) acetate a key intermediate for colon butyrate production.

16.3.5.2 Succinate, Lactate, and Ethanol

Succinate, lactate, and ethanol only figure in the human large intestine as intermediates of cross-feeding interactions between bacteria (Macfarlane and Macfarlane, 2003). Like for the end-products of colon fermentation, but perhaps more confronting and limiting, the study of their occurrence and metabolic fate in the large-intestinal ecosystem suffers greatly under the current lack of adequate *in vivo/in situ*

methods to determine their production rates (Morrison et al., 2006). Ethanol, for example, is regularly mentioned as a minor (end-)product of colon fermentation processes, but the concentration at which it occurs or its further degradation has never been investigated (Macfarlane and Macfarlane, 2003).

Lactate concentrations in the human large intestine decrease from 5 mmol (kg contents)$^{-1}$ in the cecum to 1.5 mmol (kg contents)$^{-1}$ in the sigmoid colon (Macfarlane and Cummings, 1991). Fecal lactate concentrations above 5 mM have been linked with disease conditions such as severe colitis (Vernia et al., 1988). Low lactate concentrations could be related with low large-intestinal production rates, but this seems to be contradicted by metagenomic analyses of the colon microbiome (Gill et al., 2006; Turroni et al., 2008). More likely, lactate is efficiently consumed by both propionate- and butyrate-producing colon inhabitants belonging to clostridial clusters IX and XIVa, respectively (Belenguer et al., 2006; Bourriaud et al., 2005; Duncan et al., 2004b), with butyrate production dominating at lower pH (Belenguer et al., 2007). Also, sulfate-reducing colon bacteria have been implicated in lactate removal (Flint et al., 2007).

Succinate concentrations remain constant throughout the human large intestine, with an average concentration of approximately 1 mmol (kg contents)$^{-1}$ (Macfarlane and Cummings, 1991). Considering the genetical potential present in the colon microbiome, the latter reflects efficient turnover rather than low production rates (Gill et al., 2006; Turroni et al., 2008). The metabolic fate of colon succinate is presently unknown (Walker et al., 2005). However, it should be noted that *Bacteroides* spp., possible sources of colon succinate, become more dominant at higher pH (Walker et al., 2005). The latter implies a more abundant presence of *Bacteroides* spp. in the distal colon, where the fermentable carbohydrate availability is limited (Macfarlane et al., 1992). Under these circumstances, *Bacteroide*s spp. tend to produce more propionate than succinate (Macy et al., 1978; Salyers, 1984). Succinate production by bifidobacteria – a genus that is thought to be generally represented in the proximal colon – has been demonstrated, but only in minor amounts (Van der Meulen et al., 2006a).

16.3.5.3 Hydrogen Gas Metabolism

Hydrogen gas accumulation is a determining factor in the balance and outcome of anaerobic fermentation processes (Stams, 1994). High partial H_2 pressure has been reported to affect growth rates and polysaccharide fermentation and SCFA production by H_2-producing microorganisms (Rychlik and May, 2000).

As many colon bacteria – including butyrate-producing members of the *Firmicutes* division – produce H_2 during carbohydrate degradation, the latter has important consequences for metabolic processes within the colon ecosystem (Duncan et al., 2002b, 2006; Schwiertz et al., 2002).

The two main enzymatic complexes linked with H_2 production by intestinal bacteria involve a pyruvate:ferredoxin-oxidoreductase and a NADH:ferredoxin-oxidoreductase, both coupled with a hydrogenase (Macfarlane and Macfarlane, 2003). While the latter enzymatic route is thought to be the most common among colon bacteria, it is – in contrast with the former – endergonic and requires low partial H_2 pressure to be thermodynamically feasible. Under conditions of high partial H_2 pressure, intestinal bacteria are forced to equilibrate their redox balances through the production of more reduced metabolites, including succinate, lactate, butyrate, and ethanol (Bourriaud et al., 2005). The formation of more oxidized end-products, such as acetate, is considered energetically more interesting, as it is usually associated with ATP production (Macfarlane and Macfarlane, 2003). In the colon ecosystem, H_2 is either released as gas or consumed by H_2-utilizing species, in casu methanogenic Archaea, sulfate reducers, and acetogens (Macfarlane and Cummings, 1991).

Notwithstanding the fact that acetogens show a lower affinity for hydrogen gas than methanogens or sulfate reducers, acetogenesis has been reported to provide a substantial part of the colon acetate reserve (Miller and Wolin, 1996). Acetogenesis is thought to be the dominant H_2-consuming process in the proximal part of the human colon, where acetogens might be favored by the slightly acidic conditions (Bernalier et al., 1996a; Macfarlane and Cummings, 1991). Recently, efficient cross-feeding has been demonstrated *in vitro* between *R. intestinalis* (a clostridial cluster XIVa butyrate producer) and *R. hydrogenotrophicus* (an acetogen) when growing on xylan (Bernalier et al., 1996b; Chassard and Bernalier-Donadille, 2006). The substrate is initially degraded by *R. intestinalis*, producing CO_2 and H_2, which in turn serve as substrates for *R. hydrogenotrophicus*. The latter produces acetate, an indispensable co-substrate for butyrate production by *R. intestinalis*, resulting in xylan degradation without net H_2 production (Chassard and Bernalier-Donadille, 2006).

Concerning methanogens and sulfate reducers, it is not clear whether both groups of bacteria can coexist or are mutually exclusive (Doré et al., 1995; Flint et al., 2007). Competition for hydrogen gas between sulfate-reducing and methanogenic bacteria may explain why some individuals produce methane while others do not. In fecal slurry, sulfate reducers outcompete methanogens, but the outcome of this competition is thought to be highly sulfate-dependent

(Gibson et al., 1988). However, little is known about the availability of sulfate in the human colon (Macfarlane and Cummings, 1991). As the dominating route for hydrogen gas disposal in a given individual influences the competitive balance between other colon bacteria, the result of competition for H_2 might have serious implications for the large-intestinal ecosystem (Robert and Bernalier-Donadille, 2003). In addition, sulfate reduction has potential deleterious consequences on gut health via the formation of toxic sulfide, which has been implicated with the development of ulcerative colitis (Flint et al., 2007; Pitcher et al., 2000).

16.4 Ecological Background of Colon Inulin-Type Fructan Fermentation

More than a decade of intensive research has radically altered both scientists' and consumers' perception of the role and importance of the human colon microbiota and the large-intestinal ecosystem. The fundamentals of this new perception can be summarized as follows: (1) host health and well-being are influenced by the colon microbiota (Macfarlane and Cummings, 1991; Roberfroid, 2005b), (2) the nature of a healthy or balanced colon microbiota is definable (Louis et al., 2007; Macfarlane et al., 2006), and (3) the composition and/or metabolic activity of the colon microbiota can be influenced (transiently) through changes in the diet (Rastall et al., 2005). Notwithstanding the fact that the large-intestinal ecosystem remains largely unexplored (Duncan et al., 2007b; Eckburg et al., 2005), different strategies to influence host health by managing the composition and/or activity of the colon microbiota through the diet have emerged from these understandings (Duncan et al., 2007a; FAO/WHO, 2001; Gibson and Roberfroid, 1995; Makras et al., 2004; Rastall et al., 2005). Although hard to define, the goal of such dietary interventions is to establish an optimally balanced colon microbiota, which is generally believed to be predominantly saccharolytic, comprising significant numbers of bifidobacteria and lactobacilli (Macfarlane et al., 2006).

A well-established strategy to alter the colon ecosystem consists in the use of prebiotics, selectively fermented non-digestible food ingredients that allow specific changes in the composition and/or activity of the gastrointestinal microbiota, which confer benefits upon host well-being and health (Gibson et al., 2004; Gibson and Roberfroid, 1995). At the origin of the development of the prebiotic concept lies the observation of the stimulative effect of inulin-type fructans on the fecal *Bifidobacterium* population during *in vitro* experiments (Gibson and Roberfroid, 1995; Gibson and Wang, 1994; Wang and Gibson, 1993), an observation that was

later largely confirmed by *in vivo* trials (Roberfroid, 2005c). Although the prebiotic properties of other non-digestible food ingredients have been acknowledged by now (Gibson et al., 2004), oligofructose and inulin still remain best studied and have gained themselves a status of model prebiotics (Bosscher et al., 2006; Roberfroid, 2005c).

Inulin-type fructans are linear *D*-fructose polymers linked by β(2–1)-glycosidic bonds (F_m-type), often with a terminal glucose moiety that is linked by an α(1–2)-glycosidic bond (GF_n-type), as in sucrose (*Figure 16.3*). They are present in significant amounts in several fruits and vegetables (e.g., onion, banana, garlic, leek, chicory) (Makras et al., 2004). On an industrial scale, inulin-type fructans are usually extracted from chicory roots (Roberfroid et al., 1998).

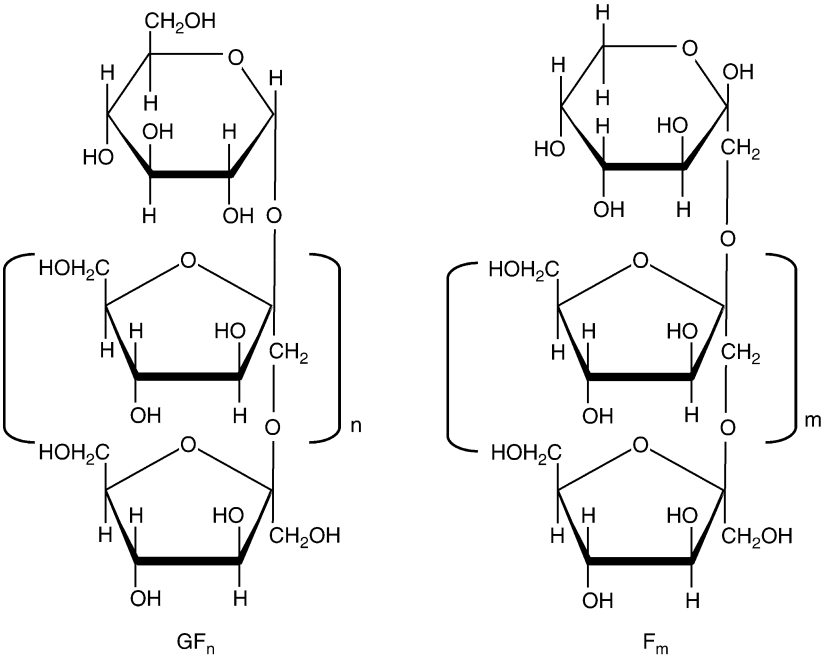

Figure 16.3
Chemical structure of inulin-type fructans. F, fructose; G, glucose. In the present manuscript, the terms inulin and oligofructose – obtained through partial enzymatic hydrolysis of chicory inulin – are used to refer to two distinct fractions of native chicory inulin, both containing F_m- as well as GF_n-type polymers. The DP of the oligofructose fraction varies between 2 and 10 with an average DP of 4, whereas that of inulin can be 60 or even more, with an average DP exeeding 23.

Inulin-type fructans are neither digested nor absorbed in the human upper gastrointestinal tract (Molis et al., 1996). They reach the colon virtually intact, where they are selectively fermented by the large-intestinal microbiota and mainly converted not only to SCFA, such as acetate, propionate, and butyrate, but also to other organic acids (e.g., lactate) and gases (H_2 and CO_2) (Alles et al., 1996). Although the physiological effects of inulin-type fructan consumption are extensively documented, their impact on the colon ecosystem is less understood. The most pronounced effects of their availability, as substrates of microbial fermentation, on the colon ecosystem are an increase in bifidobacterial numbers, the so-called bifidogenic effect (Gibson et al., 1995; Gibson et al., 2004; Roberfroid et al., 1998), and an enhancement of large-intestinal butyrate production, the so-called butyrogenic effect (Campbell et al., 1997; Le Blay et al., 1999; Morrison et al., 2006). These bifidogenic and butyrogenic effects are considered beneficial for the host's health, but their combination is remarkable, as bifidobacteria are not able to produce butyrate (Makras et al., 2006; Van der Meulen et al., 2006a; Van der Meulen et al., 2004).

16.4.1 The Bifidogenic Effect

Although stimulation of bifidobacteria by inulin-type fructans has been studied extensively for more than 15 years through both *in vitro* and *in vivo* trials, some rather elementary questions still await satisfactory answering (Macfarlane et al., 2006). It is, for example, not clear how bifidobacteria [<3% of the large-intestinal microbiota (Frank et al., 2007)] manage to get the upper hand in the highly competitive human colon ecosystem when inulin or oligofructose are added to the diet, while it has been demonstrated that also *Lactobacillus* spp. (Goh et al., 2006; Kaplan and Hutkins, 2000, 2003; Makras et al., 2005), *Roseburia* spp. (Duncan et al., 2002b, 2006; Falony et al., 2006), *Bacteroides* spp. (Van der Meulen et al., 2006b), and some enterobacteria (Hartemink et al., 1997) can use at least oligofructose as a substrate for fermentation (Huebner et al., 2007). It has been suggested that (some) *Bifidobacterium* spp. are capable of intracellular or cell-associated degradation of oligofructose, providing them with a fermentation advantage (Falony et al., 2006, 2009a; Schell et al., 2002; Van der Meulen et al., 2006b). Non-bifidobacterial species, such as *Bacteroides* spp., are thought to degrade oligofructose extracellularly, making them less competitive when growing on this substrate (Falony et al., 2006; Van der Meulen et al., 2006b). Moreover, as

not all bifidobacteria are able to degrade inulin or even oligofructose to the same extent (Huebner et al., 2007; Rossi et al., 2005), it seems likely that not all bifidobacterial species benefit in the same way of the presence of inulin-type fructans as energy sources in the colon. However, most studies concerning the bifidogenic effect of inulin-type fructans tend, unfortunately, to consider the bifidobacterial colon population as a whole, not taking into account the interspecies differences that exist between various bifidobacteria (Macfarlane et al., 2006). Recently, the existence of four phenotypically distinct clusters among bifidobacteria, based on their inulin-type fructan breakdown capacities, has been revealed, probably reflecting niche-specific adaptation (Falony et al., 2009b). This rather limited variation has been hypothesized to influence the susceptibility of various bifidobacteria toward prebiotic stimulation by inulin-type fructans and their fitness to compete for these substrates in a complex environment such as the colon ecosystem.

Recent *in vitro* studies focussing on detailed kinetic analyses of oligofructose degradation by bifidobacteria as well as *Lactobacillus paracasei* subsp. *paracasei*, *Ba. thetaiotaomicron*, *Bacteroides fragilis*, and *R. intestinalis* have revealed remarkable differences between the bifidobacterial breakdown mechanism and that of species belonging to other genera (Falony et al., 2006; Makras et al., 2005; Van der Meulen et al., 2006b, 2004). Most bifidobacteria seem to degrade oligofructose in a preferential way, i.e., preferring the DP 2 fraction over the DP 3 and 4, only initiating the breakdown of a longer chain length fraction when shorter fractions are depleted. Furthermore, only a limited number of bifidobacteria appears capable of degrading the inulin fraction (Falony et al., 2009b; Rossi et al., 2005). Non-bifidobacterial species exhibit simultaneous degradation of all fractions, often combined with the release of large amounts of free fructose in the fermentation medium, as has been revealed *in vitro* for *L. paracasei* subsp. *paracasei* 8700:2 (Makras et al., 2005) and *Ba. thetaiotaomicron* LMG 11262 (Van der Meulen et al., 2006b). It has been suggested that accumulation of breakdown products of inulin-type fructans can be linked to extracellular degradation, which might prove to be less efficient than internal or cell wall-bound degradation in a highly competitive ecosystem such as the human colon (Falony et al., 2006; Van der Meulen et al., 2006b). The appearance of free fructose and shorter fractions of oligofructose in the growth environment, which become available to opportunistic competitors that are not able to break down inulin-type fructans themselves (Flint et al., 2007; Louis et al., 2007), seems to be inherent to extracellular degradation. Bifidobacteria capable of intracellular degradation would not suffer from this drawback, which would explain their selective

growth stimulation on inulin or oligofructose (Falony et al., 2006; Rossi et al., 2005; Van der Meulen et al., 2006b).

16.4.2 The Butyrogenic Effect

Many of the health-promoting effects attributed to oligofructose and inulin are at least partially due to their influence on the production of SCFA by the colon microbiota (Delzenne et al., 2002; Guarner, 2005; Nyman, 2002; Pool-Zobel, 2005; Weaver, 2005). In humans, colon fermentation of inulin-type fructans generally does not lead to a significant increase of fecal concentrations of SCFA or to a change in molar proportions of acetate, propionate, and butyrate (Nyman, 2002). The latter can be explained by the very efficient colon SCFA absorption (Macfarlane and Cummings, 1991; Macfarlane and Macfarlane, 2003). Only drastic dietary interventions appear to be reflected in colon SCFA concentrations (Walker et al., 2005). In contrast, *in vitro* and animal studies do show an enhancement of SCFA production by inulin and oligofructose (Djouzi and Andrieux, 1997; Wang and Gibson, 1993). In particular, fermentation of inulin-type fructans by the colon microbiota seems to cause an increase in butyrate formation (Campbell et al., 1997; Le Blay et al., 1999; Morrison et al., 2006). This butyrogenic effect was already known in the case of resistant starch fermentation (Brouns et al., 2002; Scheppach and Weiler, 2004). Butyrate is of key importance for colon health: it is not only the preferred energy source for the large-intestinal epithelium (Macfarlane and Cummings, 1991), but it also has important effects on the development of and the gene expression in intestinal cells (Mariadason et al., 2000; Scheppach and Weiler, 2004). In addition, it is thought to play a protective role against colorectal cancer and colitis (Hague et al., 1996; Hamer et al., 2008).

The link between consumption of inulin-type fructans, the bifidogenic effect, and the increase of butyrate production, the butyrogenic effect, in the colon has remained unclear for several years. Bifidobacteria produce lactate, acetate, formate, ethanol, and even minor amounts of succinate, but can not produce butyrate (Falony et al., 2006; Van der Meulen et al., 2006a). Furthermore, evidence of direct degradation of inulin-type fructans by butyrate-producing colon bacteria is scarce (Duncan et al., 2006; Duncan et al., 2003; Falony et al., 2006). Although it has become clear that the stimulatory effect of inulin-type fructans exceeds the large-intestinal *Bifidobacterium* population and affects other genera, including butyrate-producing bacteria belonging to the clostridial cluster

XIVa (Duncan et al., 2003; Langlands et al., 2004), it is generally believed that such species do not play a major part in colon fructan breakdown.

Cross-feeding between starch-degrading bifidobacteria and lactate-converting, butyrate-producing colon bacteria has been demonstrated (Duncan et al., 2004b). Similarly, recent studies regarding the combined bifidogenic/butyrogenic effect of inulin-type fructans focussed mainly on cross-feeding between bifidobacteria and butyrate-producing colon bacteria belonging to clostridial cluster XIVa. In the case of inulin-type fructans, two main types of cross-feeding have been demonstrated: one involving short oligosaccharides and monosaccharides released by *Bifidobacterium* spp. from the prebiotic substrate; the other one by means of consumption of end-products of bifidobacterial fructan fermentation such as acetate and lactate (Belenguer et al., 2006; Falony et al., 2006). *Eubacterium hallii* DSM 17630, not capable of fructan degradation, has been shown to efficiently convert lactate and acetate produced by *B. adolescentis* DSM 20083, when growing in coculture on oligofructose (Belenguer et al., 2006). *Anaerostipes caccae* DSM 14662^T survived in a coculture with *B. longum* BB536, when growing on oligofructose, by taking advantage of acetate produced and fructose released by the latter strain during substrate breakdown (Falony et al., 2006). *R. intestinalis* DSM 14610^T joined *B. longum* BB536 in oligofructose degradation, but only after the latter strain initiated breakdown and started to produce acetate, an indispensable co-substrate for growth of the former strain (Falony et al., 2006). The occurrence of such or related cross-feeding interactions is inevitable when inulin-type fructans or other prebiotics are added to the diet and make their way to the large intestine as substrates for colon fermentation. Prebiotic fermentation will inherently indirectly affect other groups of colon bacteria than the ones targeted, complicating any attempt to predict the full extension of their effects (Flint et al., 2007). Notwithstanding the often underestimated complexity of the impact of fermentation of prebiotics on the colon ecosystem, the bifidogenic effect and its associated beneficial consequences on human health and well-being, linked with for example inulin-type fructan consumption, remain beyond dispute (Bosscher et al., 2006).

Although the *in vitro* demonstration of cross-feeding between primary inulin-type fructan degraders and butyrate-producing colon bacteria is convincing, these model mechanisms can not be translated to the colon ecosystem without caution. Many factors contributing to the *in vivo* ecological background of colon inulin-type fructan breakdown that remain at present insufficiently explored will undoubtably complicate this simplified *in vitro* vision on interspecies interactions (*Table 16.2*).

Table 16.2

Ecological implications of colon inulin-type fructan degradation: unanswered questions (Bosscher et al., 2006; Roberfroid, 2005a; Rossi et al., 2005; Van der Meulen et al., 2006b)

Which bifidobacteria are responsible for inulin-type fructan degradation? It is well documented that not all bifidobacteria are capable to degrade inulin-type fructans. Moreover, bifidobacterial inulin degradation is rare, indicating that bifidobacteria merely act as secondary degraders of oligofructose. Metabolite production is species-dependent: while some bifidobacteria produce lactate and acetate when growing on oligofructose, others produce acetate, formate, and ethanol solely. Furthermore, bifidobacterial metabolism is subject to drastic changes as a function of substrate degradability and fermentation rate. However, it remains presently unknown which bifidobacterial species play a key role in fructan degradation nor what their metabolic end-products are in the highly competitive colon environment.
What is the effect of increased H_2 production? Colon fermentation of inulin-type fructans results in an increase in gastrointestinal H_2 production. Changes of intestinal partial pressure of H_2 are thought to affect the metabolism of large groups of colon bacteria, including butyrate producers belonging to clostridial clusters IV and XIVa. Moreover, it may favor H_2-consuming microorganisms, including methanogens and sulphate reducers, in the colon environment. The extent and the implications of these changes in the colon microbiota are currently unknown.
What is the location of oligofructose and inulin degradation? Degradation of oligofructose is generally thought to take place in the proximal colon, while fermentation of the longer inulin chains would take place in the more distal parts of the colon. Composition of bacterial populations evolves alongside the large intestine, with less bifidobacteria and clostridial cluster IV and XIVa butyrate producers thought to be present toward the distal colon. However, it is not known what the effect of this translocation of fermentation site is on the nature and metabolic end-products of inulin-type fructan degradations.
What is the role of *Bacteroides* spp. in inulin degradation? For many years, *Bacteroides* spp. have been considered as unable to degrade inulin-type fructans. Recently, it has been shown that abundant *Bacteroides*, including *Bacteroides thetaiotaomicron*, are able to grow on oligofructose. However, the nature of the breakdown process seems different from bacteroidal starch degradation, as it is accompanied by the release of large quantities of monosaccharides in the extracellular environment. The ecological implications of colon bacteroidal fructan degradation are at present unknown.

16.5 Conclusion

Due to its complexity, its proximity, and its close relatedness with human health, the human colon ecosystem has been fascinating microbiologists for many decades (Dethlefsen et al., 2006; Flint et al., 2008; Macfarlane and Cummings, 1991; Turroni et al., 2008; Zoetendal et al., 2006). Throughout the years, several

promising, innovative technologies have been introduced in the field of gut microbiology (Macfarlane and Macfarlane, 2004; Turnbaugh et al., 2007). Although many of these techniques have radically altered the dominating views on large-intestinal ecology, others have never fulfilled the expectations that accompanied them. Many of the latter, including molecular fingerprint approaches, have been presented as being able to abolish laborious and time-consuming microbiological methods involving plating, isolating, and subsequent identifying, that have been current practice in gut microbiology research from the early days on. However, these methods failed to comply with the three main necessities that still dominate human colon research: enumeration, identification, and functionality determination of bacterial species and groups.

Given the current lack of knowledge concerning the composition of the human colon microbiota, it is not surprising that the journey toward a functional understanding of the microbial colon community has only just begun. The recent isolation and cultivation of an ecologically crucial group of colon inhabitants, namely butyrate-producing large-intestinal bacteria (Barcenilla et al., 2000), leading to the identification of the source of colon butyrate (Pryde et al., 2002), has been a wake-up call for many gut microbiologists. Although the shallow diversity and wide-spread functional redundancy among colon bacteria makes it unnecessary to isolate every individual species and strain, it is now generally accepted that colon microbiology suffers from the lack of characterized representative species. Nowadays, a revival of the interest in the metabolic characterization of individual colon bacteria can be noted, especially regarding *Firmicutes* species (Duncan et al., 2007b). However, metabolic profiling is only the first step in niche identification. Areas including spatial distribution, bacteriocin production, biofilm formation, quorum sensing, and horizontal gene transfer remain presently largely unexplored (Flint et al., 2008; Macfarlane et al., 2000; Xu et al., 2007).

The Human Microbiome Project, a world-wide attempt to increase understandings of the microbial components of the human superorganism, offers some promising opportunities to research on microbial and ecological interactions within the gut microbiota. Although the project focuses on the relationship between the human host and the microbial community as a whole, the metagenomic approach lifts up the cover of the black box and facilitates the study of bacterial interactions (Handelsman, 2008; Turnbaugh et al., 2007). Moreover, in an early stage of the project, it includes an emphasis on isolation of previously uncultured gut bacteria, to extend current genome libraries. Indeed, a full understanding of the colon ecosystem will only emerge from combining information

from molecular tools for bacterial enumeration, monitoring of gene expression, and tracking of metabolites with physiological fermentation data obtained from (co)culturing representative gut isolates.

List of Abbreviations

DP Degree of polymerization
SCFA Short-chain fatty acids

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17 Genomics of Probiotic Bacteria

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17.1 Introduction

17.1.1 Probiotic Bacteria and Probiotic Genome Sequencing Projects

Probiotic bacteria from the *Lactobacillus* and *Bifidobacterium* species belong to the *Firmicutes* and the *Actinobacteria* phylum, respectively. Lactobacilli are members of the lactic acid bacteria (LAB) group, a broadly defined family of micro-organisms that ferment various hexoses into primarily lactic acid. Lactobacilli are typically low G + C gram-positive species which are phylogenetically diverse, with over 100 species documented to date. Bifidobacteria are heterofermentative, high G + C content bacteria with about 30 species of bifidobacteria described to date. Lactobacilli and bifidobacteria are both autochthonous (naturally occurring) and allochthonous (transient) residents of the gastrointestinal tract (GIT) when delivered as probiotic cultures. Lactobacilli and bifidobacteria are used widely as probiotics; live micro-organisms which, when administered in adequate amounts confer a health benefit on the host (Reid et al., 2003). *Lactobacillus* species have long been associated with the production of fermented foods, including dairy products, vegetables, meat, and sourdough bread. Their desirable rapid acidification also contributes to flavor, texture, and nutrition. The addition of bifidobacteria to foods has been more recent where they are purposely added because of their reported health benefits and probiotic properties. Despite the low dominance of lactobacilli in the GI micro-ecology compared to the colonic associated bifidobacteria, they represent a major component of the microbiota residing in the small intestine. Consequently, certain strains of *Lactobacillus*, particularly those of human origin, have been exploited as probiotics. Consumption of probiotics is targeted towards carriage of these strains into the intestinal tract; hence these bacteria demonstrate properties such as bile and acid resistance.

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In addition, bifidobacteria and some lactobacilli have the capabilities to ferment oligosaccharides which are not digested by humans and hence can confer a growth advantage for these beneficial bacteria in the GIT.

The first bacteria and free living organism to be sequenced was *Haemophilus influenzae* in 1995. Since then biology has been revolutionized with the sequencing of the human genome and more than 749 bacterial genomes (NCBI). The human microbiome project is currently underway internationally. The increasing interest and application of LAB and bifidobacteria as probiotics in tandem with improvements in sequencing technology and costs have resulted in genomic analysis of many probiotic strains (› *Tables 17.1* and › *17.2*). In addition, probiotic bacteria from the bifidobacteria and LAB groups have received much attention especially with the establishment of the Lactic Acid Bacteria Genome Consortium (LABGC) in the US and the sequencing of relevant industrial strains (Liu et al., 2005). *Bifidobacterium longum* NCC2705 was the first *Bifidobacterium* strain sequenced (Schell et al., 2002) followed shortly by the sequencing of *Lactobacillus plantarum* WCSF1 (Kleerebezem et al., 2003). To date the sequence of at least ten probiotic cultures are publicly available, with more in progress (› *Tables 17.1* and › *17.2*). This review provides an overview of probiotic-related genome features and functional genomic studies that have linked genes to traits that are elucidating the mechanisms of probiotic action for both commensal and probiotic bacteria. Interspecies heterogeneity and niche-specialized adaptation among lactobacilli and bifidobacteria, as revealed by comparative genome analysis, are also discussed.

17.2 General Genome Features of Probiotics

17.2.1 Lactobacilli

Recently, major advances in genomic characterization of probiotic lactobacilli have allowed the study and comparison of the genetic content of these organisms and provided insights into evolution, physiology, metabolism, and interaction with host tissues. Fourteen sequenced *Lactobacillus* strains are now available and the following; *L. acidophilus*, *L. casei*, *L. gasseri*, *L. johnsonii*, *L. salivarius*, *L. plantarum* and *L. reuteri* are considered probiotic cultures (› *Table 17.2*). The G + C content of these species ranges from 33% for *L. salivarius* to 46% for *L. casei*. The majority of the genomes are approximately 2 Mb in size, whereas *L. plantarum* and *L. casei*, used as both starter cultures and probiotics, have larger

Table 17.1

Genome sequencing projects of probiotic bacteria (*Cont'd* p. 684)

Species	Strain	Size (Mb)	Genbank accession no./ Status	Institution	Reference/ source
Bifidobacteria					
B. animalis subsp. *lactis*	DN-173 010	1.94	Not public	Danone Vitaole, INRA, France	Liu et al. (2005)
B. animalis subsp. *lactis*	BB-12	~2.0	Not public	Chr. Hansen, Denmark	Liu et al. (2005)
B. animalis subsp. *lactis*	HN019	~2.0	ABOT00000000	Fonterra, New Zealand	(NCBI)
B. breve	Yakult	2.35	Not public	Yakult, Japan	Liu et al. (2005)
B. breve	M-16V	~2.3	Not public	Morinaga Milk Industry, Japan	Liu et al. (2005)
B. breve	UCC3003	2.42	Not yet published	University College Cork, Ireland	Liu et al. (2005)
B. longum	DJO10A	2.38	CP000605	University of Minnesota, USA; JGI	Lee et al. (2008)
B. longum	NCC2705	2.26	AE014295	Nestle Research Center, Switzerland	Schell et al. (2002)
B. longum	BB536	~2.4	Not public	Morinaga Milk Industry, Japan	Liu et al. (2005)
B. longum biotype infantis	M-63	~2.9	Not public	Morinaga Milk Industry, Japan	Liu et al. (2005)
Lactobacilli					
L. acidophilus	NCFM	1.99	CP000033	NC State University, California Poly. State University, USA	Altermann et al. (2005)
L. casei	BL23	~2.6	FM177140	INRA/CNRS, Caen University, France	Liu et al. (2005)
L. casei	Shirota	3.04	Not public	Yakult, Japan	Liu et al. (2005)
L. casei	DN-114 001	3.14	Not public	Danone Vitapole, INRA, France	Liu et al. (2005)
L. delbrueckii subsp. *bulgaricus*	DN-100 107	2.13	Not public	Danone Vitapole, France	Liu et al. (2005)
L. gasseri	ATCC33323	1.89	CP000413	JGI., LABGC, Fidelity Systems Inc., USA	Azcarate-Peril et al. (2008)

Table 17.1

Species	Strain	Size (Mb)	Genbank accession no./ Status	Institution	Reference/ source
L. johnsonii	NCC533	1.99	AE017198	Nestle Research Center, Switzerland	Pridmore et al. (2004)
L. plantarum	WCFS1	3.31	AL935263	Wageningen Centre for Food Sciences, The Netherlands	Kleerebezem et al. (2003)
L. reuteri	ATCC55730	~2.0	Not public	BioGaia, Swedish University of Agricultural Science, Sweden	Liu et al. (2005)
L. salivarius subsp. *salivarius*	UCC118	1.83	CP000233	University College Cork, Ireland	Claesson et al. (2006)

chromosomes of 3.31 and 2.89 Mb, respectively (*Table 17.2*). Another general genome feature of these strains is the presence of pseudogenes. Lactobacilli used as starter cultures in milk such as *L. delbrueckii* subsp. *bulgaricus* (*L. bulgaricus*) and *L. helveticus* contain significantly more pseudogenes in their genomes than do probiotic cultures (*Table 17.2*). In the case of *L. salivarius*, nearly one-third of its pseudogenes are located on the first megaplasmid described in lactic acid bacteria, pMP118 (Claesson et al., 2006). This megaplasmid encodes important features such as a locus for bacteriocin production, a bile salt hydrolase, and two genes that complete the phosphoketolase pathway (PKP), officially reclassifying this organism as a facultative heterofermenter (Claesson et al., 2006). In fact, plasmids account for 15% of the genome of *L. salivarius*, which is not the case with other sequenced probiotic lactobacilli (*Table 17.2*). All sequenced probiotic strains harbor prophage or phage remnants, while the dairy-associated *L. helveticus* and *L. bulgaricus* are the only sequenced strains that do not harbor prophage or phage remnants even though their dairy processing environment is a major reservoir for bacteriophages (*Table 17.2*).

The principal source of energy production in lactobacilli is carbohydrate fermentation via the glycolytic Embden-Meyerhof pathway (EMP) or/and via the PKP. *In silico* genome analysis confirmed that all species except the obligate heterofermentative *L. brevis* ATCC367 and *L. reuteri* F275 have a complete EMP pathway (Goh and Klaenhammer, 2008). Furthermore, *L. salivarius* has all the key enzymes for gluconeogenesis that may activate during glucose starvation, thereby conferring

Table 17.2

General genome features of publicly available lactobacilli and bifidobacteria genome sequences (*Cont'd* p. 686)

Species/strain	Strain	Accession number	Size (Mb)	% GC	Proteins	Plasmids	Prophages / phage remnant	Pseudogenes	Application	Reference/ source
Lactobacilli										
L. acidophilus	NCFM	CP000033	1.99	34.7	1864	0	0/3	10[a]	Probiotic	Altermann et al. (2005)
L. casei	ATCC 334	CP000423	2.89	46.6	2776	1	1/0	82	Starter Culture, Probiotic	Makarova et al. (2006)
L. casei	BL23 (ATCC393)	FM177140	3.1	46.3	3044				Probiotic	NCBI
L. gasseri	ATCC 33323	CP000413	1.89	35.3	1755	0	1/1	48	Probiotic	Azcarate-Peril et al. (2008)
L. johnsonii	NCC 533	AE017198	1.99	34.6	1821	0	2/1	0	Probiotic	Pridmore et al. (2004)
L. plantarum	WCFS1	AL935263	3.31	44.5	3009	3	2/2	42	Starter Culture, Probiotic	Kleerebezem et al. (2003)
L. reuteri	F275	CP000705	2	38	1900	0	2/0	39	Probiotic	Morita et al. (2008)
L. salivarius subsp. *salivarius*	UCC118	CP000233	1.83	33	1717	3	2/2	49	Probiotic	Claesson et al. (2006)

Table 17.2

Species/strain	Strain	Accession number	Size (Mb)	% GC	Proteins	Plasmids	Prophages / phage remnant	Pseudogenes	Application	Reference/ source
L. fermentum	IFO 3956	AP008937	2.1	51.5	1843			0		Morita et al. (2008)
L. brevis	ATCC 367	CP000416	2.3	46.1	2185	2	1/0	50	Starter Culture	Makarova et al. (2006)
L. delbrueckii bulgaricus	ATCC 11842	CR954253	1.86	49.7	1562	0	0/0	270	Starter Culture	van de Guchte et al. (2006)
L. delbrueckii subsp. *bulgaricus*	ATCC BAA-365	CP000412	1.86	49.7	1721	0	0/0	192	Starter Culture	Makarova et al. (2006)
L. helveticus	DPC4571	CP000517	2.08	37.7	1610	0	0/0	217	Starter Culture	Callanan et al. (2008)
L. sakei subsp. *sakei*	23K	CR936503	1.88	41.3	1879	0	0/1	30	Starter Culture	Chaillou et al. (2005)
Bifidobacteria										
B. longum	DJO10A	CP000605	2.38	60.2	1990	2	1	0	Probiotic	Lee et al. (2008)
B. longum	NCC2705	AE014295	2.26	60.1	1727	1	1	0	Probiotic	Schell et al. (2002)

[a]Confirmed by sequencing

a possible competitive advantage on *L. salivarius* in the GIT (Claesson et al., 2006). In addition, all species lack key enzymes for biosynthesis of most if not all vitamins and cofactors. However, uniquely among lactobacilli *L. reuteri* CRL1098 is capable of synthesizing cobalamin (vitamin B12) (Morita et al., 2008). In contrast to bifidobacteria, most *Lactobacillus* species lack *de novo* synthetic capability for various amino acids, with the exception of *L. plantarum*, which has complete biosynthetic pathways for all amino acids except branched-chain amino acids (Kleerebezem et al., 2003). To compensate for this deficiency genomes of probiotic cultures encode for a wide array of amino acid/peptide uptake systems and peptidases (usually organized in operons) to acquire and utilize exogenous nitrogen sources (Goh and Klaenhammer, 2008). The *L. johnsonii* genome encodes a pair of tandem aminopeptidases (LJ0176/LJ0178) with exclusive homology to corresponding enzymes in other GI lactobacilli, streptococci, *B. longum* and *Bacteroides fragilis*, indicating adaptive evolution to use peptide structures commonly encountered within the GIT (Pridmore et al., 2004). In addition, *L. acidophilus* and *L. johnsonii* possess putative cell wall-bound proteinases that are likely to participate in degradation of polypeptides from food substrates in the host diet and/or host glycoproteins such as mucin (Pridmore et al., 2004). The majority of probiotic species have a larger proportion of genes devoted to carbohydrate and amino acid transport and metabolism (*Table 17.3*). Lactobacilli of the GIT utilize the phosphoenolpyruvate (PEP)-dependent phosphotransferase systems (PTS) with 20 to 30 PTS transporters on average encoded in each genome, plus permeases of the major facilitator superfamily and several ATP-dependent binding cassettes (ABC) sugar transporters (Goh and Klaenhammer, 2008).

17.2.2 Bifidobacteria

Bifidobacterium strains have not been sequenced to the same extent as lactobacilli, while numerous sequencing projects are under way (*Table 17.1*). To date three commensal or probiotic strains have been sequenced and this information is publicly available in the NCBI database (*Table 17.2*). *B. longum* NCC2705 was the first publicly available sequenced strain (Schell et al., 2002). Prior to its release less than 50 bifidobacteria protein sequences were available in the Genbank database demonstrating the importance of this genome sequence for bifidobacteria biology (Klijn et al., 2005). In general, the sequenced bifidobacteria genomes range in size from 1.9 to 2.9 Mb and have a G + C content of around 60%

Table 17.3

COG function of protein coding genes from probiotic genomes. Data obtained from IMG v2.6 released by JGI (http://img.jgi.doe.gov/cgi-bin/w/main.cgi)

COG Function	*B. longum* DJO10A (%)[a]	*B. longum* NCC2705 (%)	*L. acidophilus* NCFM (%)	*L. casei* ATCC 334 (%)	*L. gasseri* ATCC 33323 (%)	*L. johnsonii* NCC 533 (%)	*L. plantarum* WCFS1 (%)	*L. reuteri* F275 (%)	*L. salivarius* UCC118 (%)
Amino acid transport and metabolism [E]	8.9	9.5	6.9	6.3	5.1	5.8	6.9	7.0	6.2
Carbohydrate transport and metabolism [G]	9.7	9.5	9.0	8.1	8.3	9.1	9.3	5.4	6.6
Cell cycle control, cell division, chromosome partitioning [D]	1.2	1.3	1.2	1.0	1.3	1.2	0.9	1.0	1.4
Cell motility [N]	0.1	0.1	0.3	0.1	0.3	0.3	0.2	0.4	0.3
Cell wall/membrane/ envelope biogenesis [M]	3.3	4.2	4.9	3.6	5.0	5.2	4.7	4.3	5.1
Coenzyme transport and metabolism [H]	2.6	2.8	1.6	2.0	1.7	1.8	3.0	4.2	1.9
Defense mechanisms [V]	1.9	2.4	2.0	2.4	2.3	2.3	1.6	1.5	1.5
Energy production and conversion [C]	2.6	2.7	3.4	3.5	2.9	2.9	3.5	3.6	3.1
Function unknown [S]	4.6	5.1	7.4	7.1	8.3	7.9	7.0	7.7	7.8
General function prediction only [R]	8.3	9.7	9.8	9.3	9.6	9.6	10.6	10.2	8.5
Inorganic ion transport and metabolism [P]	3.0	3.5	3.5	3.2	3.4	3.6	3.9	3.1	2.8

Intracellular trafficking, secretion, and vesicular transport [U]	0.8	0.9	1.0	0.8	1.2	1.2	0.8	1.2	1.2
Lipid transport and metabolism [I]	1.9	2.1	1.8	2.0	1.6	1.7	2.1	2.2	2.2
Nucleotide transport and metabolism [F]	3.5	3.8	4.1	2.9	3.5	3.5	3.0	4.3	3.4
Posttranslational modification, protein turnover, chaperones [O]	2.8	2.9	2.3	1.9	2.4	2.6	1.8	2.4	2.4
RNA processing and modification [A]	0.1	0.1	0.0	0.0	0.0	0.0	0.0	0.0	0.0
Replication, recombination and repair [L]	6.7	5.6	6.1	7.3	6.2	6.4	4.5	9.7	6.8
Secondary metabolites biosynthesis, transport and catabolism [Q]	0.5	0.5	0.4	0.8	0.3	0.4	1.0	1.2	0.8
Signal transduction mechanisms [T]	2.8	3.3	3.0	2.8	2.6	3.1	3.2	2.9	2.8
Transcription [K]	6.6	7.3	6.4	6.2	6.6	6.4	8.3	5.9	5.9
Translation, ribosomal structure and biogenesis [J]	6.7	7.4	7.3	5.4	7.9	7.8	5.0	7.1	6.8
Total percentage of protein coding genes	**78.6**	**84.7**	**82.4**	**76.7**	**80.3**	**82.5**	**81.4**	**85.5**	**77.7**

[a]Percentage of protein coding genes related to each COG function

(*Table 17.2*). Compared to lactobacilli, bacteriophage propagation is not well studied in relation to bifidobacteria, but analysis of both *B. longum* genomes revealed the presence of prophage (Lee et al., 2008; Schell et al., 2002). In addition, a prophage element has been described in the genome of the probiotic *B. breve* UCC2003 (Ventura et al., 2005). All three prophage were integrated in a tRNA *met* gene and exhibited homology with genes of double stranded bacteriophage related to bacteriophage infecting mostly low G + C content bacteria (e.g., *Lactococcus lactis*) (Ventura et al., 2005). The Blj-1 prophage is the first reported inducible prophage from bifidobacteria, whereas the prophage elements from *B. breve* UCC2003 and *B. longum* NCC2705 appear defective (Ventura et al., 2005).

B. longum is a strict fermentative anaerobe with mild aero-tolerance (Schell et al., 2002). Based on *in silico* analysis, *B. longum* NCC2705 has the ability to synthesize at least 19 amino acids from ammonia, phosphoenolpyruvate, fumarate, oxaloacetate and oxoglutarate. All genes required for the biosynthesis of pyrimidine and purine nucleotides are present (Schell et al., 2002). In addition, genes encoding enzymes required for folic acid, thiamine and nicotinate synthesis are present while genes for pyridoxine, riboflavin and biotin synthesis are missing (Schell et al., 2002). Analysis of genes from both *B. longum* NCC2705 and *B. longum* DJO10A revealed that approximately nine percent of the annotated genes from these strains encode for enzymes involved in amino acid transport and metabolism (*Table 17.3*). In addition, over nine percent are also involved in carbohydrate transport and metabolism. *B. longum* utilizes the fructose-6-phosphate shunt for carbohydrate fermentation and encodes all the gene homologs required to uptake fructose, galactose, *N*-acetyl-glucosamine, *N*-acetyl-galactosamine, arabinose, sucrose, ribose, lactose, xylose, cellobiose and melibiose for this pathway (Schell et al., 2002). *B. longum* also encodes for more than 40 glycosyl hydrolases with substrate affinity for di-, tri- and higher order oligosaccarides and eight MalEFG-type oligosaccharide transporters which contribute to the ability of *B. longum* to utilize a diversity of complex carbohydrates in the GIT (Schell et al., 2002). As observed for lactobacilli many of these traits are organized in operons, consisting of a repressor, transporter and single or numerous glycosyl hydrolase genes (Schell et al., 2002). As is the case with some lactobacilli some of these operons may be implicated in the degradation of complex host glycoproteins (Pridmore et al., 2004; Schell et al., 2002).

In the case of *B. breve* UCC2003, 40 glycosyl hydrolases have also been identified by *in silico* analysis (Ventura et al., 2007). These glycosyl hydrolases are suspected to act both in and outside the cytoplasm, contributing to their immediate surroundings in addition to bifidobacteria cellular function (Ventura et al., 2007). Like lactobacilli, bifidobacteria encode peptidases which may

provide additional amino acids from the GIT. *B. longum* encodes for greater than 20 peptidases and also more than 25 ABC transporters, in addition to long chain fatty acid acyl-CoA synthetases, which may play a role in fatty acid utilization in the GIT (Schell et al., 2002). These genetic traits revealed by genome sequencing illustrate that bifidobacteria have adapted to the GIT niche.

17.3 Probiotic Features as Revealed Through Genome Sequencing and Functional Genomics

17.3.1 Unusual Carbohydrate and Prebiotic Metabolism

The ability of intestinal microbiota to utilize dietary plant polysaccharides, that escape host digestion, provides a competitive advantage for the persistence and colonization in the lower GI tract. Prebiotic sugars, such as fructooligosaccharide (FOS) and raffinose, have been used as non-digestible functional food ingredients to selectively promote the growth of lactobacilli and bifidobacteria that are regarded as beneficial microbes among the intestinal microbiota. Recent work on the genetic basis of FOS utilization by lactobacilli revealed diverse catabolic pathways among different species (Barrangou et al., 2003; Goh et al., 2006; Saulnier et al., 2007). The *L. acidophilus msm* operon encodes an ABC transporter and a cytoplasmic β-fructofuranosidase that mediate the uptake and intracellular hydrolysis of FOS. In *L. plantarum*, the FOS utilization pathway is encoded by a sucrose gene cluster which involved a sucrose PTS and an intracellular beta-fructofuranosidase. *L. casei* also has a putative sugar operon that resembles the operon architecture and sequence of the *fos* operon in *L. paracasei*. Based on the functional analysis of the *fos* operon in *L. paracasei*, the FOS utilization pathway of *L. casei* is predicted to involve a putative cell-surface anchored β-fructosidase and a fructose/mannose PTS that mediate extracellular FOS degradation and subsequent uptake of fructose moieties. *L. acidophilus*, *L. plantarum*, and *L. johnsonii* are also capable of utilizing raffinose (Klaenhammer et al., 2005). Microarray transcriptional analysis of *L. acidophilus* grown on raffinose identified a putative raffinose operon that encodes an ABC transporter and an alpha-galactosidase involved in raffinose catabolism (Barrangou et al., 2006).

Bifidobacteria also digest complex carbohydrates such as resistant starches, raffinose, lactulose, FOS and galacto-oligosaccarides (GOS) which are not digested by the host and are found in the distal part of the GIT. Genome analysis revealed the presence of a FOS operon in *B. breve* UCC2003, consisting of a putative permease, a conserved hypothetical protein, and a β-fructofuranosidase

(Ryan et al., 2005). The β-fructofuranosidase gene (*fos-B*) shared 83% and 84% homology with genes in *B. longum* NCC2705 and *B. longum* DJO10A, respectively (Ryan et al., 2005). Transcriptional analysis revealed that these genes in *B. breve* UCC2003 are transcribed as a 2.6 kb tricistronic operon which was induced by sucrose and Actilight (a commercial source of short chain FOS made from sucrose), but not glucose, fructose, a combination of sucrose and fructose, or a combination of glucose and sucrose, indicating transcription control dependent on carbohydrate availability (Ryan et al., 2005). In addition, a novel FOS-degrading enzyme (Fos-C) with an affinity for only the β-(2–1) glycosyl bonds between glucose and fructose moieties was reported (Ryan et al., 2005).

Additional physiological studies confirmed the capabilities of bifidobacteria to use complex carbohydrates of host derived compounds such as mucin in addition to plant derived carbohydrates such as pectin and plant oligosaccarides (Klijn et al., 2005). With the exception of *L. reuteri*, *L. brevis*, *L. bulgaricus*, *L. sakei*, and *L. fermentum*, the genomes of all other sequenced *Lactobacillus* species encode at least one putative neopullulanase that may potentially hydrolyze pullulan, a linear polysaccharide consisting of maltotriose units linked by alpha-1,6-glucosidic bonds. A survey of 42 *Bifidobacterium* strains for α-amylase and pullulanase activity strains demonstrated different capabilities of bifidobacteria to ferment plant sugars such as potato starch, potato amylopectin and pullulan (Ryan et al., 2006). Nineteen of the 42 strains were capable of degrading potato starch and of these 11 demonstrated the ability to ferment amylopectin and pullulan. Either amylopullulanase (type II pullulanase) or type III pullulan hydrolase activity was identified in the 11 strains. The majority (five) of these were *B. breve* strains, including the sequenced strain *B. breve* UCC2003. Analysis of the *B. breve* UCC2003 genome identified a gene encoding for a bifunctional amylopullulanase (Ryan et al., 2006). This result demonstrated the varying capabilities among bifidobacteria strains to degrade plant-based sugars and indicates the need to study each probiotic candidate individually.

17.3.2 Polysaccharides Biosynthetic Gene Clusters

Putative polysaccharide gene clusters are present in most sequenced *Lactobacillus* strains except for *L. reuteri*, *L fermentum*, and *L. brevis*. The cell surface exopolysaccharide (EPS) gene clusters in the closely related *L. acidophilus*, *L. johnsonii*, and *L. gasseri* genomes are highly conserved, although the gene organization is inverted in *L. gasseri* due to chromosomal inversion (Azcarate-Peril et al., 2008;

Klaenhammer et al., 2005). All three *eps* gene clusters are located within a low G + C content region with a transposase gene located in the downstream region, suggesting that the *eps* clusters were acquired via horizontal gene transfer (HGT) from a common source. Several *eps* core genes exhibit weak or no sequence similarity between each other, despite analogous putative functions, indicating variation in the carbohydrate profiles of the EPS produced that may provide unique surface signatures in these strains (Klaenhammer et al., 2005; Pridmore et al., 2004). Notably, the *L. gasseri* genome also encodes an unusually high number of glycosyltransferases that may contribute to the complexity of the EPS polymers synthesized (Azcarate-Peril et al., 2008).

The genomes of *L. salivarius*, *L. sakei*, and *L. bulgaricus* each contains two distinct *eps* gene clusters, whereas *L. plantarum* has four different surface polysaccharide gene clusters (Chaillou et al., 2005; Claesson et al., 2006; Kleerebezem et al., 2003; van de Guchte et al., 2006). Sturme et al. (2005) recently showed that the expression of one of the surface polysaccharide locus of *L. plantarum*, *cps2* (lp_1197 to lp_1211), is regulated by an *agr*-like two-component regulatory system (2CRS), designated as *lam* for *Lactobacillus agr*-like module, that is involved in quorum-sensing. In *L. salivarius*, the *eps* cluster 1 (LSL_0977 to LSL_0997) showed little synteny with other *eps* clusters; whereas cluster 2 (LSL_1547 to LSL_1574) shared high sequence similarity with the surface polysaccharide gene clusters in *L. plantarum*, with an overall low GC content (Claesson et al., 2006). Two surface polysaccharides gene clusters are present in the *L. sakei* genome (LSA1571 to LSA1585; LSA1510 to LSA1513) that encode proteins for the biosynthesis of polysaccharide-linked techoic acids and the translocation of the polysaccharides to a surface component. These polysaccharide gene clusters were predicted to promote adherence of *L. sakei* to meat surfaces or GI mucosa (Chaillou et al., 2005). In both sequenced strains of *L. bulgaricus*, the *eps* clusters 1 and 2 are in close proximity to each other, with relatively conserved flanking regions (Makarova et al., 2006; van de Guchte et al., 2006). The *eps* cluster 1 of ATCC 11842 (Ldb1937 to Ldb1957) and ATCC BAA-365 (LBUL_1800 to LBUL1815) shared conserved sequence and gene organization, whereas the genes in cluster 2 showed significant sequence variation between both strains. Makino et al. (2006) recently demonstrated that the high molecular weight acidic EPS fraction isolated from *L. delbrueckii* subsp. *bulgaricus* OLL1073R-1 was able to induce interferon-gamma production *in vitro*, and increased natural killer cell activity *in vivo*. Hence, the diversity of *eps* clusters in *L. bulgaricus* may potentially contribute to the rheological properties as well as the immunostimulative characteristics of the displayed or secreted EPS. In a

similar context, *L. casei* Shirota has a *cps* gene cluster which encodes for the biosynthesis of high-molecular mass polysaccharide (PS-1) (Yasuda et al., 2008). Exposure of heat-killed *cps* mutant cells to mouse macrophage cells and spleen cells resulted in higher induction of TNF-α, IL-12, IL-10, and IL-6 compared to the parent strain. In addition, the CPS-deficient mutants appeared to enhance LPS-stimulated IL-6 production by mouse macrophage-like cells, which otherwise was suppressed by the parent strain. Thus, the cell wall-associated PS-1 of *L. casei* Shirota is involved in modulation of host immune response, specifically by downregulating the immune response from monocytes and macrophages directed against its own immunoreactive components as well as other inducers.

EPS production has also been reported for bifidobacteria although the biological significance is not fully understood. EPS may function in bile and acid tolerance, to mediate adherence to human cells, or conversely to avoid host recognition (Ventura et al., 2007). However, a recent study by Salazar et al. (2008) indicated the potential role of EPS as a substrate for intestinal bacteria. Sequence analysis of *B. longum* NCC2705 revealed two potential EPS regions with divergent G + C content both flanked by IS elements (Schell et al., 2002). The 21.3 kb *eps* cluster of *B. breve* UCC2003 which contains 17 putative EPS related genes is also flanked by IS elements (Ventura et al., 2007) indicating their acquisition by HGT. One of the regions in *B. longum* NCC2705 is implicated in the biosynthesis of a teichoic acid-linked rhamnose-containing EPS as it includes genes encoding for polysaccharide export, glycosyl transferase genes, rhamnose biosynthesis genes and homologs of unknown EPS-related proteins (Schell et al., 2002). Sequencing of additional strains of bifidobacteria from both commensal and probiotic groups will shed light on the role of EPS in relation to bifidobacteria.

17.3.3 Stress Response

17.3.3.1 Acid Stress

A diverse number of mechanisms for regulating intracellular pH homeostasis have been established in Gram-positive bacteria, including F_1F_0-ATPase proton pumps, amino acid decarboxylation, general stress proteins and chaperones that repair and degrade damaged DNA and proteins. Additional mechanisms also include production of alkaline compounds, modification of cell membrane composition, and regulation of transcription via alternative sigma factors and

2CRS's. The *L. plantarum* genome encodes an F_1F_0-ATPase, ten sodium-proton antiporters, and three paralogous alkaline-shock proteins for maintenance of intracellular pH homeostasis (Kleerebezem et al., 2003). A recent transcriptional study of *L. plantarum* showed that lactic acid specifically induced the expression of several cell surface proteins of unknown function, Clp protease, catalase, excinuclease, squalene synthase, and phytoene synthase (Pieterse et al., 2005). The authors proposed that some of these proteins may be responsible for altering the cell surface properties and increasing membrane rigidity to cope with acid stress. A more recent investigation on the global response of acid shock in late-logarithmic phase of *L. reuteri* ATCC 55730 cells showed induction of genes encoding the ClpL chaperone, a putative esterase and a phosphatidylglycerophosphatase involved in peptidoglycan and cell membrane biosynthesis. Phage-related genes, one of which encodes a putative cell wall degradation protein were also induced (Wall et al., 2007). Functional analysis of ClpL and esterase mutants demonstrated the importance of these genes in early response to acid shock, and possibly survival during transit through the GI tract. In *L. acidophilus*, the *atp* operon that encodes the F_1F_0-ATPase was induced when logarithmic phase cells were subjected to acid stress at pH 3.5 with hydrochloric acid (Kullen and Klaenhammer, 1999). In addition, *in silico* genome analysis identified several amino acid decarboxylases, a cation transport ATPase, and the chaperone Ffh that are likely involved in intracellular pH regulation in *L. acidophilus* (Altermann et al., 2005). Azcarate-Peril et al. (2004) recently confirmed that genes encoding for amino acid decarboxylation, including an amino acid antiporter, an amino acid permease, an ornithine decarboxylase, and a transcriptional regulator were associated with acid tolerance in *L. acidophilus*. Interestingly, pre-adaptation (pH 5.5 for 1 h) of each of all four acid-sensitive mutants restored the ability to survive during challenge at pH 3.5, indicating that these genes have indirect roles in acid adaptation.

Acid stress in relation to bifidobacteria is not as well studied and in fact very little is known about this type of stress response. Bifidobacteria encode F_1F_0-ATPase proton pumps in addition to general stress response molecular chaperone proteins such as GroEL and DnaK (De Dea Lindner et al., 2007; Klijn et al., 2005). As with lactobacilli, protease encoding genes such as *clpP* have also been identified which may play a role in acid stress (De Dea Lindner et al., 2007). A survey by Ventura et al. (2007) revealed chaperone-encoding genes; *groEL*/*groES*, *dnaK*, *grpE*, *dnaJ1* and *dnaJ2* in addition to the protease encoding gene *clpP* and the *Clp*-ATPase genes *clpB* and *clpC* in bifidobacteria. Bifidobacterial acid resistance is a desirable trait as strains must pass through the stomach to the GIT. A recent

study induced acid resistance in numerous bifidobacteria strains by incubating strains at pH 2 in order to isolate acid resistant isolates (Collado and Sanz, 2007). This exposure appeared to evoke a general stress response as these isolates were also more resistant to bile, salt and heat (Collado and Sanz, 2007). Acid resistance has also been studied in *B. longum* and its acid-pH resistant mutant. Comparison of protein maps from both strains identified nine proteins where modulation was changed in the mutant. These proteins were involved in carbohydrate and amino acid transport and metabolism, energy production and conversion, and cell envelope biogenesis (Sanchez et al., 2007).

17.3.3.2 Osmotic Stress

In silico genome analysis revealed that *L. plantarum* has at least three systems for the uptake and biosynthesis of osmoprotectants glycine-betaine/carnitine/choline (Kleerebezem et al., 2003). The trehalose utilization (*tre*) locus in the *L. acidophilus* genome encodes a PTS (*treB*) and a trehalose-6-phosphate hydrolase (*treC*) that has been functionally associated with cryoprotection by both uptake and hydrolysis of trehalose (Duong et al., 2006). Interestingly, inactivation of either *treB* or *treC* rendered the cells unable to utilize trehalose as a carbon source or cryoprotectant upon freezing and thawing cycles. Based on these observations, the authors proposed that the uptake of trehalose as well as its subsequent hydrolytic products/metabolic intermediates contribute to its cryoprotectant effect. As is the case for acid stress, osmotic stress in relation to bifidobacteria is not well studied. Molecular chaperones that have also been induced in relation to heat stress (with the exception of *dnaJ2*) were expressed in *B. breve* UCC2003 after exposure to salt (De Dea Lindner et al., 2007).

17.3.3.3 Oxidative Stress

The *L. plantarum* genome encodes a large number of proteins that are involved in the survival of oxidative stress, including catalases, peroxidases, thioredoxins, NADH oxidases, and glutathione reductases. It has been shown that the overexpression of thioredoxin enhanced resistance against oxidative stress (Serrano et al., 2007). Gene expression profiling further demonstrated that the overexpression of thioredoxin triggered the transcription of 16 genes encoding proteins in purine metabolism, protein synthesis, stress response, and a manganese

transporter that were also induced under hydrogen peroxide stress, suggesting that thioredoxin plays a role in a signal transduction cascade involved in the oxidative stress response. In addition, at least five putative manganese transporters were identified in the *L. plantarum* genome, which supported previous findings that Mn^{2+} ions serve as an important scavenger for oxygen radicals as well as cofactor for manganese-dependent catalase.

B. longum NCC2705 does not encode any superoxide dismutase genes, catalases, or genes with homology to the manganese catalase described in *L. plantarum*. However, Klijn et al. (2005) exposed *B. longum* NCC2705 cells to oxidative stress and among the upregulated genes was a potential ATPase that could be involved in manganese transport. Additional upregulated genes included one oxidoreductase (BL1626), a glutaredoxin homolog (BL0668), a subunit of an alkylhydroperoxide reductase (BL0615) and a thioredoxin reductase like gene (BL0164) (Klijn et al., 2005), implying their involvement in the oxidative stress response of *B. longum* NCC2705.

17.3.3.4 Bile Stress Response and Bile Tolerance

To date, bile salt hydrolases (BSH) or choloylglycine hydrolases have been found exclusively in human isolates, or species that encounter bile salts in their commensal habitats, such as *Lactobacillus*, bifidobacteria, *L. monocytogenes*, *Enterococcus faecalis*, and *Bacteroides* (Begley et al., 2006; Elkins et al., 2001). Consistently, no BSH-encoding genes were identified in the sequenced strains of *L. bulgaricus, L. helveticus*, and *L. fermentum*. The remaining sequenced strains possess at least one putative BSH-encoding gene, with *L. acidophilus*, *L. plantarum*, *L. johnsonii*, and *L. brevis* having two or more BSH-encoding genes. Although *L. plantarum* has four putative *bsh* genes, combinatorial mutation analysis confirmed that only *bsh1* encodes a functional BSH, whereas the remaining *bsh* genes encode for proteins exhibiting penicillin acylase activity (Lambert et al., 2008). Interestingly, the multiple BSHs within any given strain shared weak sequence similarity among each other (Goh and Klaenhammer, 2008). It has been speculated that the presence of multiple BSHs may provide a wider range of bile salt specificities, or exhibit differences in the mode of action depending upon bile shock or bile adaptation, which would ultimately enhance the survival of microbes in fluctuating host environments (Begley et al., 2006). For example, functional analysis of the BshA and BshB of *L. acidophilus* showed that each BSH demonstrated different substrate specificities of bile salts based on the steroid

nucleus of bile salts or the amino acid side chain present in the bile salt molecules (McAuliffe et al., 2005). Overall, the BSH orthologs in species of the *L. acidophilus* complex and *L. reuteri* are more closely related to each other than to the BSHs in other lactobacilli (Goh and Klaenhammer, 2008). Meanwhile, the BSHs from *L. plantarum* (Bsh1), *L. casei* (LSEI_0412), and the pMP118-encoded BSH in *L. salivarius* (LSL_1801) shared higher sequence similarity to the BSH orthologs from *L. monocytogenes* and *Enterococcus faecium*. In fact, no sequence homology of the BSH from *L. casei* (LSEI_0412) was detected with all identified BSHs in lactobacilli at the time of analysis. These observations indicate that cross-species lateral transfer of BSH-encoding genes may be a common trend within the GI microbial community (Goh and Klaenhammer, 2008).

Recent genetic studies of bile stress response in *L. acidophilus*, *L. plantarum*, and *L. reuteri* based on *in vitro* and *in situ* environments (Bron et al., 2004b; Bron et al., 2006; Pfeiler et al., 2007) have broadened the view on the molecular mechanisms of bile tolerance in GI lactobacilli. Studies by Bron et al. (2004b; 2006) using *alr* (encoding alanine racemase) complementation-based genome-wide promoter screening and microarray transcriptional analysis showed that the genes coding for cell envelope biosynthesis, putative bile exporters, and proteins associated with acid and oxidative stress responses were induced in the presence of porcine bile. Notably, two of the bile responsive genes were previously shown to be induced during *in vivo* passage of *L. plantarum* through the mouse GI tract (Bron et al., 2004a). Similarly, microarray gene expression studies of *L. reuteri* ATCC 55730 upon exposure to Oxgall bile showed induction of genes associated with cell envelope stress, oxidative stress, protein denaturation and DNA damage, whereas genes involved in substrate transport and metabolism were downregulated (Whitehead et al., 2008). Disruption of the bile responsive genes encoding ClpL, an esterase or an unknown protein increased, the mutants' susceptibility to bile. In *L. acidophilus*, whole-genome expression analysis in response to oxgall bile revealed the upregulation of a 7-kb operon consisting of eight genes encoding a 2CRS, a transporter, a putative oxidoreductase, and four hypothetical proteins (Pfeiler et al., 2007). Mutations within the 2CRS, the transporter, and one of the hypothetical proteins abolished bile resistance, whereas mutation in the oxidoreductase and another hypothetical protein enhanced bile tolerance, indicating a dual role of this operon in bile tolerance as well as bile sensitivity. Interestingly, genes involved in lactose and galactose utilization and surface adherence factors were also identified as members of the bile response regulon (Pfeiler et al., 2007). These observations led to the speculation that bile may act as an environmental signal and location indicator which triggered the expression of

genes likely important for nutrient uptake from milk and promoting interaction with the host epithelium.

Bile resistance in bifidobacteria has been shown to vary widely across strains (De Dea Lindner et al., 2007; Vernazza et al., 2006). Isolation of strains after exposure to bile salts indicated a cross resistance phenotype with acid resistance (De Dea Lindner et al., 2007). Interestingly this was also observed when bifidobacteria were exposed to acid (see above) implying a common non-specific stress response to acid and bile that conferred tolerance to these strains. In the study by Sanchez et al. (2007), the bile salt hydrolase gene was constitutively down-regulated in the acid-pH mutant and wild type in response to acidic pH. This trait in addition to osmotic, acid and heat tolerance is important for the selection of industrially relevant *Bifidobacterium* strains. Further genome sequencing and additional development of molecular tools to further investigate bifidobacteria will hopefully elucidate important unknown mechanisms of stress resistance in bifidobacteria.

17.3.4 Cell Surface Factors

As the importance of probiotic and commensal interactions with the host GIT has been realized, efforts have rapidly focused on identification of potential cell surface factors (› *Figure 17.1*). Host interactions mediated by adherence factors may promote pathogen exclusion, mucosal integrity, and host immunomodulation. Adherence factors are typically proteins or polysaccharides that are displayed on the cell surface. The number of proteins with predicted signal peptides and/or transmembrane domains for each probiotic species is shown in › *Figure 17.2*. The role of mucus-binding proteins (Mub) in intestinal mucin adherence was first described in *L. reuteri* 1063, when Roos and Jonsson (2002) identified a 358-kDa surface-associated Mub that selectively adhered to intestinal mucin glycoprotein. *In silico* detection of putative Mub encoded in the *Lactobacillus* genomes showed a clear bias toward their predominant presence in GI-associated lactobacilli (Boekhorst et al., 2006a). No Mub-encoding gene was identified in the genomes of *L. casei*, *L. bulgaricus*, *L. helveticus*, *L. sakei*, and *L. fermentum*. The fact that MUB domains were found exclusively in intestinal lactobacilli suggest that these proteins mediate specific interactions or functions between these microbes and their hosts.

L. acidophilus has thirteen putative proteins that contain one or more MUB domains, with only three that have both a predicted signal peptide sequence and a cell surface anchor LPxTG motif (Tallon and Klaenhammer, unpublished data).

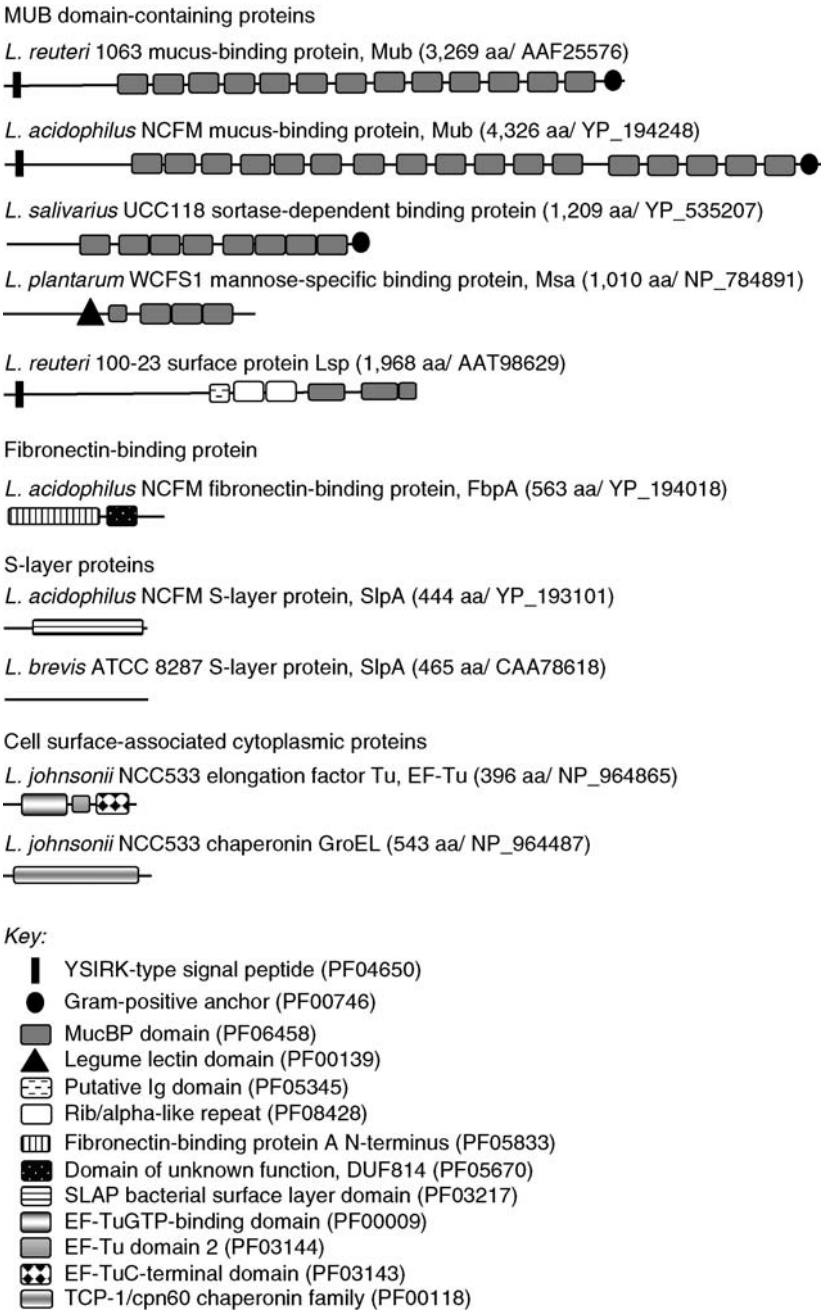

Figure 17.1

Adherence factors that have been functionally characterized in sequenced lactobacilli. Conserved domains and organization are depicted based on the Pfam database

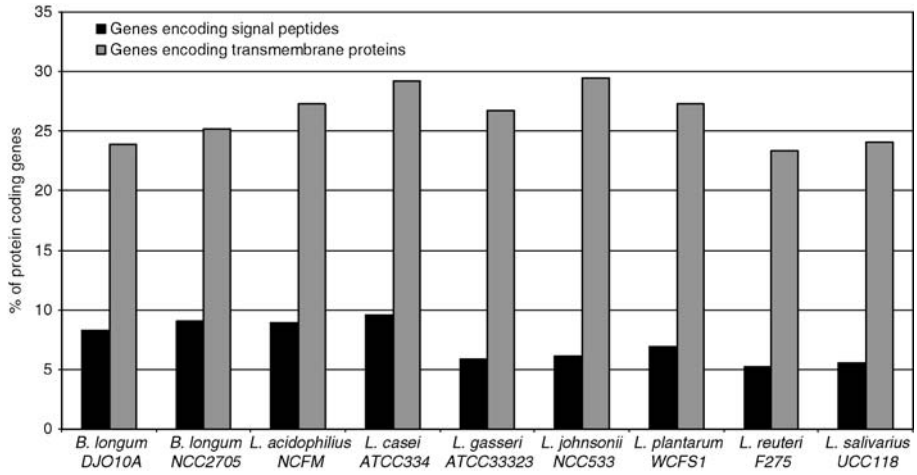

Figure 17.2
Percentage of protein coding genes with a signal peptide or transmembrane domains. Obtained from IMG v2.6 released by JGI (http://img.jgi.doe.gov/cgi-bin/w/main.cgi)

One of these Mub proteins, encoded by LBA1392, is composed of 4,326 residues and represents the largest protein encoded in the genome (*Figure 17.1*) (Altermann et al., 2005). Insertional inactivation of the LBA1392 gene locus resulted in a significant reduction in adherence to Caco-2 epithelial cells *in vitro* (Buck et al., 2005). Among the GI-associated lactobacilli, *L. gasseri* has the highest number (fourteen) of putative Mub proteins (Azcarate-Peril et al., 2008). These Mubs contain 6–12 MUB domains, and shared sequence similarity to the Mub's from *L. reuteri* 1063 and *L. acidophilus* (encoded by LBA1392). In *L. plantarum*, one of its four predicted Mubs was recently recognized as a lectin-like mannose-specific adhesin, Msa (Pretzer et al., 2005). The cell surface-anchored Msa protein has two MUB domains similar to those of the *L. reuteri* 1063 Mub and a ConA-like lectin domain, of which both domains have a predicted role in binding to mannose-containing receptors on the mucosal surface. Bioinformatic analysis of the predicted secretome in *L. salivarius* has identified three sortase-dependent Mub homologs, Mbp-1, LspA, and LspC (van Pijkeren et al., 2006). The *lspA* mutant exhibited reduced ability to adhere to HT29 epithelial cells. Meanwhile, the *mbp-1* appeared to be a pseudogene, whereas no transcription of *lspC* was detected *in vitro*. According to van Pijkeren et al. (2006), *lspA* may be acquired via lateral gene transfer due to its high GC content (40%) relative to the genomic GC content (33%). The *L. johnsonii*

genome encodes three surface proteins that harbor MUB domains which share significant sequence similarity to the Mub of *L. reuteri* 1063 (Pridmore et al., 2004). Interestingly, the amino acids repeat regions of two of the Mubs, LJ0047 and LJ1839, showed moderate sequence similarity to the N-terminal region of cell surface bull sperm binding protein SP18 or HR44 of *Homo sapiens*.

Putative fibronectin-binding protein (Fbp)-encoding gene appears to be conserved in all sequenced *Lactobacillus* genomes (Goh and Klaenhammer, 2008). Interestingly, *L. brevis* also has an additional copy of *fbp* gene (LVIS_0267) in which the deduced protein shared sequence similarity to a hypothetical protein in *L. plantarum* and putative Fbp from *Enterococcus* and *Listeria* species. BlastP analysis showed that the Fbp from the *L. acidophilus* group are more closely related to each other (77–88% sequence identity) than to the Fbp in species belong to other phylogenetic groups (Goh and Klaenhammer, 2008). Buck et al. (2005) showed that the FbpA of *L. acidophilus* contributes to Caco-2 epithelial cells adherence *in vitro*. *L. plantarum* also has a large 3,360-residue surface protein, Sdr, with a near perfect Ser-Asp repeat of 1,600 residues (Boekhorst et al., 2006b; Kleerebezem et al., 2003). A similar Ser-Asp repeat was found in the protein, ClfB, of *Staphylococcus aureus*, which is involved in fibrinogen adhesion (Hartford et al., 1997). The three putative glycosyltransferase genes present at the vicinity of *clfB* were predicted to be involved in the biosynthesis of mucin-like structures that coat the cell surface or mediate interaction with host cell mucins.

Major cell surface structures such as surface layer proteins (SLPs), techoic acids (TA), and lipotechoic acids (LTA) have also been implicated as mediators of adherence and immunomodulation. S-layers are paracrystalline arrays of proteinaceous subunits that are abundant on the cell surface of most eubacteria and archaea. The SlpA of *L. brevis* ATCC 8287 has been shown to adhere to various human cell lines, fibronectin and laminin via a specific receptor-binding region (de Leeuw et al., 2006). It has also been demonstrated that the dominant S-layer of *L. acidophilus*, encoded by *slpA*, contribute to epithelial cell adherence (Buck et al., 2005). The isogenic SlpA$^-$ mutant not only has altered cell morphology, but also exhibited a significantly reduced ability to bind to Caco-2 cells. Nonetheless, it was speculated that decreased adherence might result from loss of adherence factors potentially anchored to the S-layer. By using monoclonal antibodies Granato et al. (1999) identified LTA as a non proteinaceous cell surface component in *L. johnsonii* that was involved in the adhesion to Caco-2 cells. A recent study also showed that a *dltB* isogenic mutant of *L. plantarum* with altered D-alanylation of LTA significantly enhanced the induction of IL-10, and

consequently reduced proinflammatory response in a murine colitis model (Grangette et al., 2005). In a study using *in vivo* expression technology (IVET), Walter et al. (2005) showed that one of the genes specifically induced by *L. reuteri* 100–23 in the murine gut encodes a 300-kDa surface protein, Lsp. Inactivation of the *lsp* gene reduced the ability of the mutant to adhere to mouse forestomach epithelium *in vivo* and *ex vivo*, as well as reduced its overall ecological performance in the murine gut.

Perhaps one of the most interesting findings was the role of elongation factor Tu (EF-Tu) and GroEL in adherence and immunomodulation of *L. johnsonii* (Bergonzelli et al., 2006; Granato et al., 2004). These studies represent the first reports describing the cell surface localization of EF-Tu and GroEL in LAB. Both EF-Tu and GroEL preferentially adhered to intestinal epithelial cells and mucin at pH 5.0, a pH value that more closely resembles the physiological conditions in the small intestine (Blum et al., 2000). Furthermore, the recombinant EF-Tu and GroEL were able to elicit a proinflammatory response by inducing soluble CD14-dependent IL-8 secretion in HT29 epithelial cells. Recombinant GroEL expressed from *L. johnsonii* and other Gram-positive bacteria was also capable of specifically promoting aggregation of *Helicobacter pylori*. Aside from these functionally characterized cell surface factors, the genome of *L. johnsonii* also encodes a diverse repertoire of adhesion factors, some of which are unusual in LAB (Pridmore et al., 2004). Among the 42 predicted cell surface lipoproteins, two display weak sequence similarity to a $CD4^+$ T cell-stimulating antigen of *L. monocytogenes* and a saliva-binding protein from *S. sanguinis*, respectively. In addition, two sets of genes that collectively resembled the fimbrial operon of *Streptococcus gordonii* may encode for the biosynthesis of cell surface glycosylated fimbrial proteins similar to the Fap1 fimbrial adhesion of *Streptococcus parasanguis* and GspB platelet binding protein of *S. gordonii*. Genome analysis also revealed a putative cell surface protein that shared moderate sequence similarity to IgA proteases of pathogenic streptococci. *L. johnsonii* also has a fructosyltransferase homolog that was conserved among other *L. johnsonii* strains and *L. gasseri*, suggesting these species may potentially produce fructan polysaccharides that contribute to adherence.

The genomes of *B. breve* UCC2003, *B. longum* NCC2705 and *B. longum* DJO10A encode all the expected components of the gram-positive cell secretion machinery (MacConaill et al., 2003). However, as was reported for *L. plantarum,* these strains are missing *secDF* and the twin-arginine translocation pathway (Kleerebezem et al., 2003; MacConaill et al., 2003). This pathway functions to export folded proteins implying that these *Bifidobacterium* strains and

L. plantarum exports unfolded proteins. The role of the EPS cluster as discussed above is not fully understood but it was suggested that it may contribute to evading host recognition, cell adhesion, and bile and acid tolerance by helping bacteria to withstand stomach acids and bile salts, hence facilitating establishment within the GIT (Ventura et al., 2007). Additional cell surface factors identified in bifidobacteria genomes include glycoprotein binding, fimbriae-like structures (BL0675) and serpin-like protease inhibitors, discussed below.

Actinomyces naeslundii is a bacterium found in the oral cavity of animals and humans that has a gene designated *fimP*, which shares homology with BL0675 (Klijn et al., 2005). More recent blast analysis with BL0674 and BL0675 identified homologs in *B. longum* DJO10A (98 and 42%, respectively) and to a lesser extent (30–40%) in the gut microbes *Eubacterium dolichum* DSM 3991 and *Ruminococcus torques* ATCC 27756, which have been sequenced as part of the human microbiome project (NCBI). The function of these genes still needs to be determined. The function of BL0108, a putative serpin protease inhibitor with homologues in eurkaryotes that protect against elastases was confirmed by Ivanov et al. (2006). This protection may be an important trait for commensal bifidobacteria in the GIT. Homologs of this protein from *B. longum* NCC2705 are also found in *B. longum* DJO10A (98% homology) and *B. dentium* ATCC 27678 (45% homology). More recently, using proteomic techniques, Yuan et al. (2008) identified an EF-Tu adhesion-like factor protein from *B. longum* NCC2705 expressed *in vivo* in a rabbit intestinal model. Interestingly this factor has also been described in *L. johnsonii* where it was shown to be involved in adherence and immunomodulation (Bergonzelli et al., 2006; Granato et al., 2004) and therefore may also contribute to the attachment of *B. longum* in the GIT.

17.3.5 Bacteriocin Biosynthesis and Immunity

LAB produce a diverse of bacteriocins that, based on their structure and mode of action, are classified into Class I, II, III, or IV (Klaenhammer, 1993). The ability of *lactobacilli* and bifidobacteria to produce bacteriocins may contribute to their competitive fitness in their habitats. Moreover, bacteriocin biosynthesis is a desirable characteristic for strain selection as it serves as an important mechanism of pathogen exclusion in fermented foods as well as in the GI environment. Bacteriocin biosynthetic gene clusters are found in both GI-associated as well as industrial LAB, including *L. brevis*, *L. casei*, and *L. bulgaricus*. *L. johnsonii* NCC533 has a putative operon (LJ0763b to LJ0775) that potentially encodes

the production of lactacin F, a two-component class II bacteriocin (Pridmore et al., 2004). The putative lactacin F operon showed conserved sequence similarity and synteny with the previously characterized lactacin F operon in *L. johnsonii* VPI11088 (Allison et al., 1994). The putative lactacin F operon is composed of genes encoding the bacteriocin peptide pore complex (*lafAX*), an immunity component (*lafI*), a 2CRS, and two ABC exporters. However, the lactacin F operon in NCC533 is not functional due to the presence of an IS element within the histidine kinase gene of the 2CRS, which interferes with the production and regulation of lactacin F synthesis (Pridmore et al., 2004).

A unique 58-gene *pdu-cbi-cob-hem* genomic island was recently identified in *L reuteri* JCM1112^T genome that encodes the biosynthesis of reuterin (encoded by the *pdu* operon) and the required cofactor, cobalamin (encoded by the *cbi-cob-hem* cluster) (Morita et al., 2008). Comparison of the corresponding gene loci in *L. fermentum* and *L. plantarum* revealed a conserved region flanking the *pdu-cbi-cob-hem* gene cluster in *L. reuteri* and an overall low GC content of the gene cluster, suggesting that this genomic island was likely acquired by *L. reuteri* via HGT. The *gupCDE* gene cluster within the *pdu* operon encodes a functional glycerol dehydratase which catalyes the conversion of glycerol to reuterin. Using two-dimensional nuclear magnetic resonance (2D-NMR) with $^{13}C_3$-labeled glycerol, Morita et al. (2008) showed that reuterin was synthesized *in vivo* in gnotobiotic mice mono-associated with the wild type strain, but not with the *gupCDE* mutant. The typical presence of glycerol in the intestine further led to the authors' speculation that the functional *pdu-cbi-cob-hem* genomic island represents an evolutionary adaptation of *L. reuteri* in the GI tract.

The pMP118 megaplasmid of *L. salivarius* harbors a gene cluster that encodes for the biosynthesis of a class IIB bacteriocin, ABP-118 (Claesson et al., 2006; Flynn et al., 2002). ABP-118 is a wide spectrum salivaricin that has been shown to inhibit species of *Bacillus*, *Listeria*, *Enterococcus*, and *Staphylococcus* (Flynn et al., 2002) and protects mice from *Listeria monocytogenes* infection, *in vivo* (Corr et al., 2007). The production of ABP-118 is inducible by the AbpIP inducing peptide, which is part of a three-component regulatory system (3CRS) that also includes a histidine kinase and a response regulator. Meanwhile, a 9.5-kb polycistronic region that encodes the production of lactacin B (LBA1791 to LBA1803) was identified in the genome of *L. acidophilus* (Altermann et al., 2005). The *lab* operon consists of 12 genes that are organized in three clusters encoding a 3CRS, a putative ABC transporter, and three unknown proteins, respectively (Dobson et al., 2007). Functional analysis of the gene clusters has established that the LabT (LBA1796) is essential for the export and thus

functionality of lactacin B. Furthermore, production of lactacin B was inducible by a synthetic peptide deduced from LBA1800, indicating that this gene, along with *labK* and *labR*, encodes components of a 3CRS that presumably plays an important role in signaling and regulation of lactacin B production.

A recent review by Cheikhyoussef et al. (2008) describes the production and application of bacteriocin and bacteriocin like compounds from bifidobacteria. To date only five bacteriocins have been purified and named from bifidobacteria; bifidin, biflong, bifidocin B, bifilact Bb-12 and bifilong Bb46 (Cheikhyoussef et al., 2008). None of these bacteriocins have been studied extensively but of these five, bifidocin B shares homology with the class IIA LAB bacteroicins. This bacteriocin has been shown to inhibit select gram positive but not gram negative bacteria, suggesting that gram negative bacteria lack a specific receptor or membrane component. Further work indicated that lipoteichoic acid (absent in gram negative bacteria) was involved in binding of the bacteriocin to the cell membrane. Genes encoding for bacteriocin production and immunity were found to be encoded on an 8 kb plasmid (Cheikhyoussef et al., 2008). In contrast, bifilact Bb-12 and biflong Bb46 demonstrated inhibition of both gram negative and gram positive bacteria in a species dependent manner (Cheikhyoussef et al., 2008). More recently, as a result of genome sequencing, a lantibiotic has been described from *B. longum* DJO10A, the first in bifidobacteria (◉ *Figure 17.3* and discussed below) (Lee et al., 2008).

17.4 Evolutionary and Comparative Genomics of Probiotics

17.4.1 Lactobacilli

Sequencing of 20 LAB has expanded the understanding of genome evolution and demonstrated that loss and decay of ancestral genes has played a key role in the evolution of *Lactobacillales*. The transition of LAB to nutritionally rich niches, such as the GIT and milk, has resulted in parallel genome reduction and gene acquisition that would presumably enhance the ecological fitness of these bacteria. This hypothesis is supported by observations that the majority of sequenced lactobacilli isolated from nutrient-rich habitats lack complete biosynthetic pathways, but have gained genes for acquisition of exogenous nutrient sources (Goh and Klaenhammer, 2008; Makarova et al., 2006). For example, genes encoding for peptidases, amino acid transport proteins and genes involved in

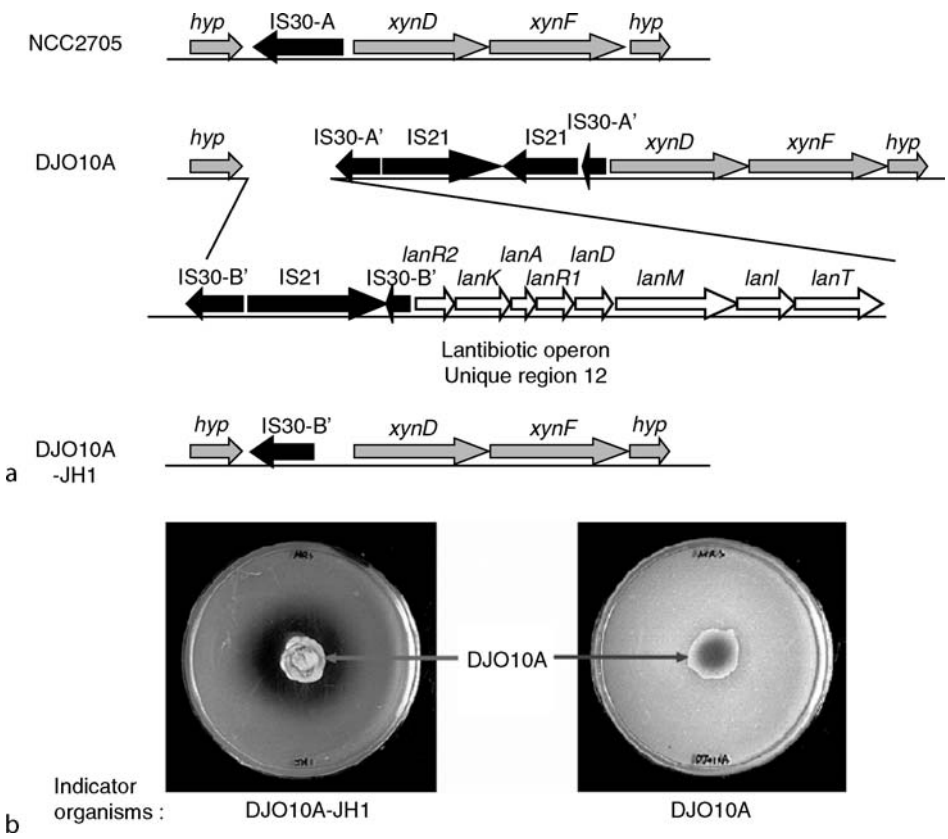

Figure 17.3

Lantibiotic production by *B. longum* DJO10A. (a) Organization of the lantibiotic encoding unique region of *B. longum* DJO10A and the corresponding genome locations in strains NCC2705 and DJO10A-JH1. (b) Bioassay for lantibiotic production by *B. longum* DJO10A with strains DJO10A and DJO10A-JH1 as indicator bacteria (modified from Lee et al. *BMC Genomics* 2008 9:247)

the metabolism and transport of carbohydrates were duplicated reflecting the milk and GIT niche adaptation after the divergence of *Lactobacillales* (Makarova and Koonin, 2007). Comparative analysis between GIT-associated species *L. acidophilus*, *L. gasseri*, and *L. johnsonii* and the dairy species *L. bulgaricus* and *L. helveticus* revealed selective pressure from niche-specific adaptation on the genome evolution of these species (Callanan et al., 2008; Makarova et al., 2006; van de Guchte et al., 2006). Specifically, the absence or decay of genes encoding carbohydrate utilization, cell surface proteins, adherence factors, and bile salt hydrolases occurred in *L. bulgaricus* and *L. helveticus*, reflecting the adaptation

of these organisms to milk. In addition, reconstruction of the gene content over evolution revealed that extensive loss of ancestral genes has occurred in *L. bulgaricus*, *L. johnsonii* and *L. gasseri*, whereas gene loss in *L. plantarum* and *L. casei* was counterbalanced with new and paralogous genes from HGT and gene duplication events (Makarova et al., 2006). These differences also increased the importance of genes retained by these species, as important contributors to probiotic functionality in the GIT.

In addition, the most metabolically diverse probiotic LAB sequenced to date, *L. plantarum* harbors a larger 3 Mb genome and a low G + C content region or 'lifestyle adaptation island' encoding sugar transport and metabolism genes that was likely to have been acquired by HGT (Kleerebezem et al., 2003). Additional examples are the *eps* clusters in the acidophilus complex (Altermann et al., 2005), the reuterin genomic island in *L. reuteri* (Morita et al., 2008), various cell surface factor-encoding genes in *L. johnsonii* (Pridmore et al., 2004) and genes involved in sugar metabolism in bifidobacteria (Ventura et al., 2007). The presence of additional mobile genetic elements such as phage in lactobacilli and bifidobacteria provide opportunities to access niche-specific genes in the GIT gene pool that may promote ecological fitness of probiotics and benefits to the host. Putative lysogenic conversion genes recently identified in the prophage of *L. plantarum* were predicted to encode proteins that may play a role in host immunomodulation (Ventura et al., 2003). The availably of sequenced LAB genomes including nine lactobacilli facilitated a comprehensive comparative genome studies by Makarova and Koonin (Makarova et al., 2006; 2007). As part of this study *Lactobacillales*-specific clusters of orthologous protein coding genes (LaCOGs) specifically for *Lactobacillales* were determined allowing a tighter and more relevant comparison of genomes for *Lactobacillales*. These LaCOGs demonstrated excellent coverage for new genomes with up to 90% of genes assigned to a LaCOG (Makarova and Koonin, 2007). Additional analysis described the conserved gene core of *Lactobacillales*; in the 12 genomes analyzed 18% (567 genes) of LaCOGs were identified that were shared among the strains. The majority of genes were from COGs involved in cell processes such as replication, transcription and translation, whereas, 50 genes had a general function or unknown function prediction (Makarova et al., 2006; Makarova and Koonin, 2007). Only two genes had no orthologs outside *Lactobacillale,* a gene containing a peptidoglycan binding domain and a highly conserved protein with an unknown function (Makarova and Koonin, 2007).

The *L. acidophilus* group has the most sequenced representatives, *L. acidophilus*, *L. gasseri*, *L. johnsonii*, *L. bulgaricus*, and *L. helveticus*, which

show overall large genome synteny (Klaenhammer et al., 2005). Sequencing of 16S rRNA genes confirmed the clustering of the *L. acidophilus* complex separate from *L. plantarum*, *L. sakei* and *L. salivarius* (Berger et al., 2007). Unsurprisingly, the intestinal associated species *L. acidophilus*, *L. johnsonii*, and *L. gasseri* shared extensive similarity in gene content and order over the length of the genome. In particular, *L. johnsonii* and *L. gasseri*, demonstrated extensive synteny, however there is a reversal of some gene order in *L. gasseri* caused by genome inversion (Berger et al., 2007). Using a *L. johnsonii* microarray the close relationship between *L. johnsonii* and *L. gasseri* was confirmed, with the exception of several prophage genes, rhamnose biosynthesis genes and part of the lactacin F operon. In fact, approximately 83–92% of the tested *L. gasseri* strains were conserved in the *L. johnsonii* comparisons. In contrast, approximately 65% of the two *L. gasseri* strains tested and only few DNA sequences (12%, related to cellular processes) from *L. acidophilus* showed a good match to the *L. johnsonii* strain (Berger et al., 2007).

17.4.2 Bifidobacteria

To date the small number of sequenced genomes available for bifidobacteria provides limited information in regard to evolutionary studies. However, as with lactobacilli, mobile genetic elements such as insertion elements and prophage play an important role in tandem with gene loss and acquisition for the evolution of bifidobacteria. There are many examples of HGT influencing genome plasticity in both commensal and probiotic gut bacteria. As discussed above, the *eps* cluster of both *B. longum* NCC2705 and *B. breve* UCC2003 are flanked by IS elements suggesting they were acquired by HGT. In addition, the phage remnant Bbr-1 of *B. breve* UCC2003 encodes a β-glucosidase which was shown to be transcribed and may contribute to carbohydrate metabolism and hence competitiveness of this organism in the GIT (Ventura et al., 2007). Plasticity of the probiotic gene repertoire presumably allows the adaptation and subsequent increased ecological fitness of these intestinal lactobacilli and bifidobacteria which underlies their potential.

While few bifidobacteria strains have been sequenced to date, there are a number of interesting and important studies that provide insights into the comparative genomics of bifidobacteria. A DNA based array based on the *B. longum* NCC2705 genome was used to compare genomes of un-sequenced strains of *B. longum* biotype *longum*, *B. longum* biotype *infantis* and *B. longum* biotype *suis*

(Klijn et al., 2005). Analysis revealed seven major regions of *B. longum* NCC2705 that were not conserved in the other *B. longum* strains. Of these seven regions, four corresponded to regions of low G + C content, one represented the prophage in *B. longum* NCC2705 and the remaining two were absent from all strains but present in *B. longum* DJO10A. Hence this region was not identified in the genome comparison of these two genomes (see below) (Klijn et al., 2005). One of these latter regions contained several genes involved in carbohydrate metabolism, which is a noted trait of bifidobacteria that contribute to competitiveness in the GIT. While DNA based arrays are useful tools for comparative genomics, only regions absent in the strain used to make the array will be identified. In contrast, direct genome sequencing allows a more comprehensive comparative analysis and will be more feasible in the future with decreased sequencing costs and faster sequencing turnaround. Comparative genome analysis of *B. longum* NCC2705 and a *B. longum* strain which is known for EPS production (CRC-002) was performed using suppression subtractive hybridization (Delcenserie et al., 2008). Interestingly, genes involved in EPS biosynthesis and metabolism of carbohydrates were specific to CRC-002.

More recent comparative genomic analysis between two *B. longum* strains has enabled the identification of genes and gene regions important for intestinal bifidobacteria (*B. longum* DJO10A) compared to those of a strain grown under laboratory conditions in a fermentation environment (*B. longum* NCC2705) (Lee et al., 2008). *B. longum* DJO10A is a minimally cultured strain (20 generations) isolated from the feces of a healthy young adult, whereas *B. longum* NCC2705 is a culture collection strain. Comparative analysis revealed that both genomes are collinear and highly conserved, except for mobile integrase cassettes and IS elements. In addition, regions unique to both genomes were identified that were suggested to reflect the different histories of the two strains. Analysis revealed 248 unique regions between the genomes which where at least 10 kb in size but of these, 23 regions encoded functional units or genes from 3 to 48.6 kb (Lee et al., 2008). The majority of these regions (17) were located in strain *B. longum* DJO10A. Interestingly, these unique regions in *B. longum* DJO10A included genes encoding for oligosaccharide and polyol utilization, arsenic resistance and lantibiotic production. The highest number of unique genes in *B. longum* DJO10A belonged to carbohydrate metabolism, in particular oligosaccharide utilization. Seven of the eleven oligosaccharide utilization gene clusters are also fully (5) or partially (2) present in *B. longum* NCC2705. Further analysis by this group indicated that *B. longum* NCC2705 lost six oligosaccharide utilization gene clusters by genome reduction during adaptation

to a fermentation based environment rather than recent genetic acquisition of these clusters by *B. longum* DJO10A (Lee et al., 2008). Interestingly, *B. longum* NCC2705 does not contain any genes for polyol metabolism whereas genes involved in polyol recognition, transport, metabolism and dehydration were located in two unique regions of *B. longum* DJO10A. These gene regions were shown to be gene acquisition events based on base deviation analysis (Lee et al., 2008). The ability to withstand low concentrations of arsenic from the diet has been demonstrated with intestinal bacteria including *B. longum* DJO10A, which demonstrated a 2,000% greater survival than *B. animalis* subsp *lactis* BB12 and a 100% better survival than *Escherichia coli* K12 (Lee et al., 2008). In addition, the production of bacteriocins may confer a competitive advantage to bacteria in the GIT. Bacteriocins have not been described in many bifidobacteria (see above) and none of the lantibiotic family, until analysis of the genome of *B. longum* DJO10A revealed a 10.2 kb cluster absent from *B. longum* NCC2705 (*Figure 17.3*). This lantibiotic was only produced by *B. longum* DJO10A during growth on agar. Base deviation analysis revealed that both of these unique regions encoding arsenic resistance and the lantibiotic gene cluster may have been traits lost by *B. longum* NCC2705 as a consequence of adaptation to a fermentation environment, where arsenic or competing bacteria would not be encountered. This group also followed the fate of seven unique gene regions, predicted useful for survival in the GIT, present in *B. longum* DJO10A after 1,000 generations in typical laboratory media (Lee et al., 2008). The lantibiotic gene cluster was lost from 40% of isolates tested supporting the hypothesis that this trait was unstable over many generations in a fermentation environment. Competitive growth experiments demonstrated that *B. longum* DJO10A faired better against newly isolated competitor strains *E. coli* and *Clostridum difficile*, than did the isolate which had lost the lantibiotic gene cluster.

17.5 Probiotic Genome Expression and Niche Adaptation to the GIT

17.5.1 Lactobacilli

Gene expression of *L. reuteri*, *L. plantarum* and *L. johnsonii* has been studied in the GIT of mice (Bron et al., 2004a; Denou et al., 2007; Denou et al., 2008; Walter et al., 2003). Using IVET technology Walter et al. (2003) identified only *L. reuteri* three genes that were expressed *in vivo*. These genes encoded for a xylose

isomerases which is involved in nutrition and a methionine sulfoxide reductase gene, which is involved in the stress response. The third gene encoded for a protein of unknown function. In contrast, a subsequent study using a modified version of IVET technology identified the expression of 72 gens from *L. plantarum* in the mouse GIT (Bron et al., 2004a). Identified genes were related to carbohydrate and amino acid metabolism, stress resistance and extracellular genes potentially involved in host interactions. Studies by Denou et al. (2007) identified different *L. johnsonii* gene sets expressed in the stomach, cecum and jejunum of mice. Only 103 genes were expressed *in vitro* and in all three locations in the murine GIT, with 44% of the genes were transcribed in any of the conditions tested, *in vitro* or *in vivo*. Additional work by this group studied the gene expression of *L. johnsonii* after 5 and 12 days of gut residence to identify genes associated with the long-gut-persistence phenotype (Denou et al., 2008). Three gene loci involved in EPS biosynthesis, a mannose PTS and a putative protease were identified that were expressed *in vivo* and also related to the long-gut-persistence phenotype. In addition, deletion of the protease and mannose PTS system decreased the gut residence time in the mutant indicating the role of these gene loci in gut persistence. Interestingly, deletion of the *eps* locus resulted in a slight increase in gut persistence (van de Guchte et al., 2006).

Adaptation to the ecological niche of the human gut is evidenced by genome degradation events resulting in reduced biosynthetic capabilities for many probiotic lactobacilli. However, intestinal lactobacilli are rich in transporters allowing the uptake of sugars and amino acids directly from nutrients in the GIT. In addition, while these lactobacilli are deficient in their biosynthetic capabilities, conversely they encode crucial features which allow them to survive the harsh environment of the GI tract such as acid and bile resistance, metabolism of complex carbohydrates, and presentation of cell surface proteins that interact intimately, with the intestinal mucosa. This adaptation to life in the GI tract is further evident when genome sequences are compared between food-adapted lactobacilli such as *L. bulgaricus* and *L. helveticus*. *L. bulgaricus* is used as a starter culture in yogurt fermentations and has undergone genome decay which has allowed it to adapt to the milk environment (van de Guchte et al., 2006). Whereas probiotic lactobacilli such as *L. acidophilus* and *L. plantarum* encode for many transport and metabolic pathways that allow them to utilize carbohydrates in the GI tract, *L. bulgaricus* shows a preference for growth in lactose, encodes numerous degraded or partial carbohydrate pathways and harbors bile salt hydrolase pseudogenes, further emphasizing its niche adaptation to milk (van de Guchte et al., 2006). *L. acidophilus* and the *L. helveticus* are the most closely related starter and

probiotic cultures sequenced to date (Callanan et al., 2008). However, genome comparison has revealed that despite their similarity [75% of predicted ORFs in *L. helveticus* have orthologues in the *L. acidophilus* genome (Callanan et al., 2008)] there are important differences in gene sets reflecting their adaptation to significantly different niches. In particular, *L. helveticus* has additional genes for fatty acid biosynthesis and specific amino acid metabolism, but notably fewer cell surface factors and PEP-PTSs for sugar utilization (Altermann et al., 2005; Callanan et al., 2008). As discussed above, no functional mucus binding proteins or transporters for complex carbohydrates such as raffinose and fructooligosaccarides are present in *L. helveticus* reflecting the deep adaptation of *L. helveticus* to a milk niche. Conversely, *L. acidophilus* encodes these traits lacking in *L. helveticus* indicating the importance of these traits for probiotic functionality.

17.5.2 Bifidobacteria

Bacterial cells in the human body are estimated to be 10 times more numerous than the total number of human cells, demonstrating the intricate relationships between the microbiota and the host (Palmer et al., 2007). Microbial density is most numerous in the large intestine where cell numbers can reach 10^{11} to 10^{12} bacteria per gram of luminal contents (Whitman et al., 1998). To date there is scant information regarding bifidobacteria and genome expression in the GIT. The mode of action of bifidobacteria and their interaction with the host is still not fully understood. The sequencing of additional strains and transcriptomic analysis in the GIT or human cell systems will provide additional insights into the probiotic and commensal functionally of these bacteria. A recent study by Yuan et al. (2008) used proteomic techniques to study host induced changes in the *B. longum* NCC2705 proteome by comparing proteomic profiles of the strain grown *in vitro* and *in vivo* in a rabbit intestinal model. Up regulated proteins in the intestine reflected the adaptation of *B. longum* for the GIT niche. These included a EF-Tu adhesion-like factor (discussed above), a BSH and stress proteins. Interestingly, four proteins were identified that had post translational modifications. One of these was the LuxS protein which is involved in quorum sensing and could play an important role in the GIT for cell communication between bacteria (Yuan et al., 2008).

Sonnenburg et al. (2006) used transcriptomics to examine the responses of the probiotic *B. longum* NCC2705 and a dominant member of the human microbial flora, *Bacteroides thetaiotaomicron*, to the presence of each other, in

the intestinal habitat of germ free and colonized mice consuming a defined and consistent diet. As the genome sequence for both strains were available, they constructed a gene chip which facilitated transcriptomic analysis. In addition, they compared the response of *Bacteroides thetaiotaomicron* to two other common probiotics, *L. casei* and *B. animalis* (Sonnenburg et al., 2006). Colonization of germ free mice with both strains resulted in an expansion of genes involved in the acquisition and breakdown of polysaccharides (Sonnenburg et al., 2006). In particular, up-regulated *Bacteroides thetaiotaomicron* genes from the COG categories, carbohydrate transport and metabolism, and energy production and conversion, were identified in addition to changes in expression of 31 *Bacteroidesthetaiotaomicron* glycoside hydrolases and two polysaccharide lyases implying an expansion in the polysaccharide repertoire targeted for metabolism by *Bacteroides thetaiotaomicron* in the presence of *B. longum* (Sonnenburg et al., 2006). In addition, 31 gene homologues from a starch utilization system (SusC/D) which could confer the ability to bind and import diverse carbohydrates from the GIT were up regulated in the genome of *Bacteroides thetaiotaomicron* in the presence of *B. longum*. A lesser cohort of genes changed expression for *B. longum* after co-colonization compared to *Bacteroides thetaiotaomicron* (3.5% versus 14.2%) with the only significant difference in COG group classification being a down-regulation of genes from the carbohydrate transport and metabolism (Sonnenburg et al., 2006). Additional analysis revealed differential expression of genes involved in mannose degradation for both strains (up regulation and down regulation of mannosidases genes for *Bacteroides thetaiotaomicron* and *B. longum*, respectively) and up-regulation of genes involved in xyloside degradation and xylose catabolism by *Bacteroides thetaiotaomicron* in the presence of *B. longum* (Sonnenburg et al., 2006). These results indicate that the presence of the probiotic *B. longum* causes the commensal *Bacteroides thetaiotaomicron* to target additional polysaccharide sources in the GIT, which was shown to be independent of host genotype. These effects were also demonstrated in the presence of probiotic strain, *L. casei*.

The host epithelial response to co-colonization of *Bacteroides thetaiotaomicron* and *B. longum* was also studied using transcriptomic analysis and revealed a synergistic induction of interferon-responsive genes. Comparison of gene sets from mono colonization of each strain and co-colonization revealed that the host response to co-colonization was different to mono-colonization of each strain (Sonnenburg et al., 2006) (*Table 17.4*). Ingenuity Pathways Analysis revealed the host response to *Bacteroides thetaiotaomicron* was centered on tumor necrosis factor-α, a cytokine produced by natural killer cells, T cells and macrophages. The host response to *B. longum* however centered on γ-interferon, a cytokine

Table 17.4

Genes involved in immuno-inflammatory responses that are up-regulated in at least one colonization state; fold-increase in expression in each colonization state is relative to the germ-free state (modified from Sonnenburg et al., 2006) (*Cont'd* p. 716)

Accession No	Name	*B. thetaiotaomicron* mono-associated (fold)	co-colonized (fold)	*B. longum* mono-associated (fold)
NM_011036	Pancreatitis-associated protein	4.3	3	−34.9
NM_011260	Regenerating islet-derived 3 γ	5.2	2.5	−8.5
BB241535	Suppressor of cytokine signaling 3	7.8	4.9	1.2
BC010754	Kallikrein 6	2.9	1.8	1.5
M17962	Kallikrein 1-related peptidase b9	4.6	2.2	2
AV084904	Chemokine (C-C motif) ligand 6	5.6	1	1
NM_017399	Fatty acid binding protein 1, liver	10.7	1.5	3
AK008170	Z-DNA binding protein 1	4.6	8.1	2.3
BB132493	Radical SAM domian containing 2	1.1	4.1	1.2
NM_008332	IFN-induced protein wirh TPR2	2.8	16	1.2
NM_008330	IFN gamma inducible protein 47	2.2	6.6	1.3
NM_019440	IFN inducible GTPase 2	1.3	3.7	1.2
AK019325	IFN, α-inducible protein	2	17.1	1.2
NM_010501	IFN-induced protein wirh TPR3	1.3	3.2	−1.4
NM_008331	IFN-induced protein wirh TPR1	2.9	12.3	1.6
BQ033138	2′-5′ oligoadenylate synthetase-like 2	1.8	7	−3

Table 17.4

Accession No	Name	*B. thetaiotaomicron* mono-associated (fold)	co-colonized (fold)	*B. longum* mono-associated (fold)
NM_018738	IFN gamma induced GTPase	2.5	5.6	−1.2
BB532597	Mucin 4	2.4	6.5	2.6
NM_007398	Adenosine deaminase	1.4	2.3	2.4
BE199688	ang4, Ribonuclease A family	1.3	2	5.6
NM_011128	Pancreatic llipase-protein protein 2	−2.5	1.1	6.3

produced by T cells (Sonnenburg et al., 2006). Both tumor necrosis factor-α and γ-interferon networks were identified in the co-colonization experiments demonstrating the synergistic induction of genes involved in the innate immune system.

17.6 Summary

- Sequencing of probiotic genomes has provided knowledge and key insights into probiotic traits that help explain their functionality and niche adaptation to the GIT. In addition, key knowledge on lactobacilli and bifidobacteria evolution is being revealed.
- The elucidation of genome sequences has facilitated the development of important molecular tools, such as microarrays, which allow the comparison of as yet unsequenced strains and hence aid in probiotic strain selection. In addition, transcriptomic and proteomic technologies can be used in tandem with genome sequencing to study probiotic traits.
- The availability of genome sequences has facilitated the identification of important genes or genetic loci that can be targeted for functional analysis, facilitating the understanding of probiotic and commensal interactions in the GIT.
- Sequencing of probiotic bacteria is revealing gene features that can be exploited for delivery of bio-therapeutics to specific niches and locations in the GIT.
- Genome sequencing of autochthonous and allochthonous lactobacilli and bifidobacteria in the GIT has contributed to the understanding of the human microbiome and the complex interactions between probiotic and commensal bacteria, and with the host.

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List of Abbreviations

2CRS	two-component regulatory system
ABC	ATP-dependent binding cassettes
BSH	bile salt hydrolases
COGs	Clusters of Orthologous Groups of proteins
EMP	Embden-Meyerhof Pathway
EPS	exopolysaccaride
Fbp	fibronectin-binding protein
FOS	fructo-oligosaccaride
GIT	gastrointestinal tract
GOS	galacto-oligosaccaride
HGT	horizontal gene transfer
IVET	*in vivo* expression technology
LAB	lactic acid bacteria
LaCOGs	Lactobacillales-specific clusters of orthologous protein coding genes
LTA	lipotechoic acids
PEP-PTSs	phosphoenolpyruvate-dependant phosphotransferase system
PKP	phosphoketolase pathway
SLPs	surface layer proteins
TA	techoic acids

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18 Manufacture of Probiotic Bacteria

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18.1 Introduction

Lactic acid bacteria (LAB) have been used for many years as natural biopreservatives in fermented foods. A small group of LAB are also believed to have beneficial health effects on the host, so called probiotic bacteria. Probiotics have emerged from the niche industry from Asia into European and American markets. Functional foods are one of the fastest growing markets today, with estimated growth to 20 billion dollars worldwide by 2010 (GIA, 2008). The increasing demand for probiotics and the new food markets where probiotics are introduced, challenges the industry to produce high quantities of probiotic cultures in a viable and stable form. Dried concentrated probiotic cultures are the most convenient form for incorporation into functional foods, given the ease of storage, handling and transport, especially for shelf-stable functional products. This chapter will discuss various aspects of the challenges associated with the manufacturing of probiotic cultures.

18.2 Selection of Strains

For a strain to be considered probiotic, it should adhere to certain criteria as follows. Preferably the microbes should have GRAS (Generally Regarded As Safe) status, have a long history of safe use in foods, be non-pathogenic, and acid and bile tolerant (Morgensen et al., 2002). Probiotics are described as "live microorganisms which, when administrated in adequate amounts, confer a health benefit on the host" (FAO/WHO, 2001). However, there is no general consensus as to whether probiotics should be viable in all cases to exert a health benefit, with some studies demonstrating that non-viable probiotic bacteria can have a beneficial effect on the host (Ouwehand and Salminen, 1998; Salminen et al. 1999). While most probiotic products are developed for the dairy industry, they are also used in non-dairy foods, such as energy bars, dietary supplements and pet food.

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Selection of the right probiotic strain is crucial and in general probiotic bacteria need to be viable in the product at the end of shelf-life for a product to be commercially successful. This requirement poses challenges during processing and following ingestion. For example, the bacteria are exposed to different technological stresses, such as acid, osmotic, cold, heat and drying stress. Probiotic strains are generally of the genera *Lactobacillus* and *Bifidobacterium*, and to a lesser extent *Pediococcus, Propionibacterium, Enterococcus, Bacillus, Streptococcus* and *Saccharomyces* (Champagne and Møllgaard, 2008).

Selecting the appropriate strain for a particular food can be divided in four categories:

- Performance in the gastrointestinal tract (GIT)
- Industrial production
- Safety of the microorganisms
- Health benefit

This chapter will focus on the first two categories. The remaining categories are described elsewhere.

18.2.1 Performance in the Gastrointestinal Tract

Following ingestion, probiotics pass through the stomach before they reach the small intestine. The acidity of the stomach is known to fluctuate, from pH 1.5 to 6.0 after food intake (Waterman and Small, 1998). In the stomach, exposure to gastric acid and the proteolytic activity of pepsin can result in viability losses. Furthermore, bile acids and pancreatin in the small intestine present further challenges to viability of probiotics during transit through the gastrointestinal tract (GIT) (Hofmann et al., 1983; Lee and Salminen, 1995).

In vitro methods have been developed to select for strains that can withstand the extreme conditions in the stomach. While there has been no consensus on the pH range that needs to be analyzed for the selection of potential probiotics, values between 1 and 5 have been studied extensively. For instance, *L. acidophilus* challenged at pH 3 showed higher acid tolerance when challenged in broth compared with phosphate buffer (Hood and Zoitola, 1988). Similarly, LAB survived better at low pH in milk than in buffered saline, and furthermore survival of LAB increased in the stomach (*in vivo*) when administered with milk (Conway et al., 1987). Collado and Sanz (2006) selected for acid resistant

bifidobacteria by prolonged exposure of human feces to low pH, and subsequently exposed the isolated pH tolerant bacteria to high bile salt concentrations and NaCl. This selection method proved very successful for the isolation of potential probiotics. Others screened *L. casei* strains for probiotic properties, such as acid and bile tolerance, adhesion to epithelial cells, antimicrobial effects and cholesterol reduction and showed there was great variation between strains (Mishra and Prasad, 2005). Similar variation was also observed in a study where thirteen spore-forming bacilli belonging to *Sporolactobacillus, Bacillus lavolacticus, Bacillus racemilacticus* and *Bacillus coagulan*s species were screened for resistance to acidic conditions and bile. Only five bacteria were capable of growth in MRS adjusted to pH 5, and all *Bacillus coagulans* and *racemilacticus* tolerated bile concentrations over 0.3% (Hyronimus et al., 2000). Propionibacteria have been increasingly used in functional foods. *In vitro* assays for the transit tolerance of these bacteria showed strain specific resistance for acid (pH 2.0–4.0). All propionibacteria tested survived simulated small intestinal conditions (Huang and Adams, 2004). For simulating the small intestine, bile salt concentrations between 0.15 and 0.3% have been recommended (Goldin and Gorbach, 1992), which agrees with the physiological concentration in the GIT. Following initial acid tolerance screening in modified MRS (pH 3.0), it was reported that one *Bifidobacterium breve* strain showed better survival capabilities when exposed to 0.5% pepsin, 1% pancreatic, and better adhesion properties out of 35 bifidobacteria strains tested, including *B. infantis, B. longum, B. bifidum, B. adolescentis, B. breve, B. animalis, B. asteroids, B. globosum* and *B. pseudocatenulatum* species (Liu et al., 2007).

In addition to overcoming the stresses encountered in the stomach and small intestine, adherence to epithelial cells is considered a desirable probiotic trait (Guarner and Schaafsma, 1998). Several studies have shown that probiotics can adhere to epithelial cells *in vitro*, with the most common cell lines used for these experiments being HT-29, HT-29MTX, Caco-2 and Int-407 lines (Bernet et al., 1994; Fernandez et al., 2003; Sarem et al., 1996). HT-29, Caco-2 and Int-407 cell lines have the characteristics (morphology and physiological) of normal human intestinal cells. HT-29MTX is a mucus excreting form of HT-29. These cell lines have been cultured for the analysis of the adhesion of enteropathogens, and also for the analysis of the adherence of probiotics. Although adherence is deemed an important probiotic trait, not all probiotics have been proven to colonize the intestine *in vivo*. *Lactobacillus rhamnosus* GG has been reported to remain in the human GIT for approximately 1 week after administration is discontinued (Alander et al., 1999; Goldin and Gorbach, 1992).

It has been shown that some probiotics do not have to be viable to prevent adherence of pathogens to epithelial cells. For example, heat killed *Lactobacillus acidophilus* inhibits the adherence of diarrheagenic *Escherichia coli* to Caco-2 cells *in vitro*, by competitive exclusion (Chauviere et al., 1992). Several studies have shown that probiotics, mainly lactobacilli and bifidobacteria, can prevent or minimize adhesion of pathogens (Bernet et al., 1994; Neeser et al., 1989).

Other approaches for selection of probiotics are screening for antimicrobial activity and ability to stimulate the host immune response. Animal studies have shown that the immune response is up-regulated when probiotics are consumed (Galdeano and Perdigon, 2006). These assays are very useful, but time consuming and costly to perform as a screening method. On the other hand, selecting antimicrobial activity against a certain pathogen, using common microbiological methods is a more feasible selection method. The relatively low cost of genome sequencing has opened the way to functional genomic studies of a variety of probiotics, providing insight into the molecular basis for probiotic traits as production of antimicrobial compounds, adhesion or adaptation to the environment. These developments could lead to novel probiotic screening methods on a genomic level (Dellaglio et al., 2005; Schell et al., 2002).

18.3 List of Commercial Strains

Food products supplemented with probiotics are gaining popularity worldwide, and manufacturers are increasingly developing new probiotic products. *Table 18.1* shows a list of some commercial available probiotic cultures and their manufacturers. A list of some probiotic dairy foods and the strains they contain is given in *Table 18.2*.

18.4 Growth Media and Conditions

To produce probiotics in adequate amounts, the growth media need to be optimized for the specific strain aiming for increased biomass yield and reduction of productions costs. There are two types of fermentation media used commercially; i.e., synthetic and dairy based media. When using synthetic

Table 18.1

Manufacturers/suppliers of probiotic cultures (*Cont'd* p. 730)

Manufacturer	Probiotic Strain
Cargill (Minneapolis, USA)	*Bifidobacterium animalis* subsp. *lactis* Bf-6
	Lactobacillus johnsonii La-1
	Lactobacillus johnsonii La-19
	Lactobacillus paracasei LCV-1
Cell Biotech Europe (Copenhagen, Denmark)	*Bifidobacterium bifidum* BF2
	Bifidobacterium breve BR2
	Bifidobacterium infantis BT
	Bifidobacterium lactis BL2
	Bifidobacterium longum BG3
	Enterococcus faecium EF1
	Lactobacillus acidophilus LH5
	Lactobacillus casei LC1
	Lactobacillus rhamnosus LR3
	Lactobacillus plantarum LP1
	Lactococcus lactis SL1
	Pediococcus pentosaceus PP
	Streptococcus faecalis SFL
	Streptococcus thermophilus ST3
Cerbios-Pharma SA (Lugano, Switzerland)	*Enterococcus faecium* SF68
Chr. Hansen (Hørsholm, Denmark)	*Bacillus licheniformis* and *Bacillus subtilis* BioPlus 2B
	Bifidobacterium lactis BB12
	Lactobacillus acidophilus LA5
	Lactobacillus paracasei subsp. *paracasei* CRL-431
	Lactobacillus reuteri RC-14
	Lactobacillus rhamnosus GG
	Lactobacillus rhamnosus GR-1
	Streptococcus thermophilus TH-4
Danisco (Copenhagen, Denmark)	*Bifidobacterium animalis* subsp. *lactis* B-420
	Bifidobacterium lactis HN019
	Lactobacillus acidophilus La-145
	Lactobacillus acidophilus NCFM
	Lactobacillus rhamnosus HN001
DSM (Heerlen, The Netherlands)	*Bifidobacterium* LAFTI B94
	Lactobacillus acidophilus LAFTI L10
	Lactobacillus casei LAFTI L26

Table 18.1

Manufacturer	Probiotic Strain
Morinaga (Tokyo, Japan)	*Bifidobacterium breve* M16V
	Bifidobacterium infantis M-63
	Bifidobacterium longum BB536
	Bifidobacterium pseudolongum M-602
	Enterococcus faecium FA5
	Lactobacillus acidophilus LA5
	Lactobacillus acidophilus LAC361
	Lactobacillus gasseri LAC343
	Lactobacillus plantarum LP83
	Lactobacillus rhamnosus LCS742
Valio (Helsinki, Finland)	*Lactobacillus rhamnosus* LGG

media in the food industry, the bacteria have to be extensively washed, before addition to the product. This prevents flavor carry-over from the media, and there may be regulations in place that will prevent from adding bacteria grown on synthetic media (Abu-Taraboush et al., 1998; Ventling and Mistry, 1993). Milk or yogurt based media are more suitable for use in the food industry. An additional advantage of using a natural medium is that the probiotics do not have to be separated from the medium, while a disadvantage is that only growth promoting supplements that have no adverse effect on the final product quality can be used. Probiotics in general require a large amount of growth factors, and thus the growth media can become very complex and expensive. There is not one ideal medium for all probiotics. Even within a species, there can be differences in optimal growth conditions. When probiotics are applied in functional foods, the type of energy source used in the fermentation greatly influences the probiotic performance of the product (Carvalho et al., 2003; Mattila-Sandholm et al., 2002). It is therefore suggested to grow the starter cultures on the same sugar that will be present in the final product matrix, and thus for dairy products, lactose is the preferred energy source. This will prevent a long adaptation phase, since when bacteria have to switch from glucose to lactose as an energy source, there is the need for induction of β-galactosidase. There has been extensive research on the optimization of growth conditions for lactobacilli and bifidobacteria, and the following section details the cultivation of these strains.

Table 18.2

Commercial probiotic dairy products

Product	Manufacturer	Probiotic cultures
ABC	Sitia YOMO	*L. acidophilus, B. lactis, L. casei*
Actimel	Danone	*L. casei* DN-114 001
Active-più	Parmalat	*B. lactis*
Activia	Danone	*B. bifidum*
Activia yogurt	Danone	*B. animalis* DN173 010
Align	Procter and Gamble	*B. infantis* 35264
BEBA	Nestlé	*S. thermophilus* and *B. lactis*
Bifidus yogurt	Morinaga	*B. longum* BB536
Bio Profit	Valio	*L. rhamnosus* LC-705, *Propionibacterium freudenreichii* subsp. *shermanii* JS
Biospega	Spega	*L. acidophilus, B. lactis*
Chamyto	Nestlé	*L. paracasei*
Culturelle	Valio	*L. rhamnosus* GG
DanActive	Danone	*L. casei* DN114 001
Femdophilus	Chr. Hansens	*L. reuteri* RC-14, *L. rhamnosus* GR-1
Florastor	Biocodex	*Saccharomyces boulardii*
Fyos	Nutricia	*L. paracasei*
Gaio	MD Foods plc	*Enterococcus faecium,* K77D *L. salivarius*
Gefilac	Valio	*L. rhamnosus* GG
Good Start Natural Cultures	Nestlé	*B. lacti*
Joie	Yakult	*L. casei* Shirota
LC1	Nestlé	*L. johnsonii*
Nu Trish	Chr. Hansens	*B. lactis* Bb-12, *L. acidophilus* La5, *L. casei CRL-431*
Rolly	Snow Brand	*Bifidobacterium* subsp.
Snow yogurt + 2	Snow Brand	*L. acidophilus* SBT2062
Stonyfield Farm yogurts	Biogaia	*L. reuteri* ATCC 55730
Teddy	Fattoria Scaldasole	*B. lactis*
TopOntbijt	Coberco	*B. lactis* Bb-12 *L. acidophilus* LA5
Vifit	Campina	*L. rhamnosus* GG
Viili	Valio	*Lc. lactis* subsp. *Cremoris*
Vitality	Müller	*L. acidophilus* LA5, *B. lactis* Bb-12
Yakult	Yakult	*L. casei* Shirota, *B. breve*
Yogurt	Meiji	*L. delbrueckii* subsp. *bulgaricus* 2038
YoMi	Danisco	*B. lactis, L. acidophilus*
Yo-plus yoghurt	General Mills Inc.	*B. lactis* Bb-12

18.4.1 Bifidobacteria

Bifidobacteria are anaerobic bacteria and have strict requirements for nutrients in the media; they are however not as fastidious as lactobacilli. Bifidobacteria can be grown in semi-synthetic media containing only lactose, the free amino acids cysteine, glycine and trypthophan, several vitamins, nucleotides, and some minerals (Gomes and Malcata, 1999). In general, the media bifidobacteria contain nutritive growth supplements, and have low oxidation/reduction and pH value between 5.0 and 8.0, with the optimal pH being between 6 and 7, growth temperature between 37 and 41°C, with no growth above 45° and below 25°C (Gomes and Malcata, 1999; Kearney et al., 2008).

Most of the media used for the growth of bifidobacteria are derived from *Lactobacillus* media. The media described in *Table 18.3* are in general used for the growth of bifidobacteria. By supplementing the media with certain antibiotics, they can be made selective for bifidobacteria, and this has recently been reviewed by Kearney et al. (2008) and Roy (2001).

Synthetic media can be supplemented with special growth factors that increase the yield and growth performance of bifidobacteria. There is however, not one optimum medium for bifidobacteria, and therefore an optimum medium should be developed for each strain (Hartemink and Rombouts, 1999). Many approaches for the optimization of media for bifidobacteria have been taken.

Table 18.3

Culture media used for bifidobacteria, adapted from Roy (2001)

Media	Name	Additive
MRS	De Man Rogosa Sharpe	–
TPY	Tryptone Phytone Yeast	–
BL	Glucose Blood-Liver	–
CLB	Columbia	–
LCL	Liver Cysteine Lactose (Blaurock)	–
RCM	Reinforced Clostridia Medium	–
mMRS	Modified MRS	L-cysteine HCL, 0.05%
mMRS + blood	Modified MRS	L-cysteine HCL, 0.05% Sheep blood 10 ml
X-α-Gal	MRS	X-α-Gal
mBL	Modified BL without blood	L-cysteine HCL, 0.05%
mRCM	Modified RCM	Lactose 1.0% Human blood 50ml
RCPB	RCM	Prussian Blue 0.03%

Table 18.3 presents some common bifidogenic supplements used for growth promotion of bifidobacteria. Ideally, the media should have a low redox potential to cysteine, cysteine-hydrochloride or ascorbic acid are routinely used as reducing agents (Calicchia et al., 1993; Shah, 1997). Furthermore, β-glycerophosphate in combination with cysteine increased growth for *B. infantis* and *B. bifidum* in milk but had no effect on *B. longum* ATCC 15708 (Roy et al., 1990). Milk components such as whey and casein have been shown to have growth promoting capabilities. Petschow and Talbot (1990) reported increased growth of *B. bifidum serovar pennsylvanicus* and *B. longum* by addition of whey proteins (α-lactalbumin and β-lactoglobulin). Casein hydrolysates promoted growth for *B. infantis, B. breve* and *B. longum* (Proulx et al., 1994), while Rasic and Kurmann (1983) reported that extracts from potatoes, carrots and corn had an increased growth effect on bifidobacteria. Other reported growth factors include threonine, peptone, trypticase, dextrin, maltose and short chain fatty acids (Modler, 1994; Pacher and Kneifel, 1996). Yeast extracts were found to be effective growth promoters, and are generally added between 0.1 and 0.5% (v/v) (Gomes and Malcata, 1999). However, an earlier study showed that *B. infantis* did not exhibit enhanced growth when the medium was supplemented with 0.25% yeast extract, while there was more acid produced (Roy et al., 1990).

18.4.2 Lactobacilli

Lactobacilli require in general complex media containing a range of nutrients. A typical medium for *L. acidophilus* requires low oxygen tension, fermentable carbohydrate, proteins, vitamins, nucleic acid derivatives, unsaturated fatty acids and minerals such as magnesium, manganese and iron. It is reported that LAB prefer peptide bound amino acids rather than the free form (Benthin and Villadsen, 1996). By increasing the thiol groups in the media, using whey, an enriched milk flavor and increased growth of lactobacilli was obtained and furthermore, addition of peptone and trypsin promoted acid production (Kurmann, 1988). Growth can be inhibited by low pH during fermentation, and to prevent premature inhibition a buffer, such as phosphate buffer can be used to neutralize the acid production during fermentation. There is however, a limit to the amount of phosphate that can be added to the media given that high concentrations can be inhibitory because of the binding of metal ions, such as magnesium, calcium or manganese, which are essential for bacterial growth (Boyaval, 1989). Therefore, the phosphate concentration has to be adjusted to the strain used (Wright and Klaenhammer, 1983).

Experimental design was used to optimize the growth media for *L. acidophilus*, and optimal conditions were found to be pH 6.0, 30°C, 40 g/l glucose, 20 g/l peptone, 20 g/l yeast extract, 5 g/l sodium acetate and 3 g/l sodium citrate (Taillandier et al., 1996). Using response surface methodology, Liew et al. (2005) studied the combined effects of glucose, yeast extract, vitamins and pH on growth of *L. rhamnosus* ATCC7469. Optimal conditions included pH of 6.9, 1.28% vitamins, 5.0% glucose and 6.0% yeast extract. Using a similar approach, it was shown that by adjusting the inoculum size and by addition of the prebiotics, fructooligosaccharide and maltodextrin, growth and acid production of *L. casei* ASCC292 were increased (Liong and Shah, 2005).

Milk supplies the majority of the nutrients required for growth of lactobacilli. The composition of milk is typically, 87% water, 4.7% lactose, 3.8% fat, 33% protein 0.2% citrate, 0.6% minerals (Heller, 2001). Several studies have found that lactobacilli can grow up to 10^8–10^9 CFU/ml when grown in milk. Stationary phase is often reached after 24 h fermentation at 37°C, with pH between 3.9 and 4.4 (Gonzalez et al., 1993; Prajapati et al., 1987). There are several ways to improve growth of lactobacilli in milk, e.g., the milk can be supplemented with growth factors or if higher numbers are required after a certain time, the inoculum size can be increased. Supplements, such as manganese, acetate, fatty acids (e.g., oleic acid), tomato juice (Babu et al., 1992), casein powder (Miller and Puhan, 1981), whey protein (Marshall et al., 1982) or simple fermentable sugars (e.g., sucrose, fructose) (Srinivas et al., 1990) have been found to promote growth of lactobacilli. Furthermore, growth of *L. acidophilus* was optimized by adjusting skim milk media with 0.5% yeast extract and 1.0% glucose (Rana and Gandhi, 2000). Basal MRS media used for enumeration of *L. acidophilus* from yogurt can be optimized with supplements of maltose, salicin, raffinose or melibiose instead of dextrose (Hull and Roberts, 1984).

Although lactobacilli tolerate oxygen, they grow better with low oxygen concentration. Ascorbic acid was used by Dave and Shah (1998) as an oxygen scavenger and promoted growth and stability of *L. acidophilus*, while supplements of whey powder, whey protein concentrates and acid casein hydrolysates resulted in improved growth for *L. acidophilus* and bifidobacteria in yogurt.

18.4.3 Alternative Media

Probiotics are mainly grown in bovine milk, but studies have shown that they can also grow well in milk of other species, including camel (Abu-Taraboush et al., 1998), buffalo (Murad and Fathy, 1997) and goat (Gomes and Malcata, 1998).

However, Gomes and Malcata (1998) reported that *L. acidophilus* had insufficient growth in goat milk. Most of the research has been performed on bovine milk and that is the focus of this chapter.

Growth of lactobacilli and bifidobacteria is often slower in milk than in synthetic media. This is believed to be due to the low proteolytic activities of bifidobacteria (Dave and Shah, 1996; Klaver et al., 1993). Another factor affecting the growth of bifidobacteria negatively in milk is the generally low beta-galactosidase activity in bifidobacteria (Desjardins et al., 1991). The proteolytic activity can be increased by adding high proteolytic LAB to the media, however they should not outgrow the probiotic bacteria (Klaver et al., 1993). Similar to synthetic media, milk can be optimized by adding growth promoting supplements as mentioned earlier (Champagne et al., 2005; Elli et al., 1999).

Some efforts in making a media more suitable for probiotics have led to novel probiotic products based on tomato juice, peanut milk, soy milk, buffalo whey/soy milk and rice. Certain plant-extracts have been shown to benefit the growth of probiotics. For example, *L. acidophilus* was reported to grow significantly better in soy milk than bovine milk (Mital and Garg, 1992). This has also been observed in yogurt type products based on soymilk (Murti et al., 1993; Shelef et al., 1988). However, bifidobacteria do not grow well in soy milk (Macedo et al., 1998). Others have reported that certain lactobacilli can grow substantially in vegetable juices from cabbage and carrot (Savard et al., 2003).

18.4.4 Fermentation Methods

Batch or fed-batch fermentations are the preferred production processes in the dairy industry, since continuous fermentation requires costly concentration steps; however there are new developments in this fields which will be discussed later. With batch fermentation all the substrates and inoculum are mixed in a fermenter. The fermenter is temperature and pH controlled to fit the optimum growth conditions. When the desired probiotic concentration is reached, the process is stopped, the cells are harvested and the process is repeated. Depending on the quantities required batch fermenters can be as large as 10,000 L. Fed-batch fermentation allows the addition of a limiting substrate during the fermentation, and this technique is commonly used to increase bacterial concentrations. Producing probiotic cultures in fed-batch has the advantage that less exopolysaccharides are formed and thus the product is less viscous (Champagne et al., 2007). Fed-batch can also be applied to stress the bacteria at the end of fermentation to

induce a stress response (e.g., osmotic or solvent stress response) to protect them from subsequent processing steps.

Due to the difficulties in propagating a cell line on an industrial scale, starter cultures from manufactures in the form of direct vat set (DVS) cultures are preferred in the dairy industry. DVS cultures are highly concentrated freeze dried (approximately 1×10^{11} CFU/g) or deep-frozen (approximately 1×10^{10} CFU/g) cultures that can be used to directly inoculate the fermenter (Honer, 1995; Oberman and Libudzisz, 1998).

Low pH is one of the main reasons for the growth inhibition of LAB, and thus, by controlling the pH, higher biomass yields can be obtained. In batch processes, pH control is achieved by adjusting the pH with a base (e.g., ammonium or sodium hydroxide), or using a suitable buffer [e.g., N-Tris(hydroxymethyl) methyl-3-aminopropanesulfonic acid (TAPS) or phosphate buffer]. Another solution is to co-culture another microorganism that will counteract the produced acid by LAB. McCoy (1992) described a method where urease producing bacteria are co-cultured with LAB in a urea supplemented media. As both bacteria grow, urease hydrolyses urea to acid-neutralizing ammonia. Other techniques include the use of a saturated salt solution (e.g., $CaCO_3$) that gradually dissolves and neutralizes pH as the pH drops this technique is also suitable for controlling pH in agar plates. Up to 10 times more biomass can be obtained when pH control is used in fermentation. When bacteria are cultured with pH control, the specific acidification rates are lower than without pH control, which means that a higher inoculation volume or a longer fermentation time is needed (Savoie et al., 2007).

Besides traditional (fed) batch fermentation, there are other procedures available to produce high concentrated bacterial cultures. For example, Doleyres et al. (2004a) reviewed continuous fermentation and described the potential benefits. This technology could provide high cell yield and decrease the downstream processing for concentration as reported for *B. longum* ATCC15707 (Doleyres et al., 2002b). There is however, an increased contamination risk when applying this technology on an industrial scale (Lacroix and Yildirim, 2007). Taniguchi et al. (1987) reported seven times higher concentrations of *B. longum* when using a membrane bioreactor during fermentation. This reactor has a constant feed of fresh media, while the bacteria are kept in the reactor by an ultra- or microfiltration membrane. Hence, any growth inhibitory metabolites are removed from the system allowing more bacterial growth. Corre et al. (1992) also reported higher cell yield using a membrane reactor as opposed to free cell fermentation of *B. bifidum*. Schiraldi et al. (2003) reported increased cell concentration and metabolite production in a similar membrane reactor.

Another interesting development in fermentation is immobilizing the bacteria in the fermenter. Immobilized bacteria on fruit (apple and quince) were used to produce high quantities of food-grade lactic acid (Kourkoutas et al., 2005). The immobilized cells were used in subsequent batch fermentations with no significant loss in acid producing activity. Another form of immobilization can be achieved by embedding the bacteria in gel beads. This was achieved by entrapping the bacteria in spherical polymer beads with diameters ranging from 0.3 to 3.0 mm (Champagne et al., 1994; Lacroix, 2005). The active biomass is immobilized by thermal (κ-carrageenan, gellan, agarose, gelatine) or ionotropic (alginate, chitosan) gelation. Growth is observed in radial form in the beads, and this biofilm-like growth results in high cell release into the media, as a result from collision shearing forces in the reactor (Doleyres and Lacroix, 2005). Based on these techniques, several studies have shown high productivities of probiotic biomass. Ouellette et al. (1994) produced continuously fermented skim milk using immobilized *B. infantis* on κ-carrageenan/locust bean gum gel beads. Cell counts reached 2.2×10^9 CFU/ml and maximum volumetric productivity approximately 1×10^9 CFU/ml h. In another study, *B. longum* was immobilized on gellan gum gel beads (7×10^9 CFU/g) in MRS medium supplemented with whey permeate, which led to high cell production, ranging from 3.5 to 4.9×10^9 CFU/ml for *D* (Dilution rate) of 2–0.5 h^{-1} respectively (Doleyres et al., 2002b). This study also reported the highest volumetric productivity for *B. longum*, 6.9×10^9 CFU/ml/h. However, this high concentration was reached by using dilution rate of 2 h^{-1} which would lead to a cell wash-out.

In a two-stage fermentation, the effluent of one reactor flows into a second reactor. Two-stage fermentation can increase cell numbers and viability during downstream processing. Doleyres et al. (2004a) used a continuous two-stage fermentation to produce a high concentrated mixed culture (*Lactococcus lactis* subsp. *diacetylactis* and *B. longum*). In the first reactor, both strains were separately immobilized on κ-carrageenan/locust bean gum gel beads; the second reactor received free cells from the first reactor. This setup allowed for the continuous production of high concentrated cells, while the ratio of bacteria could be controlled by temperature (Doleyres et al., 2002a, 2004a). This production method also improved the stress tolerance in further downstream processing (Doleyres et al., 2004b).

A problem in growing probiotic cultures in industry is the contamination by bacteriophages, particularly when working with raw milk products, which are used in the cheese industry. Up to 1995, bifidobacteria were thought not to be perceptive to phages, but Ventura and co-workers found phage like elements in

Bifidobacterium strains (Tamime et al., 1995; Ventura et al., 2005). To prevent phage contamination two strategies are applied: (1) perform strain rotation; the disadvantage of this is that the new strain does not necessary have the same biological traits and health benefits, (2) addition of probiotics only at the final processing step; the disadvantage of this approach is that the inoculation rate has to be much higher, since there is no probiotic growth, and this would imply higher production costs. Therefore, phage resistance could be an extra preferable probiotic selection criterion (Mattila-Sandholm et al., 2002).

18.5 Drying Strategies

Before probiotics can be supplied to the market the bacteria need to be prepared for transport and storage. Live bacteria used in functional foods are generally stored and shipped in dried form, which is preferred over frozen form, because of the ease of long-term storage and shipping without the use of specialized refrigerated containers. However, the drying process is one of the main causes of loss in viability of probiotics. Spray and freeze drying are the two main forms of drying of probiotics. Other drying methods include vacuum drying, fluidized bed drying or a combination of drying techniques. These techniques have been extensively reviewed (Champagne et al., 1991; Meng et al., 2008; Santivarangkna et al., 2007) and this section will give an overview of the parameters involved in probiotic drying.

18.5.1 Freeze Drying

Freeze drying generally yields higher probiotic survival rates compared with spray drying (Santivarangkna et al., 2007). Freeze drying consists of three main steps, i.e.,: freezing, primary drying and secondary drying. In the freezing step, bacteria are typically frozen at −196°C in liquid nitrogen. Ice is then sublimated under high vacuum in the primary drying step by increasing the temperature. Sublimation is a phase transition, from solid to gas, that occurs at temperatures and pressures below the triple point of water. Approximately 95% of the free water is removed in this step. The hydrogen-bound water is then finally removed in the secondary drying step by desorption. Generally, drying is continued until the water contents drops below 4%, promoting long-term storage and spoilage prevention. Subsequently, the product temperature is raised to ambient

temperature. It has been reported that the temperature in the primary drying step should not be higher than the collapse temperature (T_c), which is the maximum temperature preventing the structure of the probiotics from microscopic collapse (Fonseca et al., 2004a). When collapse occurs, higher residual water content is observed, activity decreases, and it takes more time to reconstitute the powder (Fonseca et al., 2004b). Freeze drying probiotic bacteria on a commercial scale is an expensive process with low yields. The drying is batch-wise operated, which can be a limiting step if large quantities are required.

Most inactivation of the bacteria takes place in the freezing step. It has been reported that 60–70% of the cells that survive the initial freezing step will survive the dehydration step (To and Etzel, 1997). During the freezing step, extracellular ice forms, leading to a large osmotic pressure across the membrane, causing the cell to dehydrate. As the temperature drops, osmotic pressure increases and the cells dehydrate until an eutectic point is reached. Slow freezing causes more shrinkage and damage to the cell compared with fast freezing, because with slow freezing the extracellular ice is formed gradually which increases the time needed to reach the eutectic point thus allowing for more dehydration and shrinkage (Fowler and Toner, 2005; Zhao and Zhang, 2005). Baati et al. (2000) reported the opposite for *L. acidophilus*, where slow freezing and slow thawing actually increased the survival from 42 to 70%. It should be kept in mind that the damage done whilst thawing can be just as severe as freezing, since both actions apply similar stresses to the bacteria. Furthermore, there were differences in growth media, growth conditions and freezing solutions, which could explain the contradicting result (Champagne et al., 2005; Meng et al., 2008). In general, it is believed that fast freezing and slow thawing will result in the highest recovery after freeze drying. The larger the membrane surface area, the more damage is done during freeze drying, and for that reason damage during freeze-drying is higher for larger rod shaped lactobacilli than for small round enterococci (Fonseca et al., 2000). Wright and Klaenhammer (1981) also reported that the smaller bacilloid rods induced by calcium had higher survival rates than large elongated filamentous bacteria. The lipid fraction of the cell membrane is the most sensitive to damage during freeze drying. Furthermore, destabilization of RNA and DNA secondary structures results in reduced functionality of DNA replication, transcription and translation (van de Guchte et al., 2002).

A number of approaches have been used to improve the viability of bacteria during freeze drying [reviewed in Meng et al. (2008), Santivarangkna et al. (2008)]. Addition of protectants such as skim milk powder, whey protein, buttermilk, trehalose, glycerol, betaine, adonitol, sucrose, glucose, lactose, commercial

cryoprotectants (e.g., Unipectine, Satialgine) and polymers, such as dextran and polyethylene glycol (Burns et al., 2008; Capela et al., 2006) is a common way of improving stability during and after drying. The disaccharide trehalose has been reported to have a protective effect on viability during freeze drying in a number of studies. Miao et al. (2008) reported a 10% increase in viability when *L. rhamnosus* LGG was freeze dried in the presence of 15% trehalose. Similar results were reported by Zayed and Roos (2004) for freeze dried *L. salivarius* supplemented with trehalose.

18.5.2 Spray Drying

An alternative inexpensive continuous drying method with high yields is spray drying (Knorr, 1998; Zamora et al., 2006). During spray drying, the feed is pumped through a heated nozzle and is atomized into small droplets (10–200 μm) by using compressed air. The inlet nozzle temperature can reach up to 200°C. The droplets are sprayed into the drying chamber where, while falling through the chamber, co- or counter-current flowing hot air dries the droplets. The dried particles are then collected at the bottom of the chamber for further processing. The time needed for the particle to reach the bottom is referred to as "residence time." During the drying process, cells are exposed to extreme temperature, which can have detrimental effects on the integrity of the probiotic bacteria. Although the temperature is very high (130–200°C), the atomizing alone is unlikely to inactivate the cells. It has been reported that *Lc. lactis* endured no injury from shear forces or heating when atomized (Fu and Etzel, 1995). In addition, the cells undergo stress due to dehydration, oxygen exposure and osmotic pressure during spray drying (Brennan et al., 1986; Teixeira et al., 1997). Cell membrane damage has been reported to be a principal reason for cell inactivation during spray drying. Furthermore, spray dried cells were more sensitive to lysozyme, penicillin, and pyronin Y, which would indicate that besides membrane damage the cell wall, RNA and DNA are also affected (Abee and Wouters, 1999; Gardiner et al., 2000).

The outlet temperature has been inversely correlated with the survival of probiotics during spray drying. Desmond et al. (2002) showed that higher survivability during drying was achieved at lower outlet temperatures. However, lower outlet temperatures are associated with reduced residence time, leading to higher water content in the resultant powder. Water content should be below 4%, which is the maximum level required for prolonged storage, stability and spoilage prevention (Masters, 1985). Setting a correct outlet temperature alone is not

enough to achieve high viable numbers, and it is the combination of among others, outlet/inlet temperature, choice of probiotic strain and growth conditions that are important. *L. paracasei* NFBC 338 was shown to have 80% survival when spray dried in RSM at an outlet temperature of 85–90°C, and it survived significantly better than *L. salivarius* UCC118 at the same conditions (Gardiner et al., 2000). Furthermore, under similar conditions (80°C outlet temperature) *L. rhamnosus* GG survived at 60% during spray drying (Ananta and Knorr, 2003).

A number of bifidobacteria were screened for heat and oxygen tolerance, and these were subsequently spray dried, and their viability assessed during storage. It was reported that survivability was best for bacteria with high oxygen and heat tolerance. *Bifidobacterium animalis* subsp. *lactis* survived over 70% in RSM (20% w/v) at an outlet temperature of 85–90°C (Simpson et al., 2005). Furthermore, *Bifidobacterium* strains that had better heat and oxygen tolerance also exhibited better stability during storage (*Figure 18.1*).

The time of harvesting the cells has also been reported to have a major effect on the viability during drying and subsequent storage. It has been shown that stationary phase cells are more stable than cells harvested in exponential phase (Corcoran et al., 2004; Teixeira et al., 1995a). *L. rhamnosus* GG cells harvested at lag, log and stationary phase showed 2, 14 and 50% survival, respectively, after

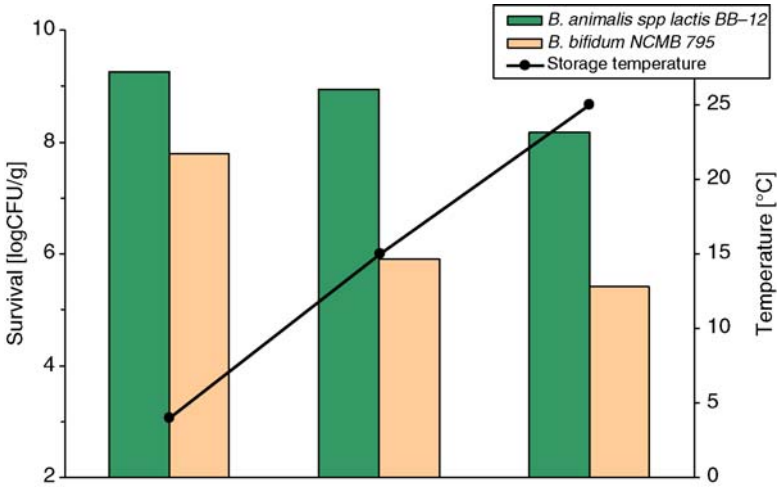

Figure 18.1

Compared storage survival of spray dried *B. animalis* subsp. *lactis* BB-12 and *B. bifidum* NCMB 795 for 30 days at three temperatures. BB-12 was more tolerant to oxygen and heat stress than *B. bifidum*. (adapted from Simpson et al. (2005)).

spray drying (Corcoran et al., 2004). Similarly, stationary phase harvested *L. delbrueckii* subsp. *bulgaricus* NCFB 1489 exhibited higher survival after spray drying compared with exponential phase harvested cells (Teixeira et al., 1995a). Prasad et al. (2003) reported that storage stability of *L. rhamnosus* HN001 was higher for stationary heat shocked bacteria than log-phase heat shocked bacteria. This could be due to certain stress mechanisms that are activated during stationary phase, and are similar to starvation conditions (e.g., glucose depletion) or acid stress.

The initial cell density can also influence cell activity after drying. Linders et al. (1998) correlated the initial cell density to the glucose fermentation activity of *L. plantarum* after spray drying. The ratio of activity before and after spray drying varied from 0.1 to 0.83 AU for initial cell concentrations of 0.025 and 0.23 g cell/g media, respectively. Therefore, a higher concentrated sample would lead to higher activity of cells after spray drying. Furthermore, it has been reported that higher initial cell concentration only marginally increased survival of *L. lactis* subsp. *lactis* after spray drying, allowing for more economical energy utilization and throughput (Fu and Etzel, 1995).

Encapsulation has proven to be an effective protection against stress endured during spray drying. By encapsulating live bacteria before drying, a protective barrier is formed around the cell, thus reducing the exposure to exterior stresses. Materials used for encapsulation include skim milk, potato starch, alginate, gum acacia, gelatine or casein. It was found that lactobacilli had increased heat tolerance when encapsulated in casein alginate beads (Selmer-Olsen et al., 1999). Lian et al. (2002) studied the survival of four encapsulated bifidobacteria in gum acacia, gelatine, and soluble starch after spray drying. Viability after spray drying was dependent on the material used for encapsulation. Besides encapsulating the bacteria in gels, spray coating the bacteria with a protective material is also an effective means of increasing viability during processing and subsequent storage. Spray-coated probiotic products survived better compared with untreated cells during their passage of the GIT, and released the biomass at predetermined sites (Siuta-Cruce and Goulet, 2001).

To further understand the processes controlling the viability during drying, genomics have been applied to investigate the role of certain genes. Desmond et al. (2004) reported that the overexpression of GroESL, a chaperone protein associated with stress response, resulted to increased heat tolerance of *L. paracasei*. Others reported that when overexpressing BetL, a betaine uptake system, the resistance to several stresses including osmo-, cryo-, baro- and chill-stress increased. Furthermore, the stability during freeze and spray drying

increased (Sheehan et al., 2006). These molecular tools are valuable for understanding the mechanisms behind stress resistance in probiotics.

18.5.3 Fluidized Bed and Vacuum Drying

Fluidized bed drying is a process where a flow of solid particles (bacteria) are dried by air that is blown through holes which causes the solid particles to be suspended and have fluid-like behavior. The particles are freely suspended in air and due to rapid heat exchange, the particles are dried (Santivarangkna et al., 2007). Operating costs of fluidized bed drying are equal to or lower than spray drying. Furthermore, the residence time can be easily extended, allowing for longer drying at lower temperatures, thus reducing the risk of heat inactivation. Yeasts have been successfully dried using fluidized bed drying (Bayrock and Ingledew, 1997a, b); it has also been applied to LAB. With fluidized bed drying, only granulated particles can be dried, therefore the bacteria must be encapsulated in support materials, such as skim milk, potato starch, alginate, or casein prior to drying. Furthermore, a fluidized bed can be used as a second drying step for the granulated particles produced during spray drying, allowing for a lower spray drying outlet temperature and higher survival of bacteria. In the fluidized bed, the powder is then dried to the desired moisture content under mild conditions.

Vacuum drying can be used to dry heat-sensitive materials, since water can be removed at low temperatures under vacuum. While freeze drying is also based on this principal, the difference is that with vacuum drying, temperatures can be kept as low as −2°C. Furthermore, since the drying takes please under vacuum, oxidation reactions can be minimized for oxygen sensitive bacteria. However, this technique has not been extensively studied for LAB. King and Su (1993) reported similar survival rates for *L. acidophilus* during freeze and vacuum drying. More recently, higher survival rates (18%) were reported, when *L. helveticus* was vacuum dried with 1% sorbitol at 43°C and 100 mbar for 12 h (Santivarangkna et al., 2006). A limitation of vacuum drying is the long drying times (10–100 h) compared with fluidized bed or spray drying, and the necessity for batch operation (Santivarangkna et al., 2007). However, this could be overcome by using continuous vacuum drying, which has been successfully applied to dehydrate materials to 1–4% moisture content in 5–10 min. Continuous vacuum dryers are available for large scale commercial product lines and are used to dry food additives, enzymes and pharmaceutical products (Hayashi et al., 1983).

18.6 Storage and Rehydration

18.6.1 Storage Conditions

The most important factors affecting survival during storage are storage temperature, oxygen, humidity and moisture content of the powder (Ananta et al., 2005; Desmond et al., 2002). High viability during processing is not correlated to high viability during storage, but the production process of probiotics does affect the storage survivability. In general, freeze dried bacteria have higher survivability when kept at low temperature and in an oxygen free environment. The survival of spray dried bacteria was also shown to be inversely correlated with storage temperature. Furthermore, by adding protective agents during growth, it has been reported that survival can be increased during storage. Selmer-Olsen et al. (1999) reported that optimal storage survival was reached when stationary phase *L. helveticus* culture was stored in non-fat milk solids or adonitol containing media. When *L. paracasei* was spray dried in milk based media containing gum acacia, over a 1000-fold increase in survival during storage for 4 weeks at 15 and 30°C, compared to milk based media alone was reported (Desmond et al., 2002). The viability of LAB during storage after freeze drying can be improved by adding supplements such as fructose, lactose, mannose, glucose or sorbitol to the growth media (Carvalho et al., 2004). However, the prebiotics inulin and polydextrose did not improve storage survival for several lactobacilli (Corcoran et al., 2004).

Oxygen can be lethal to strictly anaerobic bacteria, such as bifidobacteria. Membrane lipids can oxidize during storage, which changes the degree of unsaturated lipids, thus changing the passive permeability of the membrane leading to cell inactivation (In't Veld et al., 1992). Oxygen stress during storage can be minimized by removing any peroxide producing strains from the production process, and by adding antioxidants or free radical scavengers, such as ascorbic acid or monosodium glutamate to the media (Champagne et al., 2005). Packaging can also prevent oxygen from diffusing into the powders. Glass has been reported to be the best barrier against oxygen, while thick plastic or laminated pouches have also been used (Klaver et al., 1993; Wang et al., 2004). Furthermore, packaging under anaerobic conditions and purging material with inert gas (e.g., nitrogen) will limit the oxygen stress during storage for strict anaerobes.

Dried probiotics require different storage conditions than probiotics in liquid form. In the dairy industry, most of the products are liquid and have a shorter shelf-life than the dried products. Some bacteria used in the production

of yoghurts and milk based products have over-acidifying properties, which means that they acidify the product during storage. When making mixed cultures with probiotics, it is recommended to minimize over-acidifying bacteria, thereby increasing the chance of probiotic survival during storage (Kailasapathy and Rybka, 1997). Maintaining the moisture content at a certain level is crucial for the survivability of dried probiotics. It was reported that freeze dried bacteria have highest viability when relative vapor pressure (RVP) is below 11.4% during storage. At higher RVP, crystallization was observed which was detrimental to cell viability (Miao et al., 2008). In that study, disaccharides were used to improve storage stability. Similar results are reported elsewhere where the use of trehalose and lactose in combination with maltose improved survival during storage of dried bacteria (Zayed and Roos, 2004). Burns et al. (2008) reported the successful use of low cost buttermilk and whey for stabilizing probiotics during cryopreservation.

18.6.2 Rehydration

Dried probiotics require reconstitution before consumption and the reconstitution method can greatly affect the survival of the bacteria. The reconstitution process can be divided into four steps: wetting, submersion, dispersion and dissolving. Among these steps, wetting of the particles is very often the reconstitution controlling step. Other parameters such as powder quality, matrix properties (protective agents, wet-ability of powder, water activity (a_w), particle size), properties of the rehydration media (osmolarity, pH and energy source) and rehydration conditions (e.g., temperature of rehydration, (an)aerobic, duration of reconstitution and volume) may also significantly affect the rate of recovery to the viable state, and thus influence survival rates (Carvalho et al., 2003). The reconstitution media can have significant impact on the recovery of bacteria, with recovery varying up to 10-fold depending on the media used (Font de Valdez et al., 1985). Others reported that when milk was used as a drying matrix, the reconstitution solution had no significant effect on the recovery of dried bacteria (Teixeira et al., 1995a). It is postulated that the milk may have supplied all necessary nutrients to the cells, and thus masked the effect of any additional nutrients supplied with the different reconstitution media. When metabolizable sugars and salts were added to the media, improved recovery of freeze dried malolactic bacteria was observed (Zhao and Zhang, 2005). Similar increased viability was reported when injured *Serratia marcescens* and *Escherichia coli* were reconstituted in salt supplemented media (Wasserman et al., 1954;

Wasserman and Hopkins, 1957). Increased viability was observed when bacteria were rehydrated in solutions used for cryopreservation, providing a high osmotic pressure environment which could control the rate of rehydration, and thus avoid osmotic shock (Abadias et al., 2001). The rate of rehydration has been reported as an important factor for recovery of bacteria injured during the drying process (Kosanke et al., 1992; Leach and Scott, 1959). Two ways of controlling the rehydration rate are (1) by changing the amount of medium used for reconstitution and (2) by adding a protective matrix that lowers the a_w of the solution. Furthermore, particle size, porosity and wet-ability of the probiotic powder are factors impacting on the rate of rehydration. It is also suggested that the probiotics should be reconstituted at the optimal growth temperature of the bacteria. For example, freeze dried and spray dried bifidobacteria cultures that are reconstituted at 35–50°C and 20°C, respectively, showed increased survival compared with rehydration at 5–10°C (Wang et al., 2004). While pH is an important growth factor for LAB, little is known regarding the pH of reconstitution solution on the recovery of bacteria. Most reconstitution solutions used have a neutral pH using phosphate buffer. High recovery was reported for *Lactobacillus helveticus* within pH values of 6.0 to 7.0 (Selmer-Olsen et al., 1999).

Since the reconstitution conditions have a great influence on the recovery of dried probiotics and are strain specific, it should be optimized for each strain. Although most of the research on viability during storage and rehydration is concentrated on the ability of a probiotic to grow *in vitro*, it has been reported that non-culturable bacteria still retain a functional cell membrane typical of viable cells (Lahtinen et al., 2006). Therefore, it is desirable to use methods such as flow cytometry or real-time PCR in addition to plate counts to quantify viable or functional bacteria in products (Bovill and Mackey, 1997; Wai et al., 2000). Bio-assays are also useful tools to investigate the amount of active bacteria during and after reconstitution. Furthermore, the bacteria should retain their probiotic activity during and after storage. It has been reported that the cholesterol assimilation capabilities of *L. acidophilus* decreased significantly after storage for 21 days (Piston and Gilliland, 1994).

18.7 Cellular Stresses for Improving the Technological Properties of Probiotics

Improving the tolerance to environmental stresses by preconditioning of probiotics to a sublethal stress is a promising development for enhancing probiotic stability. For pathogens, such as *Salmonella typhimurium* and *Listeria*

monocytogenes, it has been shown that they survive a lethal pH when pre-exposed to a sublethal pH stress (Foster and Hall, 1990; Gahan et al., 1996). While undesirable for pathogens, this same adaptive mechanism has been the focus of many groups to improve survivability of probiotics during processing and subsequent storage (Desmond et al., 2001; Kullen and Klaenhammer, 1999; Walker et al., 1999). In general there are two types of defence mechanisms induced when bacteria are exposed to external stress. The first is a response to a sublethal stress, involving repair mechanisms, morphology changes or excretion of certain molecules from the cytoplasm, which will increase the tolerance to a homologous subsequent higher stress. The second is a more general mechanism, which protects bacteria to subsequent heterogeneous stress, also called cross tolerance (De Angelis and Gobbetti, 2004). One of these general stress responses can be observed when bacteria are exposed to heat, two chaperone protein complexes are formed, belonging to the 70 kDa DnaK and 60 kDa GroE families. These proteins are described as heat shock proteins (Hsp) but have also been associated with other stresses (Hartke et al., 1996). Chaperone proteins repair intracellular systems including refolding of polypeptides, assembly of protein complexes, degradation and translocation of proteins (Bukau and Horwich, 1998; De Angelis and Gobbetti, 2004).

Thermotolerance of lactobacilli was shown to increase by exposing bacteria to a sublethal heat shock followed by challenging the bacteria with a normally lethal heat shock (Desmond et al., 2001; Teixeira et al., 1994). Furthermore, it was reported that *L. paracasei* not only increased its thermotolerance in liquid media, but also showed 18-fold increased survival during spray drying compared to non-treated cells. Cross-protection was also studied; exposing the bacteria to sublethal levels of osmolarity and hydrogen peroxide increased the heat tolerance and spray drying stability of the bacteria, although to a lesser extent than heat-adaptation. In general, it has been shown that by pre-conditioning bacteria with a homologous sublethal stress its tolerance increases more than a heterogeneous stress. Similar results were reported for *Lc. lactis* subsp. *lactis,* which showed increased tolerance to a lethal heat treatment when pretreated with a sublethal heat shock compared to untreated cells (Boutibonnes et al., 1992). The treated cells also showed an increase of stress response proteins. However, pre-treatment of the cells with antibiotics that act on translation also induced the stress response proteins to be synthesized, but no extra heat tolerance was observed (Boutibonnes et al., 1992). This would indicate that the increased tolerance is attributed to factors other than synthesis of stress proteins. Desmond et al. (2004) showed that an overexpressing GroEL mutant of *L. paracasei* showed more heat resistance than the wild type, but less than a heat-adapted strain.

The heat shock induced production of GroESL chaperone complex has also been associated with better survival during freezing of *L. johnsonii* (Walker et al., 1999). Besides the upregulation of chaperone proteins following heat stress, it has been reported that membrane fluidity changed, and fatty acids were more saturated and elongated (Russell and Fukunaga, 1990).

Oxygen stress during the processing of probiotics is detrimental to strict anaerobes, such as bifidobacteria. Oxygen stress induced the protein Osp in an oxygen tolerant *B. longum* and changed membrane composition and morphology. The cellular fatty acids consisted of increased short chain fatty acids and cyclopropane fatty acid when compared with an oxygen intolerant *Bifidobacterium* (Ahn et al., 2001). Further understanding of these oxygen induced changes could increase bifidobacteria survival during processing. Furthermore, it is suggested that *L. acidophilus* adapts to hydrogen peroxide during yoghurt fermentation leading to improved storage survival (Hull et al., 1984). Storage stability of *L. rhamnosus* at 30°C was also increased when bacteria where pre-adapted with sublethal levels of heat and salt (Prasad et al., 2003).

High hydrostatic pressure is used as an alternative to heat treatment in the preservation of foods. High pressure causes protein denaturation and loss in membrane integrity leading to decrease in microbial activity (De Angelis and Gobbetti, 2004). This stress mechanism can be applied to induce a stress response. When 100MPa was applied to *L. rhamnosus* for 5–10 min, the survival was increased when exposed to 60°C compared to non-treated bacteria (Ananta and Knorr, 2003).

The acid tolerance of *L. acidophilus* was successfully increased by acid adaptation, increasing the viability of the *Lactobacillus* when exposed to normally lethal acidic media and yoghurt (Shah, 2000). Kullen and Klaenhammer (1999) have shown that several genes are up-regulated when *L. acidophilus* was pH challenged, including F_1F_0-ATPase, which is involved in the mechanism to stabilize the intracellular pH by removing excessive protons from the cytoplasm. Most of the research done on stress tolerance was in log phase cells, since these cells are most active and effects of external stress can be monitored better than stationary cells, which are more stable (Péter and Reichart, 2001). This late stationary phase induced stability may be due to starvation and stress response to low acidic conditions. When starvation induced stress response in *Lc. lactis* subsp. *lactis* was studied, the starved cells showed improved resistance to heat, ethanol, acid, osmotic, and oxidative challenges (Hartke et al., 1994).

Saarela et al. (2004) found improved acid resistance when lactobacilli and bifidobacteria were exposed to sublethal heat shock in stationary phase on a laboratory scale. However, when the same procedure was performed at pilot scale,

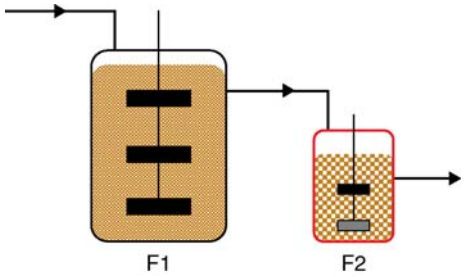

Figure 18.2
Two stage continuous fermentation setup to produce pre-conditioned bacteria with increased tolerance for downstream processing and subsequent storage. The setup consists of 2 (F1 and F2) fermenters, fermenter 1 (F1) produces a steady high concentration flow of bacteria. The effluent of F1 streams in a smaller fermenter (F2), where an external stress can be applied (e.g. heat, osmotic, acid or pressure). Since F2 is smaller, the residence time can be adjusted to provide the correct time for the stress response to be induced.

only lactobacilli showed improved resistance, indicating that up-scaling of the stress response is not straight forward. Similar cross tolerance has been reported for *L. acidophilus*. Log phase harvested cells showed increased survival when challenged with a lethal exposure to bile, NaCl or heat shock; if they were pre-conditioned with a sublethal dose of the same stress. Cross protection was also observed for different stresses tested (NaCl, heat and bile), indicating that a general defence mechanism was induced by the sub-lethal stress. In contrast to log-phase cells, stationary phase cells were inherently resistant to stress (Kim et al., 2001).

While it is not feasible to apply a heat or cold stress before drying probiotics using spray or freeze drying, respectively, since fermentation vessels are too large, there are certain fermentation set-ups that could resolve up-scaling issues. For example, a two-stage continuous fermentation can be used to induce a stress response. The first reactor produces a steady flow of high concentrated probiotics flowing into a smaller second reactor, with shorter residence time, where the stress is applied (Lacroix and Yildirim, 2007). Such developments can assist in decreasing losses during processing (*Figure 18.2*).

18.8 Summary

- The increasing demand for probiotics and the new food markets where probiotics are introduced, challenges the industry to produce high quantities of probiotic cultures in viable and stable form.

- Growth conditions have a large effect on the survivability and activity of probiotics during and after processing.
- Storage conditions, such as storage temperature, relative vapor pressure, oxygen and moisture content are very important factors to assure viable probiotics to the end of shelf-life.
- Dried probiotics are the preferred form for the ease of storage and transportation. Freeze and spray drying are the most applied drying techniques in industry.
- Reconstitution conditions such as temperature, rate of rehydration and osmolarity of the solution are vital parameters to assure resuscitation of the bacteria.
- Pre-conditioning bacteria with a sub-lethal stress can induce stress responses which can increase tolerance to subsequent stresses. This technique can be applied to increase survival during production and processing of probiotics.
- Genomics can provide insight into survival mechanisms involved during production, drying and storage of bacterial cultures, leading to the development of more efficacious probiotic products.

List of Abbreviations

a_w	Water activity
AU	Activity Unit
B.	*Bifidobacterium*
CFU	Colony Forming Units
DVS	Direct Vat Set
FAO	Food and Agriculture Organization
FOS	Fructoolisaccharides
GIT	Gastro Intestinal Tract
GRAS	Generally Regarded as Safe
L.	*Lactobacillus*
LAB	Lactic Acid Bacteria
Lc.	*Lactococcus*
MRS	de Man Rogosa Sharpe media
RCM	Reinforced Clostridium Media
RSM	Reconstituted Skimmed Milk
RVP	Relative Vapor Pressure
subsp.	subspecies
WHO	World Health Organization

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19 Some Technological Challenges in the Addition of Probiotic Bacteria to Foods

Claude P. Champagne

19.1 Introduction

In North-America, up to 93% of consumers believe certain foods have health benefits that may reduce the risk of disease (Clydesdale, 2005). Using a strict definition, limited to food and drinks that tend to make specific health claims of some kind on the packaging or in advertising, the functional foods (FF) and drinks market in the five major European markets, the USA, Japan and Australia had a combined value of 16 billion USD in 2005 (Leatherhead Food International, 2006). Dairy products account for nearly 43% of this market, which is almost entirely made up of fermented dairy products (Leatherhead Food International, 2006). Probiotics are the main bioactive component of these fermented FF and numerous economic indicators show that probiotic-enriched products are still on the forefront of innovation in the FF sector:

1. There were 523 new stock keeping units registered globally in 2007 in the probiotic foods and beverage sector (Heller, 2008).
2. The probiotic yogurt market in Latin America grew 32% annually from 2005 to 2007 (Crowley, 2008).
3. The FF market in the USA is worth 21 billion $ in 2006 and probiotic drinks and yoghurt lead the category; annual sales growth were of 6.5% between 2001 and 2006, and are expected to be at 5% between 2006 and 2011 (Anonymous, 2007).
4. In the overall USA FF market, in 2004, fresh dairy products grew by 9–10% compared to 2% for cheese (Fletcher, 2006).
5. The European food and beverage probiotics is expected to rise from its 2006 position of 62 million $ to 163 million $ by 2013 (McNally, 2007).

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6. The global retail market for probiotic dietary supplements for humans, evaluated at 1 billion $ in 2005, grew 47% between 2002 and 2005 and 32% growth expected through 2010 (Halliday, 2007).

Although supplements still enjoy strong market increases, consumers tend to prefer FF over supplements such as caplets, commonly called "nutraceuticals", as a source of health-promoting ingredients. The various yoghurt and dairy drinks clearly lead the probiotic FF market. Therefore, in examining the technological challenges in developing probiotic-based FF, the focus must be made on fermented milks. In accordance, much of this chapter will address the specific challenges linked to adding probiotics to yoghurt. This specific aspect has been covered recently (Champagne, 2009), and this chapter will therefore expand to non-dairy. In this area, soy, cereal and fruit juices constitute the trend.

A wide-ranging study of yoghurt production with 14 commercial cultures revealed that counts in probiotic bacteria could vary between 4.0×10^5 and 7.7×10^8 in fresh products (Kneifel et al., 1993). These data point to a thousand-fold variation in probiotic content, as a function of strain and production practices. The major impact of technology of probiotic bacteria constitutes a strong rationale for this chapter.

From a technological standpoint, there are many challenges in the development of a probiotic-containing food product:

1. Selection of the strain (s)
2. Preparation of the populations to add (inoculation)
3. Enable growth and/or survival during processing
4. Enable viability and functionality during storage
5. Assess the viable counts of the probiotic strain (s), particularly when multiple probiotic strains are added and when there are also starter cultures added
6. Manage the effects on sensory properties

In this chapter, the focus will be given three of these challenges: inoculation, processing and storage issues.

19.2 Inoculation of Probiotics into Foods

With the exception of very large food processors who possess their own strains, such as Danone or Nestle, food processors purchase their probiotic cultures from

specialized suppliers. Two commercial formats are mainly available: frozen pellets and freeze-dried powders.

From a technological perspective, the first point to consider is therefore the storage conditions of the unopened commercial products. It is recommended to store frozen cultures at –40°C or below. Consumer freezers which typically maintain –20°C are inappropriate for extended storage of the frozen pellets. When thawed, they must be used very rapidly. Freeze-dried cultures can be stored at higher temperatures, and a refrigerator at 4°C is considered adequate. In such conditions, probiotic and lactic cultures in practice will loose approximately 0.2 Log in viable counts over 1 year (Champagne et al., 1996). At 22°C, mortality rates are 10 times higher.

Two methods of inoculating probiotic cultures into foods and beverages exist: (1) direct vat inoculation (DVI) to the food matrix, from concentrated frozen or dried cultures and (2) liquid cultures prepared in bulk starter tanks. In bulk starter preparation, a growth medium, which typically contains dairy ingredients as well as minerals and peptones, is inoculated with a relatively small amount of cells, and the medium is incubated in appropriate temperature and time parameters to propagate the bacteria. This bulk starter is then typically added at 1–2% (v/v) of the processing milk. In large cheese and yoghurt plants, bulk starters are still frequently prepared. This is because preparing one's own bulk starter is approximately 20% less expensive than using DVI starter cultures. Nevertheless, in dairy factories as well as in most other food applications, when it comes to probiotics, DVI is generally practised. Various reasons explain this situation:

1. Immediate acidifying activity is not required. At identical population levels, fresh liquid starter cultures have shorter lag times before acidification initiates, which reduce processing time. Probiotic bacteria are not required to contribute extensively to acidification, and there is no need for immediate high specific acidifying activity.
2. Specific probiotic population levels are required and stated on the label, and this is easier to standardize with DVI.
3. No need to invest in specific tanks of probiotics. Although one could attempt to grow the probiotics in the same tank as the bulk starter, it is unfortunately difficult to obtain at high probiotic population levels in mixed cultures, and to control starter:probiotic ratios. Preparation of probiotic bulk cultures would probably require the dairy plant to add fermentation systems specifically for the probiotic bacteria.
4. Probiotic bacteria are more fastidious organisms to grow than starter cultures.

5. No need for in-plant quality control of the probiotic concentrates.
6. Greater flexibility of DVI with respect to the moment of inoculation.
7. DVI, particularly with powders, raises the concern of even distribution of the cells within the food matrix. Even distribution of the probiotics is not as critical to the quality of the fermentation as it is with starter cultures. As long as visible powder particles do not appear in the product and that the colony-forming units (CFU)/portion counts respect the claim on the label, there is room for some heterogeneity.

DVI can be carried out by simply opening the sealed packaging and adding the frozen or dried culture to the food matrix. Although it is the easiest method of inoculation, if done inappropriately it can lead to substantial losses in viability. Indeed, how a culture is thawed or hydrated can result in a tenfold variation in CFU.

19.2.1 Inoculation with Frozen Cultures

Although the use of frozen probiotics is quite common in the food industry, there is surprisingly little data on the conditions of thawing for inoculation in food matrices. When compared to freeze-dried cultures, the use of frozen probiotics is disadvantageous with respect to storage time, storage temperature and ease of sampling. When the food matrix is milk or a beverage, the frozen culture can be directly added to the liquid matrix. Unfortunately, little data are available on the effect of the medium in which the probiotics are thawed. However, there appears to be fewer problems in viability losses when they are added to the food matrix as frozen cultures than when they are dried. The cell membrane injuries to lactic acid bacteria are less pronounced in a freeze-thaw cycle than during freeze-drying (Liu and Luo, 2002). Furthermore, production parameters of the cultures can be adapted so as to enhance survival to freeze-thaw cycles (KiBeom, 2004). As a result, the ability of probiotics to survive environmental stresses does not seem highly affected by a freeze-thaw stress. Thus, lactobacilli do hot have increased sensitivity to bile salts or very low pH values following a freeze-thaw process (Alamprese et al., 2005; Fernandez-Murga et al., 2001). This would suggest that the pH of the medium in which the culture is thawed would not have a strong effect on the resulting viable count. The chemical composition of the medium can affect the subsequent lactic acid fermentation of carbohydrates by frozen-thawed

Lactobacillus plantarum. Thus, acidification was stimulated by the presence of Mn^{2+} in the medium (Raccach and Marshall, 1985). Lactic acid synthesizing capacity of *L. casei* was higher when the culture was thawed at 20°C than when thawed at 10, 30 or 40°C (Kim et al., 1993). With mesophilic lactic starters, rapid warming in a water bath at 20–45°C was better than slow thawing at 4°C (Shurda, 1980). These data suggest that thawing at a temperature close to the optimum growth temperature would be recommendable. For optimum acidifying activity, it is recommended that thawed concentrates of mesophilic starter cultures should be activated in sterile skim-milk for 1.5 h at 30°C before addition to the cheese vat (Libudzisz et al., 1977). Since a strong immediate acidifying activity is not required of the probiotic bacteria such an adaptation step might not be required. In summary, thawing temperature needs to be selected, but few other thawing parameters seem to require specific adjustments. This makes inoculation with frozen cultures rather easy and few mistakes can be made.

19.2.2 Inoculation with Freeze-Dried Cultures

A very different picture emerges with the freeze-dried cultures. Although dried cultures are much easier to ship and store than frozen ones, their use in the food processing plant is more difficult. Four parameters influence CFU counts following addition of a powder in a food matrix: the nature of the food or beverage matrix, rehydration temperature, powder-to-liquid ratios and rehydration time. With respect to the matrix, the following elements must be considered:

1. pH. It is generally best for cells to rehydrate in the 6.0–7.0 pH zone. Thus, direct addition of powder to unfermented milk (pH 6.7) would result in a higher survival level than if the powder were added in a fermented milk (pH 4.2) or a fruit juice (typically pH 3.2–3.8).
2. Salt, sugar or amino acid composition. Various studies show that the nutrient composition of the medium affects the CFU counts (De Valdez et al., 1985a; Sinha et al., 1982). Milk is better than sugar, salt or glutamate solutions (Sinha et al., 1982).

When direct addition of the dried powder in the medium results in viability losses, it might be recommendable to carry out a specific rehydration step, and subsequently blend the rehydrated cell suspension to the food.

The rehydration temperature is also critical in the inoculation process. One would assume that rehydrating at 4°C would be ideal but this is not the case. Data from various studies (De Valdez et al., 1985b; Mille et al., 2004; Sinha et al., 1982) do not point to a single "ideal" rehydration temperature. Temperatures between 30 and 37°C seem best for post hydration viabilities with thermophilic cultures, while the 22–30°C seem advisable for mesophilic bacteria. Care must be taken, however, not to go above 40°C (for mesophilic) or 45°C (for thermophilic), because this may result in denaturation of enzymes with subsequent viability losses. In the case of yoghurt-type production, where processing milk (or soy beverage) is adjusted between 37 and 45°C for the fermentation, DVI right into the processing liquid is appropriate from the perspective of temperature. In fruit juices and unpasteurized milk, which are kept cool as much as possible, a specific hydration step in a warm medium seems advisable. Osmotic choc is known to affect the viability of bacteria. Thus, it is not surprising that the ratio of powder to liquid affects post hydration CFU values. It was shown that viable counts of various probiotic and lactic cultures are higher when the powder is added into a small amount of water. A powder-to-liquid ratio of approximately 1:3 gave CFU counts between 4 and 10 times higher than at a 1:50 ratio (De Valdez et al., 1985b). When concentrated cell suspensions are needed, providing hydration conditions where milk solids are approximately 20% seems recommendable (taking into account solids in the medium and those brought by the culture itself). This would suggest that rehydration in processing milk would not be ideal, but the fact that yoghurt processing milk contains above 10% milk solids may prevent extensive problems. A last point is rehydration time. One could assume that extending the time of hydration prior to inoculation into the processing milk would result in growth initiation and higher populations in the rehydrated cell suspension. Data from De Valdez et al. (1985b) suggest that, at least when concentrated cell suspensions are involved, viability losses occur after 30 min or rehydration time. One hypothesis is that, in very high cell densities ($>10^{10}$ CFU/mL), acidification of the medium could be detrimental to viability. Presumably this is not the case when cells are in a more diluted state in foods, which is typically in the 10^7–10^8 CFU/g range.

Whatever the reasons for viability losses due to rehydration temperature, medium, solids levels or time, these data point to the need to standardize the rehydration-inoculation procedure in DVI. To avoid variability in viable counts, it seems wise to define appropriate procedures and try to keep them constant. One must also keep in mind that there are wide differences between species of

probiotic bacteria as well as between strains of the same species with respect to behavior during rehydration. It could well be found that conditions favorable to one strain may not apply to a different culture.

Finally, caution must be exercised with respect to opened packages (Champagne and Møllgaard, 2008). With DVI, inoculation requirements are often such that only a fraction of a culture package is needed. Therefore, situations occur where a commercial product is opened, the required amount is taken and the rest is stored for ulterior use. With a pellet-based frozen culture, the container must be immediately placed back in the freezer when the required amount of cell pellets has been taken. A thawed product cannot be re-frozen with satisfactory results. With dried products, the powder will absorb moisture from the air, which increases the water activity (a_w) in the product. An increase in a_w of the powder is highly detrimental to the stability of the cultures. Thus, an increase from 0.1 to 0.3 in a_w in a milk-based powder will result in only a 2% increase in moisture, but generate a tenfold decrease in storage stability (Ishibashi et al., 1985). Therefore, when a fraction of a package containing a freeze-dried powder is taken, the sachet must be closed as rapidly as possible. The "best-before" date becomes voided. In all aspects of storing and handling of commercial probiotic cultures, the "golden rule" is to follow the manufacturers' instructions.

19.2.3 Preparing Bulk Probiotic Cultures

Although DVS inoculation has many advantages, it is more expensive. As the market grows and the quantity of probiotic cultures added increases, economic considerations might tempt food processors to prepare bulk probiotic cultures. Two strategies are available: preparing pure cultures of probiotic bacteria or mixing them with the traditional starter.

The most widely used technique for the preparation of cultures is probably fermentation without pH control. Examples of pure cultures grown on soy or milk appear in *Table 19.1*. In unsupplemented milks or soy beverages the population gets above the 10^8 CFU/mL threshold, but remains under a billion CFU/mL. In order to obtain populations above 10^9 CFU/mL, growth factors must be added. If one wishes this growth supplement to be of dairy origin, casein hydrolysates seem the best choice. If non-dairy ingredients are permitted, then adding yeast, soy or fruit extracts are possible. Various peptones are also available. For high population of pure cultures, it is best to carry out a fermentation

Table 19.1

Effect of fermentation time, probiotic or streptococci cultures as well as strain pairing on the viable counts of fermented milks (Champagne et al., 2009)

Strains		Initial (CFU/mL)	Milk		Soy	
			Fermented (CFU/mL)	Fermentation time (h)	Fermented (CFU/mL)	Fermentation time (h)
Pure R0083	*S. thermophilus*	2.0×10^7	1.2×10^9	8	1.1×10^9	8
Pure ST5	*S. thermophilus*	2.3×10^7	8.7×10^8	24	8.5×10^8	24
Pure R0175	*B. longum*	2.3×10^7	3.6×10^8	24	4.7×10^8	24
Pure R0052	*L. helveticus*	1.9×10^7	2.8×10^8	24	2.8×10^8	24
Mixed R0083 + R0175	*S. thermophilus*	1.0×10^7	1.1×10^9	8	4.9×10^8	8
	B. longum	9.1×10^6	5.4×10^7	8	1.0×10^7	8
	Ratio St/Bl	1	20		49	
Mixed ST5 + R0175	*S. thermophilus*	9.6×10^6	7.4×10^8	20	1.0×10^9	20
	B. longum	9.1×10^6	6.8×10^7	20	4.7×10^7	20
	Ratio St/Bl	1	11		21	
Mixed R0083 + R0052	*S. thermophilus*	1.0×10^7	1.4×10^9	8	7.1×10^8	8
	L. helveticus	1.2×10^7	2.6×10^8	8	7.4×10^7	8
	Ratio St/Lh	1	5		10	

with external pH control. When the growth medium is appropriate, under external pH control a bifidobacteria population of 10^{10} CFU/mL can be achieved (Misra and Kuila, 1991).

Preparing a mixed starter-probiotic inoculum (in order to save on the number of bulk starter tanks) is more of a challenge. Presumably, this could be achieved in enriched growth media. With thermophilic starters, the incubation temperature is not a problem (Ostlie et al., 2005). However, with cheese starters, which are prepared in the 20–32°C range, this is another situation. Obviously, this would limit the choice of probiotics to *L. rhamnosus*, *L. casei* and *L. plantarum* strains. To enable high content of probiotic lactobacilli in a cheese starter, it was found preferable to use a whey-based medium with an extended zone pH control, and carry out the fermentation at the high end of the incubation temperature range (› *Table 19.2*).

Table 19.2

The effect of some growth parameters on the properties of the mixed starters composed of *Lactococcus lactis* subsp. *lactis* Rosell-type I, *Lactococcus lactis* subsp. *cremoris* Rosell-type II (*L. cremoris*), *Lactobacillus rhamnosus* RW-9595M, and *Leuconostoc mesenteroides* subsp. *cremoris* CAF-500 (modified from Savoie et al., 2007)

Starter property	Production parameter				
	Growth medium			pH control process	
	Milk or whey-based	Phosphate or citrate buffer	Temperature 22°C or 32°C	With or without	Zone type
Total population	NS[a]	NS	NS	With > without	6.0–5.8 > 6.0–5.2
% *L. cremoris*	Milk > whey-based	NS	22 > 32	NS	NS
% *Lactobacillus*	Milk < whey-based	Phosphate > citrate	22 < 32	NS	6.0–5.8 < 6.0–5.2
% *Leuconostoc*	Milk > whey-based	NS	22 > 32	With < without	NS
SAA[b]	NS	NS	NS	With < without	NS
IR[c]	NS	NS	NS	With <without	6.0–5.8 < 6.0–5.2

Parameters which could increase the starter content in probiotic candidate *Lb. rhamnosus* RW-9595M are those identified by % *Lactobacillus*

[a]*NS* Not statistically significant

[b]*SAA* Specific acidifying activity

[c]*IR* Inoculation rate

19.3 Growth in the Food Matrix

The most important market applications of probiotics in FF are yoghurt and fermented milks. Therefore, the challenges the probiotics face in these applications warrant a special examination. There are two types of probiotic-containing fermented products: those which involve starter cultures, such as yoghurt or cheese, and products where the probiotics are the sole microbiota. In both cases the ability of the probiotic to multiply in the food matrix will influence their final population. In yoghurt or cheese-type products, there is the additional concern of interaction with the starter cultures.

Dairy products are the main fermented products containing probiotic bacteria. Therefore, the ability to grow in milk will be addressed first. However, there are numerous novel applications appearing on the market, principally based on soy, cereal or vegetable fermentations. These substrates will also be examined.

19.3.1 Milk

Probiotic cultures can grow in milks of many species (Abu-Taraboush et al., 1998; Gomes and Malcata, 1998; Murad et al., 1997) but, most of the time, rather slowly (Klaver et al., 1993). Growth of probiotic bacteria in milk is very much strain-related (Klaver et al., 1993; Saxelin et al., 1999), but this phenomenon is not limited to probiotic bacteria. The same is observed with yoghurt (Shihata and Shah, 2002) and cheese (Heap and Richardson, 1985) starter cultures. Therefore, no clear conclusion can be reached as to which milk source is better suited for the growth of probiotic cultures. Most studies found in the literature were carried on cow milk and, unless otherwise stated, the term "milk" will refer to cow milk.

As is the case for some cheese starter cultures (Lawrence et al., 1976; Sinha, 1990), the slow growth of bifidobacteria and *Lactobacillus rhamnosus* in milk appears partially due to low lactase levels (Desjardins and Roy, 1991; Gaudreau et al., 2005).

Variations of growth rates are also function of changes which occur during the storage of milk. Milk is generally collected at the farm within 48 h, but it can be stored at the processing plant for up to 3 additional days. Such extended storage occurs when the plant has limited processing activities during the weekends. There are chemical and microbiological differences between fresh

milk and one which has been stored for 5 days, even at 4°C. These milks tend to have high counts in psychrotrophic bacteria. This microbiota will often possess substantial proteolytic and lipolytic activities. A high level of proteolysis will stimulate the growth of probiotic bacteria (Srivinas et al., 1997). Little is known, however, on the effect of lipolysis or on the fat metabolism of bifidobacteria (Tamime et al., 1995). Some data suggest that the presence of fat slightly improves milk fermentation and cell populations (Elli et al., 1999; Lanciotti et al., 2004). Fatty acids are growth factors at low levels (Partanen et al., 2001). Unless lipolysis has occurred, there are only small amounts of free fatty acids in milk (Lawrence et al., 1976). Therefore, very limited lipolysis might be stimulatory, but extensive lipolysis has the opposite effect. It cannot be recommended to use old milks because off flavors and texture problems occur in the yoghurt. However, one must consider the "age" of milk as a parameter which will affect the development of the cultures.

It must be stated, however, that the low growth rates of some probiotic bacteria in milk might erroneously be attributed to the nutritional aspects of milk. The redox level could be the limiting factor for some cultures. This is particularly the case with bifidobacteria, where deaeration favored the growth of many strains (Klaver et al., 1993). How milk is prepared is therefore critical to the growth of starter and probiotic bacteria.

The preparation of the milk for yoghurt production varies by two main parameters: solids level as well as the heating/homogenization process. Typically, the milk blend used for yoghurt manufacture will have between 10 and 15% non-fat milk solids, be heated between 80 and 95°C for 1–30 min, and homogenized at 50–70°C at pressures of 100–200 kg/cm^2 (Tamime and Robinson, 1985). This is much different from typical cheeses, where milk is not concentrated, heated at only 73°C for 15 s (pasteurization) and is not homogenized. All these parameters affect the growth of lactic cultures. In many cases, growth of lactic starter cultures is better in heated milks (Mortazavian et al., 2006) because antibacterial components are destroyed, oxygen is removed and a lower redox level is reached, limited proteolysis occurs and formic acid is produced. Little is known on the effect of milk heating on the growth of probiotic bacteria, but it can be hypothesized that trends would be similar to those observed for starter cultures. When the growth of yoghurt cultures was examined on milks heated at 85°C for 15 min or at 95°C for 5 min, the streptococci preferred the lesser-heated milk, while lactobacilli developed much better in milk previously heated to 95°C for 5 min (Kurultay et al., 2006).

Non-fat milk solids (NFMS) influence the numbers of cells in the yoghurt products as well as strain ratios (Al-Dabbagh and Allan, 1989; Alvarez et al., 1998; Torriani et al., 1996). The effect of NFMS is obviously linked to the quantity of nutrients but also to buffering ability. Therefore, increases of NFMS from 8.5 to 12–14% generally enhance viable counts in the fermented products. However, high NFMS increase the osmotic conditions which become detrimental to growth (Al-Dabbagh and Allan, 1989). Lowering of milk water activity (a_w) with sugar favors the cocci (Larsen and Añón, 1989; Shah and Ravula, 2000). Little is known unfortunately of the effect of milk solids on the ratios of probiotic bacteria in yoghurt cultures. When sugar is added to the blend, *L. acidophilus* and bifidobacteria had 1 log reductions in populations in the yoghurts with 12% sucrose, as compared to yoghurts supplemented with 8% (Shah and Ravula, 2000). However, the probiotic bacteria were similarly affected by the addition of sucrose at concentrations above 8% as were the starter cultures. If the probiotic lactobacilli do indeed react similarly as *Lactobacillus delbrueckii* subsp. *bulgaricus* (*L. bulgaricus*), then adjustments of milk solids above 14% might not be advisable.

In summary, many parameters in the formulation of the milk blend will affect the development of probiotics in yoghurt (› *Table 19.3*). This means that standardization is required in order to have reproducible results.

Table 19.3

Parameters which affect the growth of probiotic bacteria in yoghurt production

Milk blend	Fermentation	Storage
• Animal source • Pre-processing storage time of raw milk • Non-fat solids • Fat content • Growth supplements • Sugar level • Flavors and fruits • Preservatives • Heating parameters • Redox level	• Compatible starter • Form of starter or probiotic (liquid, DVI) • If dried DVI, rehydration parameters (solids, temperature, time) • Inoculation level of starter or probiotic (CFU/mL) • Moment of inoculation of probiotic • Fermentation temperature • Fermentation time	• pH (plain yoghurt and after fruit addition) • Moment of inoculation of the probiotic • *L. bulgaricus* content and activity (H_2O_2, overacidification) • Redox level. Addition of antioxidants? • Packaging, particularly with respect to oxygen permeability • Encapsulation

19.3.2 Soy

A feature of soy fermentation by probiotics is the strain-linked variability of the acidification rate. Thus, lactobacilli (Stern et al., 1977) or bifidobacteria (Garro et al., 2001; Scalabrini et al., 1998; Tsangalis and Shah, 2004; Wang et al., 2002), revealed sharp differences between strains in the rate of acid production on carbohydrates found in soy. Milk mostly contains lactose, while soy beverages contain, by order of importance, sucrose, stachyose, raffinose, glucose and fructose. Therefore, to grow in milk, the ability to use lactose is critical, but cultures have a variety of options in soy substrates. The lactobacilli and streptococci mainly use sucrose, glucose and fructose, while the bifidobacteria tend to use stachyose and raffinose (Champagne et al., 2009). The ability of bifidobacteria to assimilate the carbohydrates is a limiting factor for growth. Thus, a link was established between the ability of bifidobacteria to synthesize galactosidases as well as proteinases and their multiplication in a soy substrate (Donkor et al., 2007).

This literature shows that, for pure cultures, strain selection is essential to obtain adequate acidification rates. Even then, with pure probiotic cultures, the data show that fermentation times required to attain a pH below 4.5 are typically 10 h or more at 37°C (Champagne et al., 2009) and that this is in line with the literature (Angeles and Marth, 1971; Blagden and Gilliland, 2005; Chien et al., 2006; Kamaly, 1997; Murti et al., 1993). As in milk, probiotic bacteria tend to grow more slowly than the *S. thermophilus* cultures used for yoghurt (*Figure 19.1*).

It was mentioned earlier that, in milk, the solids level influences the populations which can be achieved in the fermented product. On an identical protein basis, milk has about a 30% higher buffering capacity than a soy beverage. This would suggest that populations obtained in soy blends would be lower than in milk due to inhibition linked to low pH. This does not seem to be the case (*Table 19.1*). However, one reason for the adequate growth in soy in this study (*Figure 19.1*; *Table 19.1*) may be the inclusion of ascorbic acid in the medium. The positive effect of antioxidants towards the growth and stability of bifidobacteria in milk (Dave and Shah, 1997), has also been reported for soy beverages (Kamaly, 1997). The reports of poor growth of *B. longum* in soy (Abd El-Gawad et al., 2005; Scalabrini et al., 1998; Tsangalis et al., 2002; Wang et al., 2002), may be related to unfavorable redox levels of the soy media as much as the lack of available nutrients for these strains.

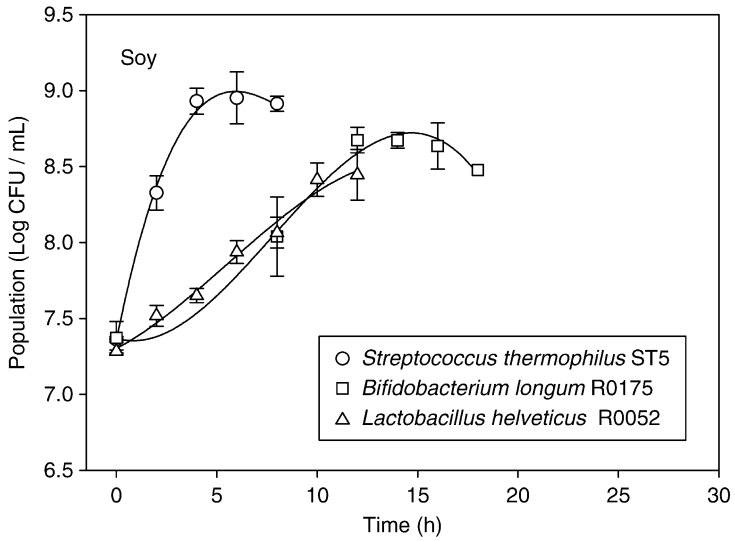

Figure 19.1
Growth of starter and probiotic cultures in a soy beverage. Inoculation levels are those presented in *Table 19.1* (Champagne et al., 2009).

19.3.3 Cereal

Food fermentations with probiotics have mainly been conducted on dairy and soy substrates. Nevertheless, cereal-based products offer many possibilities. Indeed, numerous cereal-based products in the world require a lactic fermentation, often in association with yeast or moulds (*Table 19.4*). Only a very small fraction of the current products contains proven probiotic bacteria which leave much room for innovation. The presence of yeast and moulds is an interesting feature of many of these fermentations, and this could actually be beneficial to probiotics for two reasons: these fungi assimilate oxygen and, in this aerobic condition, they assimilate lactic acid. These mixed cultures could therefore be sophisticated means of carrying out pH control as well as lowering the redox level. Not surprisingly, in one study involving oat fermentation with *L. plantarum*, the population of the lactobacilli increased from 1×10^8 to 7×10^8 when a mixed fermentation with *Aspergillus oryzae* was conducted (Patel et al., 2004).

Sourdoughs are obviously the cereal products where lactic cultures are used the most. However, the cooking step subsequently involved in breadmaking kills most probiotic bacteria. Therefore, unless encapsulation can prevent cell

Table 19.4

Lesser-known fermented cereal products which carry a lactic fermentation (modified from Gélinas et al., 2009) (*Cont'd* p. 776)

Food	Country	Ingredients	Microorganisms[a]
Adai	India	Cereal, legume	*Pediococcus* spp., *Streptococcus* spp., *Leuconostoc* spp.
Anarshe	India	Rice	Lactic acid bacteria
Arroz fermentado	Ecuador	Rice	Lactic acid bacteria, yeasts
Aya-bisbaya	Mexico	Rice	Lactic acid bacteria
Balao balao	Philippines	Rice, shrimp	*Lb. brevis*, *Ln. mesenteroides*, *S. cerevisiae*
Banku	Ghana	Maize, cassava	Lactic acid bacteria, moulds
Bhatura	India	Wheat	Lactic acid bacteria, yeasts
Bogobe	Botswana	Sorghum	*Lactobacillus* spp.
Burukutu	Nigeria	Sorghum, cassava	Lactic acid bacteria, *Candida* spp., *S. cerevisiae*
Dokla	India, Sri Lanka	Rice, chick pea, Bengal gram, fenugreek	Lactic acid bacteria, yeasts
Dosa, dosai	India, Sri Lanka	Rice, black gram	*Leuconostoc* spp., *Lb. fermentum*, *Saccharomyces* spp.
Fermented oatmeal[b] (ProViva)	Sweden	Oatmeal	*Lb. plantarum*
Hopper	Sri Lanka	Rice or wheat	Lactic acid bacteria, *S. cerevisiae*
Idli	India	Rice, black gram	*Ln. mesenteroides*, *Sc. faecalis*, *Lb. delbrueckii*, *Lb. fermenti*, *Lc. lactis*, *P. cerevisiae*, *Geotrichu candidum*, *Torulopsis* spp.
Llambazi, lakubilisa	Zimbabwe	Maize	Lactic acid bacteria, yeasts, moulds
Injera	Ethiopia	Sorghum, tef, corn, millet, barley, wheat	*Lb. plantarum*, *Aspergillus* spp., *Penicillium* spp., *Rhodotorula* spp., *Candida* spp.
Jalebi	India	Wheat, dahi	*Lb. fermentum*, *Lc. lactis*, *Lb. buchneri*, *Sc. Faecalis*
Kanga kopiro	New Zealand	Maize	*Leuconostoc* spp., *Clostridium* spp.
Kenkey	Ghana	Maize	*Lb. fermentum*, *Lb. reuteri*, *Lb. plantarum*, *P. pentosaceus*, *Lb. brevis*, *Candida* spp., *Saccharomyces* spp., *Penicillium* spp., *Aspergillus* spp., *Fusarium* spp.

Table 19.4 (*Cont'd* p. 777)

Food	Country	Ingredients	Microorganisms[a]
Khanomjeen	Thailand	Rice	*Lactobacillus* spp., *Streptococcus* spp.
Kishk, kushuk, trahanas	Egypt, Syria, Lebanon	Milk (yoghurt), wheat	*Lb. casei*, *Lb. plantarum*, *Lb. brevis*, *B. subtilis*, *B. licheniformis*, *B. megaterium*, yeasts
Kisra	Sudan, Irak, Arabian Gulf	Sorghum, millet	*Lactobacillu.* spp., *Lb. brevis*, *Lb. fermentum*, *E. faecium*, *Acetobacter* spp., *S. cerevisiae*
Kulcha	India, Pakistan	Wheat	Lactic acid bacteria, yeasts
Kwuna-zaki	Nigeria	Millet	Lactic acid bacteria, yeasts
Mahewu, mogou	South Africa	Maize	*Lc. Lactis*
Mawe	South Africa	Maize	*Lb. brevis*, *Lb. fermentum*, *Ln. mesenteroides*, *Lc. lactis*, *P. pentosaceus*, *W. confusa*, *yeasts*
Me	Vietnam	Rice	Lactic acid bacteria
Mutwiza	Zimbabwe	Maize	*P. pentosaceus*
Nan	India, Pakistan, Afghanistan, Iran	Wheat	Lactic acid bacteria
Nasha	Sudan	Sorghum, pearl millet	*Streptococcus* spp., *Lactobacillus* spp., *Candida* spp., *S. cerevisiae*
Ogi	Nigeria	Maize, sorghum, millet	*Lb. plantarum*, *Lb. brevis*, *Lb. fermentum*, *Ln. mesenteroides*, *W. confusa*, *Saccharomyces* spp., *Candida* spp.
Pozol	Mexico	Maize	*Lc. lactis*, *Lb. plantarum*, *Lb. casei*, *Lb. delbrueckii*, *Lb. fermentum*, *Clostridium* spp.
Puda, pudla	India	Bengal gram, mung bean, wheat	Lactic acid bacteria, yeasts
Puta	Philippines	Rice	*Ln. mesenteroides*, *Sc. faecalis*, *S. cerevisiae*
Rabdi	India	Maize, buttermilk	*P. acidilactici*, *Bacillus* spp., *Micrococcus* spp.
Togwa	Tanzania	Maize, sorghum	*Lb. plantarum*, *Lb. brevis*, *Lb. fermentum*, *Lb. cellobiosus* *P. pentosaceus*, *W. confusa*, *S. cerevisiae*, *C. tropicalis*

Table 19.4

Food	Country	Ingredients	Microorganisms[a]
Trahanas, tarhanas, kishk	Greece, Turkey	Wheat, sheep milk (yoghurt)	Lactic acid bacteria
Tsukemono	Japan	Vegetables, rice	Lactic acid bacteria
Uji	Kenya, Uganda, Tanzania	Maize, sorghum, cassava	*Ln. mesenteroides*, *Lb. plantarum*
Yosa[b]	Finland	Oat bran	Lactic acid bacteria, *Bifidobacterium* spp.

[a]*A. = Aspergillus; B. = Bacillus; C. = Candida; Lb. = Lactobacillus; Lc. = Lactococcus; Ln. = Leuconostoc; P. = Pediococcus; R. = Rhizopus; S. = Saccharomyces; Sc. = Streptococcus; W. = Weissella*

[b]Contains documented probiotic strains

mortality, only probiotics which synthesize a thermostable bioactive compound during leavening can be of use in bread making. In this perspective, *Lactobacillus reuteri* has potential due to reuterin synthesis (Gerez et al., 2008; Gopal et al., 2007). In this instance, the *L. reureri* cells might be inactivated by heating, but the bioactive compound might remain active. Probiotic *Bacillus* strains could be even better adapted to this breadmaking application.

Oat is the substrate that has mostly been used in studies when cereals were actually fermented by probiotic bacteria rather than lactic starter cultures (Gopal et al., 2008; Laine et al., 2003; Martensson et al., 2001; Sumangala et al., 2005). Presumably, this is because oats have demonstrated health effects as such, and enrichment with probiotics is a logical association. Populations in fermented oats can easily reach 2×10^8 CFU/g. This is slightly lower than fermented milk or soy products (*Table 19.1*). With *L. reuteri* the fermentation can even be conducted at 6°C (Martensson et al., 2002). In most applications, *L. reuteri or L. plantarum* are used.

The most comprehensive study of cereal fermentation other than oats was carried out by Charalampopoulos et al. (2002) in which malt, barley and wheat were compared as substrates. As a rule, *L. fermentum* and *L. plantarum* reached populations between 2×10^9 and 2×10^{10} CFU/g, while *L. reuteri* and *L. acidophilus* were typically between 2×10^7 and 7×10^8 CFU/g. The *L. plantarum* populations are quite satisfactory, since they are in the range obtained on milk or soy for other species (*Figure 19.1*; *Table 19.1*). Fermented malt tended to have higher populations than barley or wheat. Therefore, some probiotic cultures can very successfully be used in cereal fermentations. Again, matrix and strain selection are required.

19.3.4 Vegetables

Data in ◉ *Table 19.4* were limited to cereal products, but fermentation of vegetables offers just as much potential for the development of FF (Farnworth, 2004).

In Kimchi fermented at 4°C, there was slow development of the lactic acid bacteria, but no growth of various bifidobacteria was recorded (Lee at al., 1999). As a function of probiotic strain tested, viability losses in this study were between 1 and 4 logs CFU/g over the 10 day fermentation period. This shows that vegetable fermentation, often carried out at low incubation temperatures are a problem for the introduction of the traditional *L. acidophilus* and *Bifidobacterium* probiotic bacteria. As has been suggested in cheese ripening (Stanton et al., 1998), other species might be more appropriate. Thus, probiotic cultures of *L. rhamnosus*, *L. casei* and *L. plantarum* seem much better adapted to the vegetable substrate as well as to the incubation temperatures used during fermentation. Nevertheless, when the temperature is adjusted at 37°C, probiotic bacteria grow quite rapidly in plant-based substrates (Savard et al., 2003).

In addition to Kimchi, sauerkraut-type products (fermented cabbage, carrots, onions, cucumbers) are based on a lactic fermentation where *L. plantarum* is particularly involved. Most of these products, however, are stabilized by heating. The traditional process would therefore result in inactivation of the probiotic cultures. The use of probiotics in fermented vegetables would require low-temperature storage of the products, as in yoghurt. Furthermore, the shelf-life would be much shorter, potentially only weeks. These probiotic-containing products therefore require much different commercialization practices than the current "stabilized products" which are shelf-stable for months at room temperature. Fermented vegetables which are not heated or supplemented with preservative are subject to spoilage by yeast (Savard et al., 2002). To reduce this spoilage, inoculation with selected starter cultures must be carried out (Gardner et al., 2001). The spoilage danger is arguably greater for such fermented vegetable products than for cheese or yoghurt, because the vegetable food substrate used is not heated prior to fermentation in order to eliminate the contaminating flora.

19.3.5 Supplement Ingredients Which Affect the Growth of Probiotics

Since probiotic bacteria grow more slowly than starter cultures on milk and soy (◉ *Figure 19.1*; ◉ *Table 19.1*), it was examined if the addition of growth

supplements could selectively enhance the development of probiotic bacteria. These supplements are mainly used in processes where high biomass levels (above 10^9 CFU/g) are required.

Milk supplementation by minerals, amino acids and nucleotides have all improved the growth of *Lactobacillus johnsonii* (Elli et al., 1999) and amino acids are frequently seen as good supplements for bifidobacteria (Klaver et al., 1993). Soy beverages do not have much more free amino acids than milk (Champagne et al., 2009), and neither do most vegetables. This would suggest that high proteolytic activities would enhance probiotic bacteria growth rates and biomass levels in protein-rich substrates such as soy or milk. Surprisingly, it appears that proteolytic activity is not always the limiting factor with bifidobacteria in milk (Desjardins and Roy, 1991). Indeed, some *L. acidophilus* and *Bifidobacterium* cultures have proteolytic activities as high as those of yoghurt starters (Shihata and Shah, 2000), but still do not multiply as fast in milk. Presumably this is because another factor is more limiting to growth than the availability of amino acids or peptides. As mentioned earlier, in milk this could be due low β-galactosidase production level, while in soy it could be the low α-galactosidase production level, to assimilate the stachyose and raffinose carbohydrates. Nevertheless, in some instances involving milk, mixing a non-proteolytic *Bifidobacterium* strain with a highly proteolytic *L. acidophilus* culture will be helpful (Klaver et al., 1993) to the bifidobacteria. However, if the starter cultures are used the fermentation time is shortened and the counts of probiotic bacteria can even be lowered (Shihata and Shah, 2002).

Numerous studies show that enriching milk or soy-based substrates with growth supplements enhances the multiplication of probiotics. This constitutes an opportunity for innovation. Indeed, examples of potential supplements are extracts or juices of the following sources: yeast (Kim et al., 1995), citrus (Sendra et al., 2008), ginseng (Goh et al., 1993), tomato (Babu et al., 1992), peanut (Murad et al., 1997), soy (Yajima et al., 1992), cereals (Kyung and Young, 1993; Vasiljevic et al., 2007), honey (Ustunol and Gandhi, 2001), berries (Kailasapathy et al., 2008), mango (Kailasapathy et al., 2008), herbs (Ray-Chowdhury et al., 2008) and whey (Christopher et al., 2006). A recent trend is the addition of prebiotics, which include fructooligosaccharides (FOS) (Bruno et al., 2002), such as chicory's inulin (Aryana et al., 2007). Other potential prebiotic or non-prebiotic carbohydrates which have been added as growth supplements include lactulose (Bruno et al., 2002), oat and barley glucans (Vasiljevic et al., 2007), galactooligosaccharides (GOS) (Shin et al., 2000), starch/maltodextrins (Bruno et al., 2002) and raffinose (Martinez-Villaluenga and Gomez, 2007). In view of the

fact that many probiotics are poor users of lactose, the addition of a more readily available carbohydrate can selectively enhance the growth of probiotics during milk fermentation. Thus, addition of 5% lactulose, GOS or inulin reduced the doubling time of bifidobacteria in milk by 25–50% (Bruno et al., 2002; Shin et al., 2000). Even in soy, which contains numerous carbohydrates, supplementation with lactulose enhanced the viable counts of bifidobacteria by up to 2 log CFU/mL (Pham and Shah, 2008a). It is noteworthy to mention that this also enhances the level of bioactive compounds, such as soy isoflavones, resulting from fermentation (Pham and Shah, 2008b).

In addition to the above supplements, manufacturing processes often include artificial and natural flavors, sugars or sweeteners, and preservatives which affect probiotic bacteria (*Table 19.5*). Sucrose at less than 10% is generally not inhibitory to probiotic bacteria (Shah and Ravula, 2000; Vinderola et al., 2002). Aspartame and acesulfame sweeteners are not toxic at concentrations used in practice (Vinderola et al., 2002). Some fruit and vanilla flavors are detrimental to growth of probiotics, but this is quite variable (Vinderola et al., 2002). The preservatives natamycin and lysozyme are not a problem at the concentrations used commercially, but data show that nisin inhibits a significant proportion of probiotic cultures (Vinderola et al., 2002).

Current yoghurt products contain 1 billion probiotic cells per portion (90–150 mL) at the end of storage, which means there are approximately 10^7 CFU/mL of probiotic bacteria. Since the total bacterial population in yoghurt is typically 20–100 times that number, probiotic bacteria therefore represent only a small fraction of the microbiota. Products are increasingly seen which contain as much as 50 times more probiotic bacteria per portion and, in fact, may not contain starter cultures at all. In many cases, the product only contains probiotic bacteria. Such products will be referred to as high probiotic density (HPD) cultures. There are excellent reviews of specialty HDP products containing bifidobacteria (Roy, 2005), which even present specific manufacturing processes of commercial products (Tamime et al., 1995). Supplementation with amino acids, vitamins and minerals is critical to obtain very high bacterial populations in the HPD products. The most widely used technique for the production of HPD beverages is probably fermentation without pH control using only probiotic cultures. The population in the fermented product will depend on the buffering capacity of the substrate as well as on the addition of growth supplements. Examples of HPD products in milk or soy without pH control appear in *Table 19.1*. In milk or soy blends adjusted at 4.5% protein, but without growth supplements, the population gets above the 10^8 CFU/mL thresholds, which is 10 times higher than current

Table 19.5

Effect of some processing and storage conditions on the growth or viability of probiotic cultures in yoghurt or specialty fermented milks

Processing/ storage condition	Effect	Reference
Sugar addition (up to 16%)	Decrease in *Bifidobacterium* and *L. acidophilus* during fermentation as sugar concentration increases	Shah and Ravula (2000)
Addition of fruit or flavors	Variable. Rarely stimulatory, more often inhibitory	Vinderola et al. (2002)
Addition of preservatives	Natamycin not a problem, but nisin inhibits many strains	Vinderola et al. (2002)
Addition of starter	Less growth of the probiotics	Roy et al. (1997)
Presence of oxygen in milk	Less growth of the probiotics, particularly bifidobacteria	Klaver et al. (1993)
Freezing	Bifidobacteria show 1 log drop in low acid yoghurt (pH 5.85) but at least 2 log drop in CFU when pH is 4.47. Strain dependant. *L. acidophilus* appears to have better resistance	Laroia and Martin (1991), Modler and Villa-Garcia (1993), Mitchell and Gilliland (1983), Brashears and Gilliland (1995)
Drying	6–150 fold reduction of viable populations after drying. *Bifidobacterium* more sensitive than *L. acidophilus*	Rybka and Kailasapathy (1995, 1997)
Refrigerated storage (typically 4°C)	Bifidobacteria losses in CFU counts of up to 6 logs over 40 days, typically around 1 log. Cultures of *L. acidophilus* seem more stable. Highly affected by strain and pH.	Micanel et al. (1997), Rybka and Kailasapathy (1995), Shah et al. (1995), Roy et al. (1997), Dave and Shah (1996), Nighswonger et al. (1996), Medina and Jordano (1994)
Carbonation	No effect overall on probiotic populations during manufacture. CO_2 stimulated growth of *L. acidophilus* but production time was shorter	Vinderola et al. (2000)

probiotic levels in commercial yoghurt. However, there is little room for loss of viability during storage. In order to obtain populations above 10^9 CFU/mL, growth factors must be added. To further enhance cell counts in HPD products, the fermentation should be carried out with external pH control. When the growth medium is appropriate, a population of 10^{10} CFU/mL in bifidobacteria

can be achieved (Misra and Kuila, 1991), which is more than 100 times the probiotic population in yoghurts. With so high populations, the probiotic bacterial concentrates can be blended with a variety of juices and beverages, and the latter could constitute 90% or more of the product. This provides great latitude for innovation in HDP beverages. Obviously, prebiotics can be added at this stage in order to prepare synbiotic blends.

19.3.6 Competition with Starters

The sections above mainly examined the growth of probiotic alone in the food matrices. However, most probiotic FF are derived of existing fermented foods which contain starter cultures. Therefore, one of the main challenges in developing probiotic-containing FF is to enable growth of the probiotic culture in competition with starter cultures. Using the data in *Figure 19.1*, it can be seen that the probiotic cultures only show half the growth rate of the starter culture, in this case *S. thermophilus*. At first glance, this would suggest that, in a mixed culture, the fermentation time would be cut in half (*Figure 19.1*) and the probiotic population would only be half of that reached in a pure culture. This is not the case because of the mathematics of bacterial growth, which are exponential in nature. In a typical food fermentation, cells are inoculated at 10^7 CFU/mL and grow to 10^9 CFU/mL. This requires approximately 6 generations (multiplication cycles). Therefore, from a mathematical standpoint when one culture has half the growth rate of the other and when 6 generations are allowed, shortening the fermentation time by half would actually reduce the probiotic population by a factor of 10. In one series of assays (*Table 19.1*), this is exactly what has occurred with *B. longum* R0175. But the picture becomes more complicated when strains are changed. In selecting a very slow-growing *S. thermophilus*, one would have expected the bifidobacteria to attain the same population as in a pure culture, especially in soy, where the streptococci and bifidobacteria are feeding on different carbohydrates. An extended incubation did indeed enhance the proportion of bifidobacteria in the finished product, but not to the extent expected (*Table 19.1*). Also, when *L. helveticus* R0052 is used in the mixed culture, then the mathematics do not hold at all. These data show that the growth patterns in pure cultures cannot be used as sole indicators of the populations to be expected when they are mixed.

The starter cultures are generally less affected by the presence of the probiotic bacteria than the reverse. Counts of both *S. thermophilus* R0083 and

S. thermophilus ST5 were not significantly reduced in mixed cultures, which is in agreement with the results of Wang et al. (2002). However, there are reports that some probiotic-*S. thermophilus* pairings do not function as well (Dave and Shah, 1997; Mital et al., 1974). Evidently, probiotic strains used in the Champagne et al. (2009) study did not negatively impact growth of *S. thermophilus*, but it cannot be automatically assumed that this is will always occur.

A partial explanation for the low bifidobacteria counts in mixed cultures is that they grow rapidly during the first hours of incubation, but multiplication stops at low population levels (Samona and Robinson, 1994). This pattern is typical of protease-negative lactococci (Juillard et al., 1995). Therefore, nitrogen metabolism becomes critical in the relationship between the starter and the probiotic culture. When starters are not highly proteolytic, it can be feared that competition would occur for the assimilation of the limited amount of free peptides and amino acids in milk as noted for cheese cultures (Juillard et al., 1987). Hence, the proteolytic activities of the various strains in the blends will influence the strain ratios (Klaver et al., 1993).

Starter cultures produce additional inhibitory products to that of lactic acid. Since some probiotic bacteria are strongly affected by the redox level (Klaver et al., 1993), the production of hydrogen peroxide is particularly to be feared. *Lactobacillus delbrueckii* is a recognized producer of H_2O_2 (Villegas and Gilliland, 1998). Hydrogen peroxide strongly influences the storage stability of probiotic cultures in yoghurt (Lankaputhra et al., 1996) but it remains to be determined if H_2O_2 production during fermentation is also detrimental. Bifidobacteria which synthesize peroxidases are less susceptible to this occurrence (Shimamura et al., 1992).

Cheese starter cultures synthesize bacteriocins (Carr et al., 2002). Bifidobacteria are sensitive to nisin (Kheadr et al., 2004), but this bacteriocin is produced by lactococci in cheese starters. Many lactobacilli also produce bacteriocins which raises a concern. However, the inhibition of the growth of probiotic bacteria by bacteriocins of yoghurt starters is undocumented.

Fresh liquid cultures will initiate growth and acidification much faster than cells which were added to the processing milk frozen or dried. Therefore, another reason probiotic bacteria do not seem to compete well with starters is simply linked to the form of inoculation. Yoghurt cultures are often added in the fresh liquid form, while probiotics are inoculated in the frozen or dried state. Even if thawing or rehydration conditions of the probiotics are acceptable, the growth of the probiotic bacteria will still be delayed as compared to that of a fresh liquid starter. An option is to prepare liquid probiotic cultures

at the plant. For the reasons mentioned earlier (Section 19.2), this is not the current practice.

In addition to providing a food matrix medium favorable for the growth of bacteria a number of techniques can be used to attempt to improve the growth of probiotic cultures during the production of the FF itself: (1) select a compatible starter (2) reduce the *L. bulgaricus* content in a yoghurt starter, (3) use an oxygen-consuming *S. thermophilus* culture, (4) de-aeration of the matrix (5) inoculate the starter later in the fermentation, (6) inoculate with a sonicated starter culture having high intracellular lactase levels, (7) lower the starter inoculation level, (8) adapt the fermentation temperature. In most cases, these techniques were developed using milk for yoghurt fermentation. Lowering the fermentation temperature from 43 to 37°C in a soy substrate also favored the probiotic bacteria (Bozanic et al., 2008). Therefore, it can be hypothesized that these strategies could also extend to non-dairy substrates as well. The first three approaches are related to the composition of the thermophilic starter. In the past the probiotic culture was the one being tested for compatibility with the current yoghurt starter. The opposite may now need to be carried out. It is extremely costly to carry out clinical tests with probiotic bacteria and a given *Lactobacillus* or *Bifidobacterium* strain might be selected for its demonstrated health benefits. In this instance it is the starter strains which must now be subject to compatibility testing with the probiotic culture. Starters which produce inhibitory compounds towards the probiotic bacteria are obviously to be avoided. This is why some authors recommend to reduce or even eliminate the *L. bulgaricus* strains in the yoghurt culture (Rybka and Kailasapathy, 1995). Indeed, some *L. bulgaricus* strains are undesirable for two treasons: production of H_2O_2, overacidification of the product during storage. However, one must be careful. *Lactobacillus bulgaricus* contributes greatly to the fermentation, particularly by its proteolysis (Juillard et al., 1987; Tamime and Robinson, 1985) and acetaldehyde production, and removing this strain might affect the acidification rate as well as the flavor of the product.

Some probiotic cultures are very sensitive to oxygen, and growth will not occur even if the medium is adequate for growth. It was previously mentioned that milk could be supplemented with antioxidants to achieve the goal of reducing the redox level as well as deaeration of the matrix. Another approach is also possible: the selection on an oxygen-scavenging strain of *S. thermophilus* (Ishibashi and Shimamura, 1993) or of lactococci and *Leuconostoc* for mesophilic fermentation processes. Deaeration might not be necessary if care is simply taken not to reintroduce oxygen in the fermentation tank following pasteurization.

Indeed, heating of the yoghurt milk blend is generally carried out above 85°C for many minutes, which strongly reduces the oxygen level. To prevent oxygen reintroduction in the milk blend, pumping and agitation must be kept at a minimum, and gas flushing of the headspace of the fermentation vat can be considered. If the medium composition and redox levels are adequate, then changing the starter inoculation procedures can be successful.

Inoculating with a lower number of viable cells of the starter, for example, 10^6 CFU/mL, while maintaining the probiotic inoculation at 10^7 CFU/mL, will favor the development of the probiotic bacteria for two reasons: (1) longer fermentation time and (2) longer time for the pH to drop to acid values; the extended incubation time at pH values above 5.5 helps the growth of probiotic bacteria. In one application based on this strategy, ruptured cells are used (Shah et al., 1997). Presumably, the liberation of lactase from the ruptured starter cells added to the two factors just mentioned. Indeed, lactose assimilation is often a limiting factor in the growth of probiotic bacteria in milk, and this is an elegant method of combining inoculation and addition of lactase.

Traditionally, yoghurt fermentation is carried out between 40 at 44°C (Tamime and Robinson, 1985). In such "thermophilic" fermentations, lowering the incubation temperature to 37°C will promote the selective growth of *L. acidophilus* and bifidobacteria (Mortazavian et al., 2006). Presumably this would also be the case for *L. casei*, *L. rhamnosus and L. plantarum* cultures of which many have the ability to grow at 15°C (Carr et al., 2002). Not surprisingly, Kneifel et al. (1993) reported that some processes use a 32°C incubation temperature. The optimum growth temperature for *L. bulgaricus* is close to 43°C, while that of *S. thermophilus* is around 40°C (Beal and Corrieu, 1991). Therefore, it must be mentioned that, in yoghurt type productions, using an incubation temperature under 40°C will slow the fermentation, which has economical impacts. Furthermore, lowering the incubation temperature to 37°C not only affects the level of probiotic in the product but also the strain ratios of the yoghurt starters. Again, it must be kept in mind that such changes will affect the acidification rate (consequently the fermentation time), flavor (acetaldehyde) and acidification during storage. This being said, lower-than-traditional incubation temperatures are increasingly used in yoghurt production because the production of exopolysaccharide is sometimes enhanced under these conditions (Farnworth et al., 2007). Some cheesemaking processes, for example Emmental, also require thermophilic cultures. Presumably the same temperature changes could be applied. However, changes in processing temperature are much more difficult to apply in cheesemaking because temperature not only affects acidification rate but

also wheying off. The latter will influence the residual humidity in the cheese, a characteristic that has consequences on the evolution of ripening, in addition to all the legal considerations related to cheese denominations. In most cheese productions (Cheddar, Camembert, Blue, Gouda) mesophilic cultures are used and the manufacturing temperature typically starts at 32°C. In these situations, promoting the development of the probiotic involves increasing the production temperature at one point, rather than lowering it as in yoghurt or Emmental. Again this is complex because of the acidification and wheying off rates which must be controlled.

Interactions between lactic cultures are sometimes to the benefit of the strains. The symbiosis between *S. thermophilus* and *L. bulgaricus* is a typical example (Tamime and Robinson, 1985). It is noteworthy that enhanced acidification rate when cultures are mixed can also occur between *S. thermophilus* and probiotic *L. helveticus*, as well as *B. longum* (*Figure 19.2*). It was noteworthy that this beneficial interaction occurred in milk but not in a soy beverage (Champagne et al., 2008a, b).

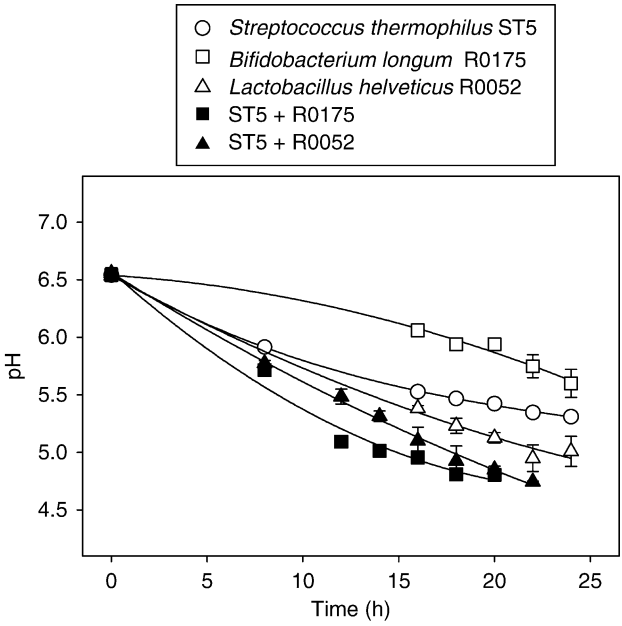

Figure 19.2
Acidification of a milk blend by pure and mixed cultures. Inoculation levels are those presented in *Table 19.1* (Champagne et al., 2009).

In summary, there are at least seven parameters of the fermentation process which will affect the development of probiotics (*Table 19.3*) and which require standardization.

19.4 Survival to Some Processing Steps

Various stressful conditions are encountered in the preparation of food and beverages. Some are presented in *Table 19.5* and more extensive lists are presented in the reviews of Champagne et al. (2005) and Roy (2005). In non-fermented processed foods and beverages, the most frequent products containing probiotic bacteria are frozen desserts and pasteurized beverages (milk, fruit juices). Therefore, survival to freezing and heating are the major processing hurdles probiotic bacteria face in the current market. The focus will be placed on these processing barriers.

19.4.1 Freezing

Frozen concentrated lactic cultures have been on the market for many years, and survival of lactic acid bacteria is therefore well studied. Factors such as fermentation parameters (temperature, growth stage, medium composition), concentration technology, freezing medium, cell density and freezing rate influence the survival of cultures. To some extent these apply to foods as well.

The acidity is the factor having the greatest effect on post freeze-thaw populations. The viability losses subsequent to freezing cells in a pH 6.6–7.0 medium are rather small, often between 20 and 60%. However, they can be 100 times higher in an acid product (yoghurt pH 4.2), to a point where it can be feared that the number of viable cells becomes insufficient to ensure the health benefit. Acidity is not only detrimental to survival to freezing, it may also enhance viability losses during storage (*Table 19.6*), and this will be further addressed. The effect of acidity can even occur before freezing. Indeed, in a study on cranberry juice (Reid et al., 2007), it was noted that simply adding the freeze-fried powder into the juice generated high viability losses. Therefore, the parameters which surround the rehydration of the cultures must be controlled. It seems ill advised to directly add a freeze-dried powder of probiotic bacteria into an acid food or beverage matrix.

Table 19.6

Factors which influence the survival of probiotic bacteria in frozen dairy desserts

Factor	Effect	Reference
Sucrose level (15–21%)	Freezing: no major effect on survival to freezing when ice cream is acid (pH 5.5)	Akin (2005)
	Storage: High concentration improves stability	
Addition of prebiotics	Addition of inulin or oligofructose improve survival to freezing even in acid ice cream (pH 5.5)	Akin (2005), Akalin and Erisir (2008)
Acidity – pH	Freezing: viability losses vary between 0.3 and 2.5 log CFU/mL during; the lower the pH the greater the viability losses.	Akin (2005), Akalin and Erisir (2008)
	Storage: the same detrimental effect of pH occurs	
Low fat-high sugar[a]	Freezing: greater survival of lactobacilli and bifidobacteria in the "low fat-high sugar" blend	Haynes and Playne (2002)
	Storage: similar survival of lactobacilli and bifidobacteria in the "low fat-high sugar" blend	Alamprese et al. (2002)
Dosage of probiotic bacteria	Freezing and storage: no effect on survival in the range studied (2×10^7 vs. 2×10^6 CFU/mL)	Haynes and Playne (2002)
Encapsulation	Freezing: enhanced survival. Alginate better than carrageenan	Sheu et al. (1993), Sheu and Marshall (1993), Tsen et al. (2007)
	Storage: enhanced stability	

[a]The low-fat formulation also had higher sugar content

Sugars act as cryoprotectants. Therefore, it is not surprising that the effect of carbohydrates, including prebiotics, have been examined in ice cream (*Table 19.6*). With respect to prebiotics, short chain fructo-oligosaccharides (FOS) seem better than inulin in improving survival to freezing and storage stability (Akalin and Erisir, 2008). In ice cream, the large carbohydrate polymers are useful as fibers, prebiotics or texturing agents. However, such large molecular weights carbohydrates do not appear as effective in protecting cells during freezing as short chain oligosaccharides.

The most promising approach to enhance the stability of probiotics in frozen desserts appears to be encapsulation. Most studies have been carried out using cells microentrapped (ME) in alginate beads (*Table 19.6*), but the commercial

spray-coated products appear even better suited for this application. It must be kept in mind that gel bead capsules can affect texture. Unfortunately, as the size of the capsule is lowered to prevent this effect of texture, the protective effect diminishes also.

The commercial inoculation practice of probiotics in ice cream is typically DVI. Not surprisingly, there does not appear to be data available on how the preparation of the probiotic cultures can be modulated to enhance survival to freezing in foods per se. As was mentioned earlier, there are numerous data on how starters can be prepared to better survive the freeze-thaw cycle of concentrated cultures, and some could apply to probiotics in food processing. For example, cryotolerance of *L. acidophilus* is greater if the cells are cultured at 30°C rather than 37 or 42°C (Wang et al., 2005). This suggests that fermented milks destined for freezing should be prepared at sub-optimal incubation temperatures. Minor cold shocks aimed at inducing stress responses could also be considered. When some food matrices are highly detrimental to survival to freezing, food manufacturers might therefore want to examine adaptation techniques for probiotic cells prior to their addition to the blend and subsequent freezing.

19.4.2 Heating

Heating is applied in many food processes. In some cases it is an integral part of the manufacturing process, such as in bread, while in other instances it is carried out to destroy pathogens or spoilage organisms.

Considerable commercial activity has emerged with unfermented beverages. In this category, unfermented milk and fruit juices are the most widely seen. As opposed to fermented milks, these beverages are stabilized by heating before packaging. The sensitivity of probiotic bacteria to heating is such that manufacturers prefer to add the cultures after the heat treatment. This practice is a concern for safety as well as spoilage reasons. Ideally, the probiotic cultures could be added into the milk and juices prior to pasteurization and they would survive the heating step. Therefore, sensitivity of probiotic bacteria to heating is a feature which deserves attention.

It must first be mentioned that the problem lies with the traditional probiotic bacteria, which are lactobacilli and bifidobacteria. There are claims that some cultures on the *Bacillus* family possess probiotic properties. Since *Bacillus* produce heat-resistant endospores, they seem appropriate in processes which carry a

heating stage under 100°C (Belvis et al., 2006). Unfortunately these cultures are far from being as documented as the bifidobacteria or the lactobacilli as far as health effects. Therefore, their attractiveness is currently limited, even in this technological situation.

Exposure to 65°C for 30 min resulted in drops in viable counts varying from 3.6 to 5 logs CFU/mL for six probiotic free cell cultures (Ding and Shah, 2007). Using these data, $D_{65°C}$ (D = time required to generate a 1 log loss of viability) values can be estimated at 6–10 min, which are in line with those of Mandal et al. (2006) who observed $D_{65°C}$ of 3 min for *L. casei*. These data suggest that viability losses of 3 logs CFU/mL would occur during a low-temperature pasteurization process (63°C for 30 min). This viability loss is much too high for the process to be economically viable, which explains the current practice of adding the cultures to milk after the pasteurization step.

However, when alginate ME cultures were exposed to the same 65°C for 30 min treatment, $D_{65°C}$ values increased twofold (11–21 min) (Ding and Shah, 2007), which was also in line with the results of Mandal et al. (2006). It was noteworthy, however, that viability losses of free and ME cultures were very similar after 60 min of incubation at 65°C. This suggests that ME bacteria were protected through reduced heat transfer to the cells, a protection which was only temporary. A result of this property is that microentrapment/encapsulation could find applications in processes which use short heating periods. It is unknown how ME cells would react to the current 73°C for 16 s milk pasteurization process. However, data of Chen et al. (2007) suggests that survival could be satisfactory because some gellan-alginate ME cultures exposed to 75°C for 1 min showed less than 0.5 log CFU/mL losses. There are also other foods where ME provided enhanced survival during heating: biscuits (Reid et al., 2007), dry sausages (Muthukumarasamy and Holley, 2006; Muthukumarasamy et al., 2006) and chocolate (Goulet and Wozniak, 2002). In summary, as was the case for the freezing stress, encapsulation has potential to protect the cells during a heat treatment.

Cells which have been subjected to a mild heating stress, for example 52°C for 15 min, synthesize stress resistance components (De Angelis et al., 2004; Prasad et al., 2003). These cells are subsequently more resistant to lethal heating conditions (Saarela et al., 2004). Such responses have been put to use to enhance survival to spray-drying, but not in food processing. The same comment as was mentioned in the section on survival to freezing could apply here. Food processors wishing to improve the survival of their cultures to a heating process could attempt to develop heat adaptation steps prior to inoculation instead of limiting their choice to DVI.

19.5 Storage

The ability to survive during processing and storage are not necessarily linked (Prajapati et al., 1987). This justifies the specific examination of factors that influence survival of probiotics during storage. Many studies have assessed the stability of probiotic bacteria in foods during storage in simulated warehouses or grocery shelves and there are reviews which discuss the stability of probiotics in various food products (Champagne et al., 2005; Roy, 2005). Therefore, this review will rather focus on the parameters which affect stability during storage.

19.5.1 Effect of Strain

As a rule, probiotic bacteria die during storage, but data show wide variations in stability. An example of such variations is presented in *Figure 19.3* which was obtained in a fruit juice blend. Frustratingly, viability during storage is very much linked to strain and species. This effect of strain has constantly been observed. Therefore, amongst all the factors which influence CFU counts

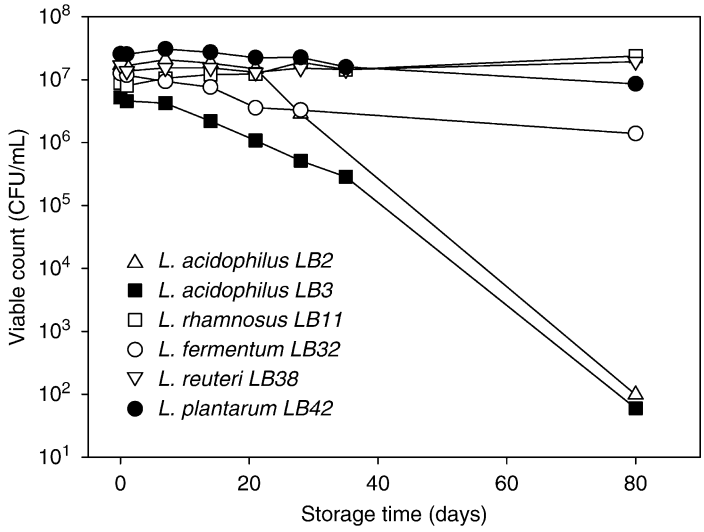

Figure 19.3
Viability of various lactobacilli in a fruit juice blend having a pH of 4.2 (Champagne and Gardner, 2008).

of probiotics in FF, the strain used in the application is probably the most important.

As a result, in the past, stability during storage was an important criterion for the selection of bifidobacteria and *L. acidophilus* strains by food processors. It still is, but the limited number of strains that have demonstrated health effects reduces the choice in cultures. If the chosen strain is unstable in a product during storage, it will be necessary to adapt the production and storage parameters to enhance its stability. In this goal, the three most important parameters are food matrix composition (acid, moisture, chemicals), oxygen level and probiotic culture format (encapsulation).

19.5.2 Effect of the Food Matrix

Since most FF are fermented products, acidity is probably the main detrimental factor on viability during their storage. This is also the case for fruit juices. In fermented milks, the problem of sensitivity to acidity is compounded by (1) the addition of fruits and (2) the continued acidification ("over-acidification"). One means of addressing the problem of fruit acidity is to package the yoghurt and the fruits separately (Koch and Carnio, 2001). To prevent a continued drop of pH during storage of yoghurt, it appears important to select lactobacilli that have weak over-acidification properties, or to reduce and even exclude *L. bulgaricus* from the starter (Kailasapathy and Rybka, 1997).

The pH of fruit juices varies considerably, with lemon being as low as pH 2.2 and oranges reaching pH 4.3. Since the optimal pH for growth of probiotic bacteria varies between 5.5 and 6.5, the acidity of fruits is obviously detrimental to viability. It was long considered that fruit beverages could not support probiotics. However, by mixing various low-acidity juices and other ingredients (such as dairy products or cereals), it is possible to obtain blends having pH levels well above pH 4.0. It was shown that some lactobacilli were quite stable in such a fruit blend (*Figure 19.3*).

In addition to pH, enriching the food matrix with various components improve the stability of probiotics during storage. Here are some examples:

1. Lowering the salt content of cheese (Gomes and Malcata, 1998) or kimchi (Lee et al., 1999)
2. Adding protein or substituting casein by whey proteins in fermented milks (Gardini et al., 1999)

3. Adding peptides in dairy products (Gomes and Malcata, 1998)
4. Adding sugar, prebiotics or honey (*Tables 19.5* and *19.6*; Ustunol and Gandhi, 2001)
5. Adding mannitol or glycerol (Kebary et al., 1998)
6. Adding antioxidants (Dave and Shah, 1997)

The last ingredient pertains to the redox level and oxygen metabolism. This will be addressed in the next section.

The addition of carbohydrates, glycerol and mannitol influences the a_w of foods. As a rule, lowering the a_w improves the stability of probiotics during storage. In cereals and powders, the a_w should be between 0.1 and 0.2 (Ishibashi et al., 1985), particularly if the product is to be stored at room temperature. This typically represents from 2 to 8% moisture in the powder.

In cheeses, most probiotic cultures lose their viability during storage. However, there are examples of species that grow in this environment. This is the case of propionibacteria in swiss-type cheese where their number increases to 10^9 CFU per g during the 4–8 weeks maturation period. *L. casei* can also grow during the ripening of Cheddar (Stanton et al., 1998). *L. rhamnosus*, *L. plantarum* and *L. reuteri* are also potential species that could at least survive in ripened cheeses or fermented milks (Antonsson et al., 2002; Rodas et al., 2002).

19.5.3 Effect of Oxygen

Oxygen in the medium can lead to hydrogen peroxide production by either the probiotic culture itself of by lactobacilli from the starter (Villegas and Gilliland, 1998). Certain probiotic cultures are very sensitive to oxygen (Dave and Shah, 1997; Meile et al., 1997), and this is presumably linked to variable levels of peroxidases (Talwalkar and Kailasapathy, 2003b), which eliminate H_2O_2. The ability of *L. bulgaricus* to produce H_2O_2 partially explains why its removal from fermented milk starter cultures has had success in improving the survival of *L. acidophilus* in fermented milks (Rybka and Kailasapathy, 1995). The problem of oxygen sensitivity is compounded by the occurrence of a synergistic inhibition of bifidobacteria by acid and hydrogen peroxide (Lankaputhra et al., 1996). This being said, if a probiotic culture does have the ability to synthesize peroxidases, it is better that the culture be added into the yoghurt at the beginning of the fermentation (Hull et al., 1984), rather than at the end with, for example, the fruits. Presumably, the synthesis of the peroxidases occurs

during the fermentation, which helps protect the probiotic cells during the subsequent storage.

Many studies have focused on the way to prevent the detrimental effects of oxygen on the probiotic cultures. In addition to adjustments in *L. bulgaricus* content in starters, the most common are the addition of anti-oxidants, such as ascorbic acid (Dave and Shah, 1997) and packaging with materials impermeable to oxygen (Talwalkar et al., 2004). De-gassing and electroreduction have proved successful in lowering the redox level and enhancing the stability of probiotics in unfermented milk (Bolduc et al., 2006).

19.5.4 The Potential of Encapsulation

Of the various methods used to enhance the survival of cells to acid and oxygen-rich environments, encapsulation seems promising (Doleyres and Lacroix, 2005). There are numerous methods on encapsulating food ingredients and the following have been applied to probiotics: microentrapment in gels, spray-coating, extrusion, spray-drying and emulsions (Champagne and Kailasapathy, 2008). Gel particles have received the most scientific attention but spray-coated cultures have been marketed to a much larger extent.

Microentrapment in gel beads was shown to be very effective for increased stability during storage in numerous foods: yoghurt (Sultana et al., 2000), unfermented milk (Truelstrup-Hansen et al., 2002), vegetable and frozen cranberry juice (Reid et al., 2007), ice cream (Sheu et al., 1993), powders (Siuta-cruce and Goulet, 2001), mayonnaise (Khalil and Mansour, 1998), cream fillings or peanut butter (Belvis et al., 2006). In yoghurt, it was first believed that ME protected against acidity, but there is evidence that the benefit of ME to probiotic stability in yoghurt is rather due to protection from oxygen (Talwalkar and Kailasapathy, 2003a).

One must nevertheless be critical of encapsulation. In one study, it was shown that the method of preparing and freeze-drying the cells had much more impact on their subsequent stability to freezing and storage than did microentrapment in a protein gel (Reid et al., 2007). Furthermore, encapsulation has been unsuccessful in enhancing the stability of probiotics in cheese (Gobbetti et al., 1998; Godward and Kailasapathy, 2003). Although cultures spray-coated with fats would seem ideal for protection against acid and oxygen, this protection only seems transient.

19.5.5 At the Consumer Level

Most studies on stability during storage have been carried out to simulate warehouse or grocery store conditions. In these instances, the packages are not opened and cells are protected from the detrimental effect of oxygen, if packaging is appropriate. One study examined the stability of probiotics during storage of a fruit juice blend at home (Champagne et al., 2008a, b). In this instance, 1 L containers were opened, sampled, and the remainder of the product stored in refrigerator. With *L. rhamnosus* R0011, there was no detrimental effect on viability due to shaking and introduction of oxygen in the product, over a 3 week storage period. This might be partially due to the addition of an antioxidant in the juice blend (ascorbic acid). It remains to be determined if more oxygen-sensitive probiotic strains will react in the same fashion.

19.6 Conclusion

Adding probiotics to fermented milks holds many challenges. In addition to the processing and storage issues which were treated in this chapter, it must be kept in mind that strain selection, enumeration of viable populations in fermented products containing starter cultures as well as impacts on sensory properties must be examined. These issues could be the subject of a chapter in itself.

But these challenges also provide the opportunity for novel products. Thus, ingredients which are added to the food matrix can be selected not only for their beneficial effect on growth and stability of the probiotics in the FF but also to provide additional health benefits. As an example, antioxidants could be used to protect the probiotics from the detrimental effect of oxygen during storage, but also for the potential risk reduction of cancer.

Another challenge industry faces is obtaining a health claim. It cannot be assumed that simply adding a given number of probiotic bacteria to a FF will enable the transfer to the FF the health attribute which demonstrated with nutraceuticals or caplets. Indeed, it has been shown that the form of the probiotic consumed strongly affects recovery levels in the gastro-intestinal tract (Saxelin et al., 2003). It can thus be foreseen that regulatory organizations will eventually demand clinical trials with the given matrix. Nevertheless, the FF industry can be optimistic. Some foods enable better survival of probiotics to the GI tract that do powders containing unprotected cells (Saxelin et al., 2003). This raises the

perspective that FF might be even better delivery formats for probiotics than non-encapsulated cells in caplets or powders.

List of Abbreviations

a_w	water activity
B.	*Bifidobacterium*
CFU	colony-forming units
DVI	Direct Vat Inoculation
FF	Functional Food
FOS	fructo-oligosaccharides
HPD	high probiotic density
L.	*Lactobacillus*
ME	microentrapped
NFMS	Non-fat milk solids
S.	*Streptococcus*
UK	United Kingdom
USA	United States of America
USD	United Stated Dollars

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20 Micro-Encapsulation of Probiotics

Jean-Antoine Meiners

20.1 Introduction

Micro-encapsulation is defined as the technology for packaging with the help of protective membranes particles of finely ground solids, droplets of liquids or gaseous materials in small capsules that release their contents at controlled rates over prolonged periods of time under the influences of specific conditions (Boh, 2007). The material encapsulating the core is referred to as coating or shell.

The majority of microcapsules have spherical shapes and their diameter varies from a few microns to 1 mm. However, some authors consider particles of even more than one millimeter as microcapsules. For the purpose of this chapter the term microcapsule refers to capsules whose aim is the protection and controlled release of the active substance, and the term microsphere, a commonly used term in the scientific literature, refers to granules, that do not have a core-shell morphology, and can be simply defined as the embedding of an active substance in a matrix (Watheley, 1996).

In general, encapsulation can be used to improve the stability of the active substance during processing and storage, mask unpleasant flavors and odors, control possible oxidative reactions, and provide controlled release at the right place and the right time. As such, it has numerous applications in the food, pharmaceutical, cosmetic, agricultural, textile, paper and paint industries. One of the earliest published inventions was the carbonless copy paper in which tiny microcapsules were fixed on the backside of a sheet of paper; these were crushed by the pressure of writing, thus releasing their dye (Green, 1957). In the areas of pharmaceuticals and chemicals, many products, e.g., enzymes, can cause health and safety hazards when manipulated in a very fine powder form, due to excessive dust formation; granulation into larger size particles and coating can be used to alleviate such handling problems (Meesters, 2006). In terms of food applications, the aims of encapsulation techniques are to improve the stability of bioactive

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ingredients during processing and storage and to prevent undesirable interactions with the food matrix (Champagne and Fustier, 2008). An example is the case of ascorbic acid, a highly reactive, water soluble and heat-labile compound, encapsulation of which can double its shelf life compared to the free form (Wilson and Shah, 2007).

Various approaches can be used to open up the microcapsules to release the active substance. These include (i) mechanical rupture of the membrane, e.g., in the case of mastication of micro-encapsulated flavours in chewing gums, (ii) exposure to high temperatures to make the coating material melt, a technology frequently used for encapsulated chemical leavening agents in baked goods, (iii) dissolution of the capsules when placed in solvents, (iv) exposure to specific pH, (v) biodegradation of the polymer coating by enzymes, (vi) diffusion of the active substance through the polymer coating, (vii) high osmotic pressure inside the microcapsule and (viii) combinations of the above (Pothakamury and Barnosa-Canovas, 1995).

In the area of microbial products, micro-encapsulation is used in order to enhance the delivery of probiotic microorganisms into foods during processing and storage, or to protect against the acidic conditions in the stomach and ensure delivery into the intestine. In addition, microbial cells can also be immobilized onto polymer matrices and used as biocatalysts in fermentation processes. The main difference between encapsulation and immobilization is that in the latter the polymer beads produced allow fast and easy diffusion of water and other fluids, and thus the cells are biochemically active (Klein and Vorlop, 1985).

20.2 Micro-encapsulation Techniques and Processes

A variety of encapsulation techniques are available and include both chemical processes, such as phase microseparation, coacervation, liposome encapsulation molecular inclusion, as well as physical processes, such as spray drying, spray chilling, prilling, spinning disk, fluidized bed coating, and extrusion. Certain steps are common to many of these processes.

Basically the initial step is to introduce the active substance inside the shell; this involves dispersion or atomization in order to position the membrane on the outside of the microcapsule and the active substance in the core.

In the case of microcapsules containing a liquid core active substance an emulsion is prepared, whose surface is polymerized in a subsequent step, the interface condensed and/or the solvent evaporated. In the section below, the different types of encapsulation methods are presented in more detail.

20.2.1 Spray Drying

Spray drying is one of the most commonly used encapsulation method, as it is a well established and cost-efficient technology; it is basically designed to evaporate water from the dry matter. The solution is injected in a hot air stream in a closed vessel and the solvent, which most of the times is water, is evaporated. The energy is absorbed to evaporate the water and consequently the powder temperature can be controlled. The residence time in the tower is one of the limiting factors; the majority of the moisture has to evaporate during the fall time in the tower of the particles (Adamiec and Marciniak, 2004). In order to be efficient, the total surface area should be as large as possible and consequently the droplet sizes small, and if possible, of the same size.

The principle of the technique is based on a spray, which is created by forcing the fluid through an orifice. The energy required to overcome the pressure drop upon exiting the orifice is supplied by the spray dryer feed pump. Pressure nozzles coupled to high pressure feed pumps, which produce pressures of as much as 3000 psig, have the advantage of producing a narrow particle size distribution, but can have severe damaging effects on a microorganism's cell structure. Despite the fact that they are less energy efficient and produce narrower particle size distribution, the two-fluid nozzle is very popular for laboratory equipment, probably since it allows working with small flow rates. The most popular nozzle type for industrial spray drying is the centrifugal atomizer, where a spray is created by passing the fluid across or through a rotating wheel or disk. The benefit for micro-encapsulation purposes is that rather large particles, up to 100 μm, can be produced this way.

20.2.2 Spray Chilling and Cooling

Spray chilling and spray cooling (or congealing), the second being operated at lower temperatures, involve mixing thoroughly the core and a molten shell material with a melting temperature well above the operating temperature (usually lipids), and atomizing through a two media nozzle into a cooling chamber in order to solidify the droplet instantaneously. One of the limitations is the speed in heat transfer of the energy freed during the re-crystallization. The use of cooling media has allowed high volume and high speed production. Another limiting factor is the difference in surface tension between the matrix and the core material. Consequently, the core is not always placed in the center of the microcapsule, which may affect the protective properties of the microcapsule (Meiners, 2004).

20.2.3 Prilling

Hawley's Condensed Chemical Dictionary says that prills are 'small round or acicular aggregates of a material'. Prilling can be a useful technology, when solidification of the shell material is an instantaneous reaction. The core and the shell material are mixed thoroughly and the dispersion is pumped to flow over a sonically vibrating dispersion head. Gravity allows the droplets to fall into the collection device for cooling or polymerization (Wu et al., 2007). More advanced and sophisticated equipment have been developed that limit considerably the residence time of the dispersion and the need for large cooling towers (Meiners, 2004).

20.2.4 Spinning Disk

A comparable technology to prilling is the spinning disk technology, which uses centrifugal forces for droplet separation.

The droplet volumes and their mutual spacing are governed by the channel geometry and the frequency of rotation. Devices exist that combine spinning disk forces with high frequency droplet separation (Chesnokov, 2001).

20.2.5 Fluidized Bed

Fluidized bed technology is based on the separation of individual particles in a gas stream and the fixation of the membrane substance by polymerization, drying or crystallization around the core. The solvation and drying steps can be avoided, which for thermo-sensitive materials may represent a major advantage. Agglomeration and retention in the filter system cause the use of the fluidized bed system to be difficult with products having strong adhesive properties (Guignon, 2002).

20.2.6 Extrusion

Micro-encapsulation by extrusion involves projecting an emulsion core and coating material through a nozzle at high pressure. It involves preparing a hydrocolloidal solution, adding the active substance and extrusing the suspension through a nozzle in the form of droplets into a hardening solution or setting bath (Krasaekoopt et al., 2003). Carbohydrate matrices in the glassy state have very

good barrier properties and extrusion is a convenient process enabling the encapsulation of active substances in such matrices (Gouin, 2004).

20.2.7 Coacervation

Coacervation consists of the separation of colloid particles from a solution, which then agglomerate into a separate liquid phase called coarcevate. A number of hydrocolloid systems have been evaluated for coacervation micro-encapsulation including among others the gelatine/gum acacia, heparin/gelatine, carageenan, chitosan, soy protein, lactogloboulin/gum acacia and the guar/dextran system (Gouin, 2004).

20.2.8 Liposomes

Liposomes are artificially made microscopic membrane vesicles consisting of one or more concentric layers of lipids. They are formed by dispersing the lipid formulation in a solvent system, decreasing the solvent volume and then redispersing the film of lipid/solvent in an aqueous phase (Bangham, 1995).

20.2.9 Inclusion Complexation

Cyclodextrins are cyclic oligomers, who have the ability to form inclusion complexes with the active substances. They are typically used for the protection of unstable and high value chemicals, such as flavours. Oil-in-water emulsions using cyclodextrins can also be subsequently spray dried (Astray et al., 2009).

20.3 Technologies used for the Immobilization and Micro-encapsulation of Microganisms

Immobilization of living cells was the first form of micro-encapsulation and was pioneered about 50 years ago for medical purposes, and (Chang, 1964). The first patent demonstrating the biocompatibility of polymers and the resistance of the encapsulated material to sterilization conditions was granted in 1965 (Mauvernay, 1965).

Immobilization of microorganisms for bioprocessing purposes became important for the development of the continuous fermentation process. Much of the

research work was done by the brewing industry using immobilized yeast cells and the dairy industry using immobilized lactic acid producing bacteria (Prevost and Divies, 1988).

The pore size of the microcapsules produced has an important effect on the cellular biochemical activities, as it controls the rate of retention/passage of undesirable metabolites (Klein et al., 1983). Different methods have been used to immobilize microorganisms, including, physical entrapment in a polymeric network, attachment or adsorption to a carrier, and membrane entrapment.

In order to entrap the biomass in microcapsules simultaneous with the membrane formation, it is important for the that droplets to be generated simultaneously with the membrane. For this purpose, specific equipment has been developed and polymers have been selected. The polymers generally form a gel under the influence of ionization or thermosetting.

For the purpose of droplet separation with a narrow size distribution, a pumping system under gravity provides a constant uninterrupted flow of the liquid. In the early droplet generators, the liquid consisted of a solution of the biomass and the dissolved polymer. Upon falling into the collection bath filled with a solution containing the ionic solution, the polymer membrane was cross-linked to form self sustaining microcapsules, encapsulating the biomass droplet within the membrane (Sheu and Marshall, 1993). The more recent versions use a nozzle designed for co-extrusion, creating a polymer network around the droplet during its fall into the collection bath. The different dripping systems can be identified according to the principles below. *Figures 20.1–20.4* depict the various types of nozzles used.

- Dripping without assistance by gravity into the collection bath
- Dripping assisted by an air stream
- Dripping assisted by an electrostatic force
- Laminar jet break up assisted by vibration
- Jet break up assisted by rotation and vibration
- Co-extrusion assisted by laminar jet break up.

The use of static mixers has also appeared to be very promising, since it allows large volume and cost efficient production. The device consists of mixer elements contained in a cylindrical (tube) or squared housing. These can vary from 6 mm to 6 meters diameter. Static mixer elements consist of a series of baffles. As the streams move through the mixer, the non-moving elements continuously blend the materials (Maa and Hsu, 1996).

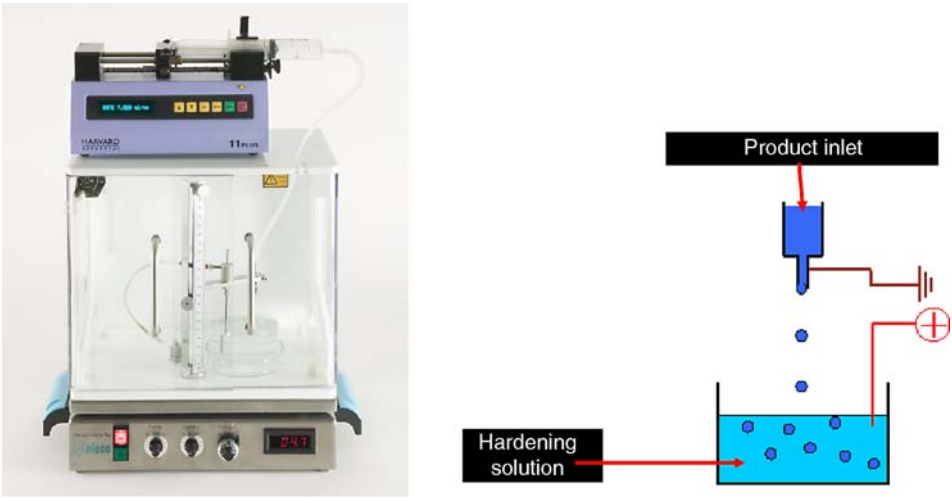

Figure 20.1
Dripping assisted by an electrostatic force (courtesy of Nisco Engineering AG).

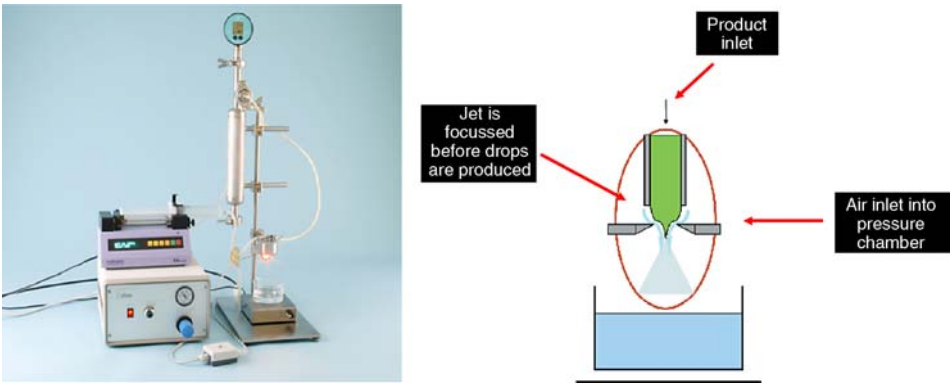

Figure 20.2
Dripping assisted by aerodynamically jetting (courtesy of Nisco Engineering AG).

20.4 Objectives for the Micro-encapsulation of Probiotics

A major function of micro-encapsulation is to provide protection against the high acidity of the gastric fluids. A microcapsule containing the probiotic must not be fractured until it passes through the stomach. Since the biological release mechanism is triggered by the higher pH in the upper intestine, a coating can be used that

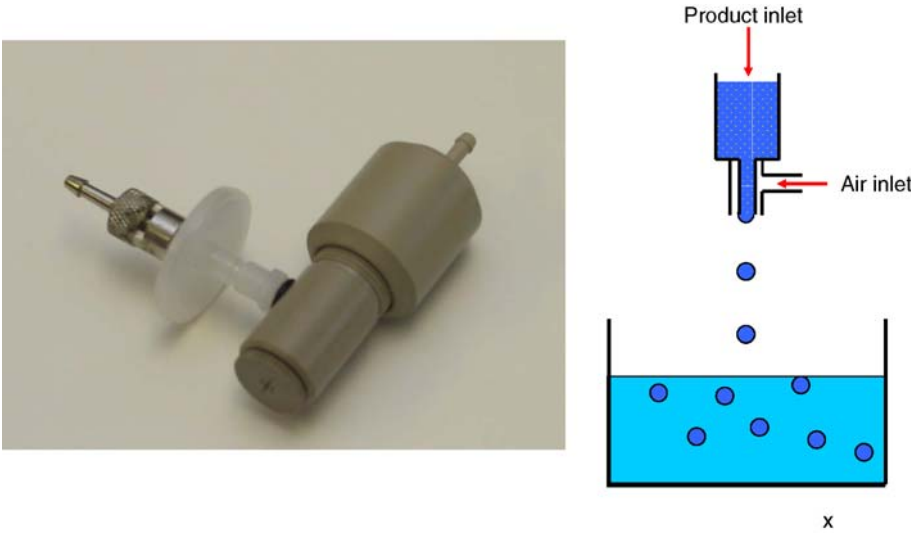

Figure 20.3
Dripping assisted by co-axial air (courtesy of Nisco Engineering AG).

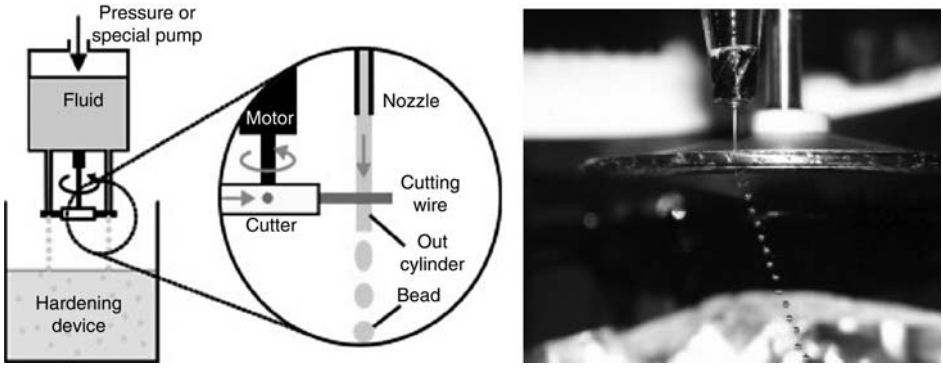

Figure 20.4
Jet cutter (courtesy of Genialab AG).

withstands the low pH and can release its content at a pH similar to that of the large intestine (e.g., pH 5.5 to 7). The survival of commercial probiotics in conditions of very low pH was recently investigated; the researchers assessed *in vitro* the survival of 32 probiotic strains, all isolated from commercially available products

in simulated gastric contents. Approximately 50% of them were viable after 20 minutes exposure; eighteen strains were further tested in respect to their bile tolerance; five of them showed poor tolerance, whereas seven of them showed moderate tolerance and the rest high tolerance (Gibson et al., 2005). The above highlight the importance of developing effective micro-encapsulation methods, especially for strains that are less robust than others. Another function of micro-encapsulation of probiotics is to enhance the viability of the cells during processing and storage. To this end, it is important to highlight that the final application of the product may dictate the use of totally different technologies, or different shell or carrier materials. For example, in the case of dried products (e.g., foods or neutraceuticals), the water content, and in particular the water activity, is one of the most important factors influencing cell survival, as it has a direct effect on the metabolic activity of microorganisms. Water activity (a_w) describes the (equilibrium) amount of water available for the hydration of materials. Water activity is the effective mole fraction of water, defined as $a_w = \lambda_w X_w = p/p_0$, where λ_w is the activity coefficient of water, X_w is the mole fraction of water in the aqueous fraction, p is the partial pressure of water above the material and p_0 is the partial pressure of pure water at the same temperature. Based on the above, a non-water permeable capsule material would be the first type of material to evaluate for using in a dried product. If the capsule material is chosen from non-ionisable polymers, the protection against the gastric acidity would likely improve too.

In addition to the above, it must be noted that the suitability of a particular encapsulation technology or material depends on the properties of the specific probiotic strain (e.g., acid tolerance, oxygen tolerance, bile tolerance). It is generally difficult to simply transfer the results from one strain to another; significant amount of experimental work is needed to identify and optimise suitable encapsulation methods. Below is a list of applications of encapsulated probiotics in the food and non-food sectors.

- Cell immobilisation (e.g., industrial fermentations)
- Micro-encapsulation of probiotics in dairy and beverage applications
- Micro-encapsulation of probiotics in dry food applications
- Micro-encapsulation of probiotics in nutritional or medical supplements

Table 20.1 lists the encapsulation technologies and the types of shell materials commonly used for the micro-encapsulation of probiotics.

Table 20.1

Technologies used for the micro-encapsulation of probiotics (Adapted from Anal and Singh, 2007)

Technology	Shell material	Micro-encapsulation steps
Spray drying	Water soluble polymers	(a) Incorporate the microorganisms into the solution
		(b) Atomization into spray
		(c) Evaporation of solvent
		(d) Separation of powder
Spray chilling	Lipids, waxes	(a) Incorporate the micro organisms into the melt
		(b) Atomizing into spray
		(c) Solidification of the coating by chilling below the melting temperature
Air suspension coating	Water insoluble and water soluble polymers, lipids, waxes	(a) Preparation of the coating melt or solution
		(b) Fluidizing the core particles
		(c) Atomizing small droplets of the coating material around the core particles
		(d) Drying, solidifying, crystallizing the coating with core in the center
Extrusion; Jet cutter; Static mixer	Water soluble polymers	(a) Active substance is dissolved into the polymer solution
		(b) Dripping the solution into the collection bath
		(C1) Cross linking the polymer with divalent ions
		(C2) Gelling the polymer by thermosetting
		(C3) Complexing with a polyelectrolyte
Co-extrusion	Water soluble polymers	(A1) Active substance is pumped to the inner nozzle port
		(A2) Polymer is pumped to the outer nozzle port
		(b) Dripping the active substance and polymer into the collection bath
		(C1) Cross linking the polymer with divalent ions
		(C2) Gelling the polymer by thermosetting

20.5 Biopolymers

It is obvious that the biopolymers applied must be biocompatible and non toxic to the cells. The most popular biopolymers for micro-encapsulation of micro-organisms are discussed below:

Alginate Alginate is one of the most used polymers for the encapsulation of microorganisms. It can be found in the cell walls and intercellular spaces of brown algae. It is a linear co-polymer with homo-polymeric blocs, covalently linked in different sequences, depending to the source of algae. Alginic acid, the free acid from alginate is the intermediate product in the commercial alginates and has limited stability. In order to make stable water soluble products alginic acid is transformed into a range of salts i.e., Ca-alginate; Na-alginate; K-alginate; Mg-alginate; NH_4-alginate.The ratio of mannuronic acid to gluconic acid and the structure of the polymer determine the properties of alginate in solution. Alginates may be prepared with a wide range of molecular weights. Alginate capsules are formed by dripping an aqueous alginate solution into a solution containing a multi-valemt cation, usually a calcium salt. The calcium ion attaches to two polymer strands by replacing the salt bond, and can thus form a very fine network.

Carrageenan Carrageenan is family of a naturally occurring sulphated poly-saccharide which fill the voids of the cellulosic structure of red seaweed. Carrageenans are made up of repeating galactose units and 3,6 anhydrogalactose, joined by alternating glycosidic linkages. They are divided into three classes, k-, i- and l- carrageenan, which have different properties. The plant source and the extraction method determine to a large extent the molecular structures and properties; e.g., carrageenans with different amounts of sulphate ester groups will associate differently with metal ions. All types are all soluble in hot water, but only the k- type is soluble in cold water. Gelation of carrageenan involves helix formation, and is induced by cooling down to room temperature from a hot solution. Potassium and calcium ions are also essential for gelling, as they stabilize the gel, prevent swelling, or induce gelation (Krasaekoopt et al., 2003).

The strength of the carrageenan capsules can be improved using locust bean gum (LBG) at a ratio of carrageenan to LBG of 2:1. LBG is part of the galacto-mannan family. It is extracted from the kernels of the carob tree; it forms a food reserve for the seeds and helps to retain water under arid conditions. LBG consists of a backbone of b-(1,4)-D- mannose units; approximately every fourth mannose unit there is a substitution by a α-D-galactose side chain. The level of substitution is important for the properties of the gum, as the galactomannan can associate and self cross-link. LBG requires heating to dissolve in water and is rarely used as a

single shell material (Rees, 1972). The increased strength of carrageenan/LBG capsules is due to synergistic effects, which are attributed to the interaction between the double stranded helix of k- carrageenan and the un-branched segments of the D- mannose backbone of the LBG molecule.

Chitosan Chitosan is a weak anionic polysaccharide consisting mainly of β(1,4) linked glucosamine units together with some N-acetylglucosamine units. It is produced by de-acetylation of chitin extracted from crustacean shells. It is positively charged and water soluble at pH below 6.5, and this enables it to form polyelectrolyte complexes with negatively charged materials, such as polyphosphates, $[Fe(CN)6]^{4-}$ and $[Fe(CN)6]^{3-}$, and citrate (Peniche et al., 2003).

Starch Starch is a polysaccharide consisting of a large number of glucose monosaccharide units joined together by glycoside bonds. Starch consists of two types of molecules, amylose (normally 20 – 30%) and amylopectin (normally 70 – 80%). Both consist of polymers of α-D-glucose units. Of the two components of starch, amylose has the most useful functions as a hydrocolloid. Chemical modification of starches, such as cross-linking, oxidization, acetylation, and hydroxypropylation may confer interesting changes in functionality. For example, octenylsuccinic acid anhydride (OSAN)-modified starches are popular for their emulsifying properties, as they contain both hydrophobic and hydrophillic groups. In addition, resistant starch, i.e., the indigestible form of starch, offers an ideal surface for adherence for the probiotic microorganisms during processing, storage and their passage through the gastrointestinal tract (GIT) (Anal and Singh, 2007). Mixing starch with κ-carrageenan, alginate, xanthan gum and low molecular weight sugars is a popular practice in micro-encapsulation as they reduce starch retrogradation. Starch derivatives mostly hydrolyzed forms such as dextrins and maltodextrins, are also frequently used as carrier material for spray and freeze drying (Anal and Singh, 2007).

Gum arabic Gum arabic is a hydrocolloid produced by the natural exudation of acacia trees. Because it is a complex mixture of molecules and the fact that the material varies significantly with the source, the investigation of the exact molecular structures still attracts considerable research interest. It is generally composed of a highly branched polysaccharide fraction consisting of galactose, arabinose, rhamnose and glucuronic acids. It also contains an arabinogalactan-protein complex in which arabinogalactan chains are covalently linked to a protein chain through serine and hydroxyproline groups (Dror et al., 2006). The protein plays an important role in the functionality of the gum. The simultaneous presence of hydrophilic carbohydrates and hydrophobic protein gives the molecule its emulsification and stabilization properties (Randall et al., 1988).

Gum arabic is used to produce gum arabic / gelatine coacervates, which have been used as encapsulation materials. The formation of coacervates is possible only at pH values below the isoelectric point of gelatine, which is approximately at pH 8. It is at these values that gelatine becomes positively charged, whereas gum arabic continues to be negatively charged. The formation of stable micro-capsules using mixtures of gum arabic and whey protein has also been reported (Weinbreck et al., 2004).

Pectin Pectin is a major cell wall component in plants, playing a role in the control of cell growth and the defence against the invasions of microorganisms. Pectins are composed of a α-D-galacturonic acid, which are interrupted by single α-L-rhamnose residues. A major difference between pectins is their content in methyl esters. The degree of esterification (DE) is defined by the number of esterified D-galacturonic acid residues. High methoxyl pectin forms gels due to hydrophobic interactions and hydrogen bonding between pectin molecules. Low methoxyl pectin forms gels in the presence of di- and polyvalent cations, which cross-link and neutralise the negative charges of the pectin molecule (Wher et al., 2004).

Gelatin Gelatin is a high molecular mass polypeptide derived from connective animal tissue, such as bone and skin. The protein chain unfolds upon melting and upon cooling it forms a coil-helix structure and entraps water, forming a reversible gel. Gelatin is extracted after acid pre-treatment and has an isoelectric point (IP) of 7–9.4; gelatin after lime pre-treatment has an IP of 4.5–5.3. The possibility to vary the IP, and thus the charge by adjusting the pH makes gelatin a favourite candidate for micro-encapsulation. However, in order for the capsules to be self sustainable cross-linking is required; and the cytotoxity of the traditionally applied organic solvents makes the process less suitable for microorganisms (Hyndman et al., 1993). However, as mentioned previously, gelatin is used due to its amphoteric nature in co-operation with anionic carbohydrates to form gum/gelatine coacervates.

Whey protein Whey protein is the protein obtained from whey during cheese making. Whey proteins are obtained by ultrafiltration, during which, the low molecular weight compounds such as lactose, minerals, vitamins and non-protein nitrogen are removed in the permeate while the proteins become concentrated in the retentate. After ultrafiltration, the retentate is pasteurized, sometimes evaporated, and then dried usually by spray drying at low temperatures in order to avoid significant protein denaturation.

Whey protein is very popular for its film forming characteristics and is used as a protective material in spray drying, resulting in a water soluble microcapsule system (Picot and Lacroix, 2004).

Other polymers There is a large choice of acid resistant polymers with designed release at intestinal pH values. Most of them have been approved for pharmaceutical purposes, while only few of those have food approval: hydroxypropyl methylcellulose; methylcellulose; ethylcellulose; hydroxypropyl methylcellulose phthalate; hydroxypropyl methylcellulose acetate succinate; poly(methyl methacrylate); carboxymethyl cellulose; polyvinyl acetate phthalate; methylcellulose phthalate; cellulose acetate phthalate; polyvinyl acetate phthalate; polyvinyl pyrrolidone; carboxypolymethylene. Most of these products are only soluble in alcohol and cannot be brought into direct contact with the culture; therefore they can be applied as an outer top coating only. Finished products are in general found in the pharmaceutical or the nutritional supplement markets.

20.6 Applications of Micro-encapsulation of Probiotics

In this section, some examples from the various studies on micro-encapsulation of probiotics are presented. The area of cell immobilization for use in fermentation systems has attracted considerable interest recently for both probiotic and non probiotic lactic acid bacteria (LAB). The advantages of immobilized cells over free cells include enhanced biological stability, high biomass concentration, increased product yields, increased product stability, and the ability to separate and re-use cells (Dervakos and Webb, 1991; Lacroix and Yidirim, 2007). As such, immobilisation techniques have the potential for enhancing the performance of probiotics and produce strains with specific physiological and functional characteristics, as well as improved technological properties.

The alginate system has been studied extensively for the encapsulation of probiotics. The research suggests that the size of the beads affects considerably cell survival under simulated gastrointestinal conditions (Lee et al., 2000; Chandramouli et al., 2004). Beads of less than 100 μm in size did not significantly protect *Bifidobacterium* cells; the protection was increased as the beads became bigger, especially for very large beads with sizes higher than 1 mm. However, such large beads cause a coarseness of texture in food systems (Hansen et al., 2002).

Starch has also been investigated as a potential encapsulation material for probiotics. Researchers have developed a micro-encapsulation technology that involves encasing bifidobacteria in the hollow core of partially hydrolyzed granular starch, which is then encapsulated with an outer coating of amylose.

This technology is designed to protect the probiotic bacteria from adverse environmental conditions during processing, in products during storage, and during passage through the upper GIT for delivery in the large intestine (Mattila-Sandholm et al., 2002).

Mixtures of various polysaccharides and in some cases proteins have also been investigated as a potential encapsulation materials for probiotics. For example, alginate was combined with pectin and whey proteins in order to protect *Bifidobacterium bifidum* during transit through the GIT. A clear improvement in cell survival was observed compared to free cells, although still a significant percentage of the cells were dying (Guerin et al., 2003). In another study alginate-starch gel beads were produced to protect *Lactobacillus acidophilus* and *Bifidobacterium lactis;* it was found that encapsulation prevented cell death from oxygen toxicity (Talwalkar and Kailasapathy, 2003). It was also found that chitosan-coated alginate beads were offering protection to various LAB strains during storage in milk (Krasaekoopt et al., 2004), as well as in simulated gastrointestinal fluids (Lee et al., 2004). In another study, a polysaccharide from kiwifruit was combined with alginate and chitosan and shown to improve the survival of *Lactobacillus rhamnosus* at low pH (Ying et al., 2007). A low-cost micro-encapsulation technique has been recently proposed, which consists of coating milk fat droplets containing powder particles of freeze dried cultures with whey protein and polymers, using emulsification and spray drying in a continuous two step process. Rigorous control of the size distribution of the different elements constituting the microcapsule is required. In particular, the size of the material dispersed in the hydrophobic phase must be larger than that of the globule (Picot and Lacroix, 2004).

The use of polymers as immobilisation matrices for probiotics during freeze and spray drying has also been investigated. It was shown that immobilization of *Lactobacillus acidophilus* with k-carrageenan could enhance the temperature tolerance of the freeze dried cells and improve their storage stability (Tsen et al., 2002). The results from other studies regarding the role of specific polymers on cell survival during storage were less conclusive, and were highly dependent on the species (Champagne et al., 1996a, 1996b).

In the case of spray drying, most of the probiotic strains do not survive well the high temperatures and the osmotic stress to which they are exposed to in the process. The temperature and phase changes, and drying, stress the cells and damage their membranes. As a result their activity is typically lost after a few weeks of storage at room temperature (Anal and Singh, 2007). One of the methods that has been proposed to circumvent this is the incorporation of

acacia gum into the drying medium; it was shown that this increased significanlty the survival rate of *Lactobacillus paracasei* during storage at a variety of temperatures (Desmond et al., 2002).

The size of the market for probiotics in animal feed applications has increased significantly after the ban on antibiotics as growth promoters. Since feed additives are generally stored under 'barn conditions', i.e., ambient temperature, oxygen and moisture, micro-encapsulation can help to improve the survival of probiotics. In this regard, and taking into account the relationship between high moisture content and death, a watertight shell is required to achieve high protection. Fat encapsulated probiotics have shown to be 'barn stable' for a period of over 2 years without refrigeration (Meiners, 2004). In this process, also called 'hot melt' process, very thin layers of molten lipids are applied to the surface of freeze dried cultures in fluidized bed equipment. After re-crystallization, very tight microcapsules are formed. The properties of the microcapsules depend on the choice of lipids, the moisture residue in the fluidizing air stream and the process parameters, such as the droplet size of the molten lipid and the temperature of the atomizing air.

20.7 Conclusion

The micro-encapsulation of probiotics has received a lot of attention, as it can help to improve probiotic survival during processing and storage, as well as during passage through the GI tract. A number of encapsulation techniques, such as spray drying, spray coating, phase separation and extrusion, a well as natural biopolymers, such as alginates, carrageenan, chitosan and pectin have been studied. Although promising at a laboratory scale many of these technologies are difficult to scale up. Further research is needed for the development of scalable and effective technologies as well as for the design of controlled release delivery systems.

List of Abbreviations

DE Degree of Esterification
GIT Gastrointestinal Tract
IP Isoelectric Point
LAB Lactic Acid Bacteria
LBG Locust Bean Gum

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21 Probiotics and Antibiotic-Associated Diarrhea and *Clostridium difficile* Infection

Christina M. Surawicz

21.1 Introduction

Diarrhea is a common side effect of antibiotics. Antibiotics can cause diarrhea in 5–25% of individuals who take them but its occurrence is unpredictable. Diarrhea due to antibiotics is called antibiotic-associated diarrhea (AAD). Diarrhea may be mild and resolve when antibiotics are discontinued, or it may be more severe. The most severe form of AAD is caused by overgrowth of *Clostridium difficile* which can cause severe diarrhea, colitis, pseudomembranous colitis, or even fatal toxic megacolon. Rates of diarrhea vary with the specific antibiotic as well as with the individual susceptibility.

Risk factors for antibiotic-associated diarrhea (AAD) include broad spectrum antibiotics, especially ampicillin or amoxicillin, cephalosporins, and clindamycin, although other antibiotics may be involved (McFarland, 1995). AAD results in longer hospital stays (8 days on an average), higher cost of care ($2,000–4,000 USD), a fivefold increase in other nosocomial infections and a threefold increase in mortality (0.7–38%) (McFarland, 1998).

The pathophysiology is not completely understood, but changes in fecal flora may result in altered carbohydrate metabolism of undigested carbohydrates with an osmotic diarrhea. The fecal flora normally ferment unabsorbed carbohydrates and produce short chain fatty acids. A change in the fecal flora could alter this fecal fermentation, resulting in changes in pH as well as changes in carbohydrate by-products which could cause an osmotic diarrhea (Clausen et al., 1991). Other possible mechanisms are reduced anaerobic flora or overgrowth of potential pathogens, such as *Staphylococcus aureus*, *Klebsiella oxytoca* or *Candida*

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(Surawicz, 2005). Finally, direct effects of the antibiotic on the intestinal mucosa could cause fluid secretion and diarrhea.

Antibiotics have a major effect on the gastrointestinal bacterial flora. The normal flora consists of over 500 distinct species of bacteria, most of which are anaerobic. Various antibiotics alter the flora in different ways; some suppress the anaerobic flora, other alter the aerobic flora (Surawicz, 2005). Thus, it makes sense that probiotics could have a role in either preventing this disruption or normalizing any effects. In fact, evidence for the efficacy of probiotics is strong for the prevention of AAD.

Most trials of probiotics to prevent AAD have used a similar study design: probiotic versus placebo in patients getting antibiotics with measurement of diarrhea as the outcome. There are only a few studies of probiotics to prevent *C. difficile* infection (CDI), which is a much smaller subset of AAD. However, evidence for the prevention or treatment of CDI is lacking with the exception of preventing further recurrences of CDI after one or more recurrences.

21.2 Antibiotic Associated Diarrhea – Prevention

There is ample data to support the role of probiotics in the prevention of AAD. Several recent meta-analyses have concluded that probiotics have efficacy. The two agents that are effective, with strong evidence to support them, are the bacteria *L. rhamnosus* GG and the yeast *Saccharomyces boulardii* (*Saccharomyces cerevisiae*). Other agents that have been studied are *Bacillus clausii*, *Bifidobacterium longum*, *L. acidophilus*, *L. delbrueckii* subsp *bulgaricus* (*L. bulgaricus*), *L. plantanum* 299v, *Enterococcus faecium* SF68, *Clostridium butyricum* MIYAIRI and mixtures of probiotics and prebiotics. However, the evidence for their efficacy is not as strong (see *Table 21.1*).

21.2.1 Specific Probiotics

21.2.1.1 Lactobacilli

These are often found in fermented dairy products and survive an acid environment by producing lactic acid. Some strains produce bacteriocins, which are rarely pathogenic. *L. acidophilus* is part of the normal flora. A study with Lactinex, a commercial product containing *L. acidophilus* and *L. bulgaricus*, in

Table 21.1

Probiotics in the prevention of AAD – evidence-based analysis. See section 21.6 for explanation of grading levels

Probiotic		
Lactobacillus rhamnosus GG	Grade A	Level 1a
Saccharomyces boulardii	Grade A	Level 1a
L. acidophilus and *L. bulgaricus*	Grade A	Level 1d
L acidophilus and L. casei	Grade A	Level 1c
L. casei, L. bulgaricus and *S. thermophilus*	Grade A	Level 1c
Bifidobacterium lactis and S. thermophilus	Grade A	Level 1c
Bifidobacterium longum	Grade A	Level 1d
Clostridium butyricum	Grade A	Level 1c
Enterococcus faecium	Grade A	Level 1d

which the product was provided to 38 children failed to show any benefit, with diarrhea in 66% of treated children and 69% of controls. One difficulty in evaluating this study is that these are very high rates of diarrhea compared to average rates, thus this study population may not be typical (Tankanow et al., 1990). In seventy nine adults, none being given Lactinex had diarrhea compared to 14% of controls ($p < 0.05$); this is a small study (Clements et al., 1983). In these two studies Lactinex appeared to prevent AAD in adults but not in children.

L. rhamnosus GG is a strain identified by Gorbach and Golden (1987) (Goldin et al., 1992). It is stable in acid and bile and also produces a bacteriocin. Bacteriocins are peptides or polypeptides that are produced by some lactic acid bacteria and have antibacterial activity against some microorganisms. Thus strains that produce bacteriocins may alter the flora. *L. rhamnosus* GG also results in lower levels of fecal β-glucuronidase which may also affect the flora. The prevention of AAD by *L. rhamnosus* GG has been shown to be effective in many trials (including several in children) as compared to placebo. However, a later randomized controlled trial did not show efficacy. Three studies of *L. rhamnosus* GG in children showed decreased rates of AAD – a total of 388 children were studied (Arvola et al., 1999; Szajewska et al., 2001; Vanderhoof et al., 1999). Szajewska et al. used *L. rhamnosus* GG to prevent nosocomial diarrhea in 81 children aged one to thirty six months of age. Out of 45 children in the *L. rhamnosus* GG group, 6.7% developed diarrhea as compared to 33.3% of 36 in the placebo group (Szajewska et al., 2001). Vanderhoof et al. studied two

hundred children among of whom 188 completed the study. The children were given either *L. rhamnosus* GG or placebo. Overall, 7/93 (7.5%) of the *Lactobacillus* group had diarrhea as compared to 25/96 (26%) in the placebo group (Vanderhoof et al., 1999). In a third study, there was less AAD compared to placebo in children treated for respiratory infections (Arvola et al., 1999).

Several studies have been conducted in adults with *L. rhamnosus* GG; in controls, diarrhea rates were 15–33% as compared to 5–7% for treated patients ($p < 0.05$). In a study of adults receiving erythromycin, those given *L. rhamnosus* GG had less AAD (Siitonen et al., 1990). However, in a large study of 267 hospitalized adults, diarrhea rates were similar, 30% with controls and 29% with *L. rhamnosus* GG (Thomas et al., 2001).

L. rhamnosus GG has also been given with other probiotics in various combinations, and studied for the effect on AAD. A milk drink containing *L. rhamnosus GG*, *L. acidophilus* La-5, and *B. lactis* BB-12 showed significant decrease in AAD (5.9%) compared to placebo (27.6%) in a controlled trial of 63 patients (Wenus et al., 2007).

In a large study in India, patients receiving preoperative antibiotics (ampicillin and cloxacillin) prior to cataract surgery were randomized to lactobacilli versus placebo. For this study, 740 cases were enrolled. There was no diarrhea in the treatment group as compared to 13% in the control group (Ahuja and Khamar, 2002).

Fermented milk containing *L. acidophilus* and *L. casei* was tested in hospitalized patients in Montreal. AAD occurred in 44 (15.9%) in the probiotic group as compared to 16 out of 45 (35.6%) in the control group. Hospitalization was two days shorter in the probiotic group (Beausoleil et al., 2007). A probiotic combination of *L. casei*, *L. bulgaricus*, and *S. thermophilus* was tested in a randomized controlled trial versus placebo in a study of 136 patients in a hospital receiving antibiotics. Seven out of fifty seven (12%) of the probiotic group developed diarrhea as compared to 19 out of 56 (34%) on placebo. This gave a number needed to treat (NNT) of five for AAD (Hickson et al., 2007).

A commercial yogurt containing *L. acidophilus*, *L. bulgaricus*, and *S. thermophilus* was tested in 202 hospitalized patients given antibiotics. There was less AAD among those people who were given yogurt as compared to placebo (13 out of 105, 12%, versus 23 out of 97, 24%) ($p = 0.04$) (Beniwal et al., 2003). However, in a general primary care practice, 369 patients who were given antibiotics were randomized to bio-yogurt, commercial yogurt, and no yogurt. In the no yogurt group, 17 of 120 (14%) developed diarrhea. In the commercial yogurt group, (a yogurt containing *S. thermophilus*, and *L. bulgaricus*) 13 of 118 (11%) developed

diarrhea. In the bio-yogurt group (a yogurt containing *L. acidophilus*, *B. animalis* and *S. thermophilus*) 9 of 131 (7%) developed diarrhea. These AAD rates (7–14%) were similar in all three groups ($p = 0.17$), suggesting no benefit from yogurt (Conway et al., 2007). A formula supplemented with *Bacillus coagulans* (incorrectly termed *L. sporogenes* by the authors) and fructo-oligosaccharides prevented AAD in infants and children (LaRosa et al., 2003).

21.2.1.2 Bifidobacteria

Bifidobacteria are components of saccharolytic bacteria in the colon, which are found in increased numbers (up to 95%) in the stools of breast fed infants, and are considered to be protective. They produce vitamins, digestive enzymes, and short chain fatty acids. There are many strains of bifidobacteria that are used as probiotics.

In a study of ten volunteers given clindamycin, an antibiotic that can cause diarrhea, co-administration of a fermented milk with *B. longum* and *L. acidophilus* resulted in less gastrointestinal discomfort (Orrhage et al., 1994). In another study of ten healthy volunteers receiving erythromycin, co-administration of *B. longum* led to a significant reduction in stool weight and frequency (Colombel et al., 1987). In a randomized controlled trial, Correa et al. studied 157 children aged six to thirty six months receiving antibiotics. Among these children, the ones who were given a daily dose of a probiotic containing *B. lactis* BB-12 and *S. thermophilus*, 13 out of 80 (16%) developed diarrhea as compared to 24 out of 77 (31%) of controls $p = 0.044$ (Correa et al., 2005). This was a decrease in incidence of diarrhea of almost 48%.

21.2.1.3 Enterococcus faecium

E. faecium is found in healthy adults and produces lactic acid. It also inhibits pathogens and resists antibiotics. In a small trial that involved adult patients who were given antibiotics, those who also received *E. faecium*, (45 patients) had less diarrhea (9%) than controls (27%) (Wunderlich et al., 1980). In another small controlled trial of this product to prevent AAD associated with drugs to treat pulmonary tuberculosis, diarrhea occurred in 3% compared to 18% with placebo (Borgia et al., 1982). Evaluation is limited by the small numbers in both of these studies.

21.2.1.4 Clostridium butyricum

C. butyricum is a Gram positive anaerobe that produces butyric acid. A *C. butyricum* product, CBM 588, is a probiotic preparation with 10^7 cfu per tablet. *C. butyricum* was used as a probiotic to prevent diarrhea in children receiving antibiotics for upper respiratory infections. For this trial, 110 children were enrolled. Diarrhea developed in 5% and 9% in the two treatment groups as compared to 59% who were given placebo $p < 0.05$ (Seki et al., 2003).

In adults receiving treatment for *Helicobacter pylori*, 3 out of 7 (43%) in the placebo group had diarrhea compared to 1 out of 7 (14%) who were given a single daily dose of CBM 588, and none in the group given the double dose (Imase et al., 2008). In patients being treated for *H. pylori*, an adjunct *C. butyricum* preparation in two different doses was associated with less diarrhea (1 out of 12, or 8.3%) as compared to controls (3 out of 7, or 43%) (Seki et al., 2003). At the end of seven days, *C. difficile* toxin was present in 2 out of 7 (29%) of controls and 1 out of 12 (8.3%) in the two treatment groups.

21.2.1.5 Saccharomyces boulardii

S. boulardii is a nonpathogenic yeast with a growth optimum of 37°C. It also resists gastric acid. It has been shown to decrease AAD in several randomized controlled trials. In children, Kotowska et al. showed that *S. boulardii* as an adjunct to the antibiotics being given to children with upper respiratory infections had significantly less diarrhea than those given antibiotics and placebo (Kotowska et al., 2005). In their study, 269 children, aged six months to fourteen years, were given antibiotics for respiratory infections. Among those given adjunct *S. boulardii*, 9 out of 119 (8%) developed diarrhea as compared to 29 out of 127 (23%) of controls. In adults, five placebo controlled and randomized trials showed significant reduction in AAD with *S. boulardii* (Adam et al., 1977; Cindoruk et al., 2007; Mansour-Ghanaei et al., 2003; McFarland et al., 1995; Surawicz et al., 1989). In a study of 388 French outpatients receiving tetracyline or a β-lactam antibiotic, diarrhea occurred in 33 out of 99 (17%) with placebo as compared to 9 out of 99 (4%) with *S. boulardii* ($p < 0.01$) (Adam et al., 1977). In a US study of hospitalized patients, diarrhea rates were 22% with placebo as compared to 9.5% with *S. boulardii* (Surawicz et al., 1989). In a study of patients receiving β-lactam antibiotics, 15% of controls had diarrhea as compared to 7% in *S. boulardii* (McFarland et al., 1995). In these three studies, there were a total of

761 adults, and controls had diarrhea rates of 17–22% as compared to *S. boulardii* treated 4–9%, $p < 0.01$.

A more recent paper studied the addition of *S. boulardii* as an adjunct to antibiotics given for the treatment of amebiasis and evaluated the incidence of subsequent AAD. In 57 patients, adjunct *S. boulardii* shortened the duration of diarrhea to 12 h as compared to 48 h with placebo ($p < 0.001$). In addition to diarrhea as an outcome, they evaluated the persistence of amebic cysts in the patient's stools. There was no shedding of amebic cysts at four weeks in the *S. boulardii* group as compared to 18% in the placebo group (Mansour-Ghanaei et al., 2003).

S. boulardii as an adjunct to antibiotic therapy for *H. pylori* resulted in less diarrhea than the controls (9 out of 62 or 14.5% vs. 19 out of 62 or 30.6%), and there was no effect on the rates of *H. pylori* eradication (Cindoruk et al., 2007).

There was a negative study of *S. boulardii* in the prevention of AAD. In 69 elderly patients, 14% of controls had diarrhea as compared to 21% of treated patients, which is an insignificant difference (Lewis et al., 1998).

21.2.2 Meta-Analyses

Several meta-analyses of probiotics in the prevention of AAD have been done, with the conclusion that probiotics prevent AAD, especially lactobacilli, odds ratio 0.39, $p < 0.01$, and *S. boulardii*, odds ratio 0.39, $p < 0.001$ (Cremonini et al., 2002; D'Souza et al., 2002). The meta-analysis by D'Souza et al. combined results from nine studies including four with *S. boulardii*, two with *L. acidophilus* and *L. bulgaricus*, one with *L. rhamnosus* GG and one with *L. acidophilus* and *B. longum*, and concluded that probiotics decreased the risk of AAD by two-thirds (odds ratio 0.37) (D'Souza et al., 2002). The meta-analysis by Cremonini et al. analyzed results from seven trials (only trials with *L. rhamnosus* GG and *S. boulardii*), with a positive benefit for probiotics, odds ratio 0.4. In both these meta-analyses, the probiotics included *L. rhamnosus* GG, *L. acidophilus*, *L. bulgaricus*, *E. faecium*, *B. longum*, and *S. boulardii* (Cremonini et al., 2002). The meta-analysis of *S. boulardii* by Szajewska and Mrukowicz included results from five trials, with an overall ratio of 0.43 and NNT of ten to treat and prevent one case of AAD (Szajewska and Mrukowicz, 2005).

A later and larger meta-analysis included 2,810 patients from 25 trials with separate studies in children and adults. Overall, in 25 trials, 13 had prevention of AAD and 12 did not. In adults seven out of sixteen showed efficacy (44%).

In children, six out of nine showed efficacy (67%) (McFarland, 2006). Thus, it makes sense to separate studies of children from adults. In this meta-analysis, three probiotics were most effective, namely, *S. boulardii*, *L. rhamnosus* GG, and probiotic mixtures of *L. acidophilus* and *L. bulgaricus*, *L. acidophilus* and *B. infantis*, and *L. acidophilus* and *B. lactis*.

A Cochrane review of probiotics for the prevention of pediatric AAD by Johnston et al. concluded that probiotics show promise (Johnston et al., 2007). The per protocol analysis gave statistically and clinically significant results but the intention to treat analysis did not. Therefore, future studies were recommend that focused on the most promising strains (*L. rhamnosus* GG, *Bacillus coagulans*, and *S. boulardii*) in doses of 5–40 billion colony forming units per day. However, routine use is not recommended because the evidence is not strong enough to support this.

In the review of probiotics in Pediatrics by Szajewska et al., the study concludes that probiotic use can be warranted when "the physician feels that preventing this usually self-limiting complication is important" (Szajewska et al., 2006).

21.2.3 Mechanisms of Actions

How do these probiotics work? There are many possible mechanisms. One is changes in the normal colonic flora. A probiotic yogurt containing *L. acidophilus*, *B. lactis* and *Lactobacillus* F19 prevented clindamycin induced changes in the fecal flora compared to controls, specifically in the placebo group. Numbers of lactobacilli and *Bacteroides* decreased compared to controls (Sullivan et al., 2003). *L. plantarum* 299v has been shown to increase the number of lactobacilli in fecal flora (Gossens et al., 2003). A multispecies probiotic given to healthy volunteers taking amoxicillin found changes in enterococci levels with less diarrheal stools in those given the probiotic than the controls (Koning et al., 2008). In these situations, changes in microbiota may benefit the host in a positive way to counteract the negative effect of changes in the microbiota induced by antibiotics.

In vitro studies of *L. acidophilus* and *L. casei* fermented milk showed inhibition of some pathogens including *Staphylococcus aureus*, *Enterococcus faecalis*, and *Listeria innocua*, and suggest that these antimicrobial mechanisms may prevent AAD (Millette et al., 2007).

Other probiotics produce antimicrobial substances such as bacteriocins. These may inhibit pathogens. They may also lower the intestinal pH and thus

stimulate growth of beneficial organisms such as lactic acid bacteria. *Lactococcus lactis* produces a bacterocin, a compound called nisin that is active against *Clostridium difficile*. This compound, lacticin 3,147 is a two-component lantibiotic from Irish kefir grain (Rea et al., 2007). Organisms may also adhere to intestinal mucosal cells, thus blocking sites for pathogens. Adherence to the mucosa may also stimulate the gastrointestinal immune system and stimulate cytokines, IgA or other substances. Possible modulation of immune activity is an exciting concept.

21.3 *Clostridium Difficile* Infection

C. difficile is a Gram positive anaerobic bacteria that can colonize in the colon, producing toxins that cause diseases. *C. difficile* infection (CDI) (previously called *Clostridium difficile*-associated diarrhea, CDAD), can range from mild symptoms to moderate or severe disease, including pseudomembranous colitis that can result in colon perforation, multiorgan failure, and death. Diagnosis of CDI relies on the detection of toxins in the stool, toxin A and/or toxin B. Its treatment involves antibiotics, either metronidazole or vancomycin. The symptoms of CDI usually improve in three to five days, and disappear in two weeks. Severe diseases may need prolonged therapy, and surgery (colectomy with ileostomy) can be lifesaving. Approximately 20% of cases will have recurrent CDI after the antibiotic course. In recurrent *C. difficile* infection (RCDI), recurrence rates increase from 40–60%. However, there are a variety of treatment approaches available.

21.3.1 Prevention of *Clostridium Difficile* Infection

As mentioned, there are limited trials of probiotics to prevent CDI. Out of four trials that evaluated probiotics in prevention of CDAD, none showed significant efficacy although none were specifically designed to treat CDAD.

One recent paper reports the use of a probiotic mixture of *L. casei*, *L. bulgaricus*, and *S. thermophilus* in milkshake form given to hospitalized patients receiving antibiotics. In this randomized controlled trial, it took two years to recruit the 135 study patients, all over fifty years of age. Furthermore, very few of the patients screened could be enrolled, only 7% of the 1,760 patients were enrolled. In this group, 7 out of 57 (12%) who were given the probiotic

developed diarrhea compared to 19 out of 56 (34%) on placebo. None of the 56 patients who were on probiotic developed CDI as compared to 9 out of 53 (17%) of controls, which was significant ($p < 0.007$). The authors concluded that the probiotic was safe and economical. A financial analysis suggested that the additional cost associated with AAD was 3,559 USD, the cost to prevent AAD was 100 USD, and the cost to prevent CDI was 120 USD (Hickson et al., 2007).

A probiotic mixture of *L. acidophilus* and *B. bifidum* was given in a capsule form to 150 elderly hospitalized patients out of which 138 completed the study with 69 in each group. The rates of diarrhea were the same in probiotic and no-probiotic groups with 15 cases each (22%). Among patients who developed diarrhea, *C. difficile* toxin was positive in 2.9% of the probiotic group and 7.25% of the placebo group. Among all patients tested for *C. difficile*, fewer were positive in the probiotic group than the controls group (46% vs. 78%). In patients who developed diarrhea, *C. difficile* toxins were detected in 2.9% of the probiotic group and 7.25% of the placebo group (Plummer et al., 2004).

The earlier studies of the probiotic mentioned above that were given to patients undergoing antibiotic therapy for *H. pylori* analyzed also changes in fecal flora. In the probiotic group, there were more *Bacteroides* species, enterococci were similar in both groups, and the placebo group had more facultative aerobes and enterobacteria. Diarrhea was not reported. The placebo group had increased antibiotic-resistant enterococci (Madden et al., 2005).

In a trial of *S. boulardii* to prevent AAD, no one in the probiotic group had *C. difficile* toxin compared to two in the placebo group (Can et al., 2006). However, these numbers are too small to draw conclusions. In this randomized controlled trial of 151 patients, there was less diarrhea in the *S. boulardii* group (1 out of 73 or 1.4%) as compared to placebo (7 out of 73 or 9%).

In a study of *C. butyricum* to prevent AAD during *H. pylori* therapy, 1 out of 7 (29%) in the control group developed *C. difficile* toxin A in one week as compared to 1 out of 7 (14%) on standard dose therapy, and 0 out of 5 who got double dose therapy (Imase et al., 2008).

21.3.1.1 Treatment of CDI

There is no evidence that probiotics have a role either as primary therapy or as adjunctive therapy.

21.3.1.2 Meta-Analysis

A Cochrane analysis by Pillai and Nelson concludes that there is insufficient evidence to recommend probiotics as sole therapy or adjunct therapy for CDI (Pillai, 2008).

A study by Dendukuri et al. also concludes in the systematic review that there is not enough data to support routine use of probiotics to prevent or treat CDI. This calls for better randomized studies of large number of patients, and for clarifying antibiotic groups with adequate long term follow up. The authors also call for clarification of when different types of probiotics should be used, and better standardization of the dose of probiotics, and standard definitions for CDI (Dendukuri et al., 2005).

21.4 Treatment of Recurrent *Clostridium Difficile* Infection

Recurrent *C. difficile* infection (RCDI) is a *C. difficile* infection that recurs after the initial response to treatment after the antibiotic is stopped. RCDI is difficult to treat and one recurrence makes repeated future recurrences more likely.

There are a wide variety of clinical approaches because there is no uniformly effective treatment. Retreatment with metronidazole or vancomycin is required. When given in pulsed and/or tapered fashion, recurrences are less frequent. A small number of patients with severe recurrences responded to a course of vancomycin followed by two weeks of rifaximin (Johnson et al., 2007).

The notion of toxin binding resins is appealing but while they may bind toxins *in vitro*, bile salt binding resins have no proven efficacy. Because resins can also bind the treating antibiotic, if used, they should be given several hours after the antibiotic to avoid binding the antibiotic. Immune approaches include bovine antibody-enriched whey, intravenous immunoglobulin (IVIG), and a vaccine in development. Several probiotics have also been studied.

L. rhamnosus GG looked promising in case reports and small series. In small uncontrolled studies, *L.* rhamnosus GG showed decreased recurrences of RCDI (4 out of 5 adults, 2 out of 4 children, and 27 out of 32 adults) (Bennet et al., 2000; Biller et al., 1995; Gorbach et al., 1987). Early reports from a randomized and controlled trial showed benefit in the prevention of RCDI with *L. rhamnosus GG*. Among fifteen patients in each group, 1 out of 9 (11%) had RCDI with *L. rhamnosus* GG as compared to 6 out of 9 (67%) with placebo

(Pochapin, 2000). However, in a small randomized and controlled trial there was no efficacy (Lawrence et al., 2005). *L. plantarum* had modest efficacy for RCDI, with recurrences in 4 out of 11 (36%) of the treated group as compared to 6 out of 9 (66%) of the placebo group, though this study is clearly underpowered (Wullt et al., 2003).

S. boulardii decreased recurrences in patients with RCDI in one study, where 22 out of 34 (65%) recurred on placebo, as compared to 9 out of 26 (35%) given *S. boulardii* ($p = 0.04$) (McFarland et al., 1994). In a later study of 168 patients with RCDI, only the subgroup of patients with RCDI who responded to *S. boulardii* were those who had been given high dose vancomycin; 3 out of 18 or 16.7% had recurrence with *S. boulardii* as compared to 7 out of 14 (50%) on placebo, ($p < 0.05$). Of the 83 patients given low dose vancomycin, 23 out of 45 (51.1%) given *S. boulardii* had diarrhea as did 17 out of 38 (44.7%) given placebo. Of the 53 given metronidazole, 13 out of 27 (48.1%) given *S. boulardii* had diarrhea as did 13 out of 26 (50%) given placebo. These were not significant differences (Surawicz et al., 2000).

Small series and case reports reveal the efficacy of fecal bacteriotherapy for refractory RCDI (Aas et al., 2003; Persky and Brandt, 2000). This unconventional therapy appears to be very effective, but is usually the "last resort."

21.4.1 Meta-Analyses

A meta-analysis of six randomized and controlled trials of probiotics for the treatment of RCDI concluded that *S. boulardii* was effective but *L. rhamnosus* GG and *L. plantarum* were not (McFarland, 2006) (Table 21.2).

A systematic review of probiotics for the treatment of CDI in adults evaluated four studies – all small with methodologic problems. Only one study found significant benefit for *S. boulardii* to prevent RCDI, (Relative Risk, RR 0.59; 95%,

Table 21.2

Probiotic therapy in the treatment of recurrent *C. difficile* infection – evidence-based analysis. See section 21.6 for explanation of grading levels

Probiotic	Effectiveness	Comment
Saccharomyces boulardii	Grade A	Level 1a
Lactobacillus plantarum	Grade A	Level 1c
Lactobacillus rhamnosus GG	Grade A	Level 1d

Probiotics in the treatment of RCDI in terms of effectiveness

CI 0.35–0.98) (Pillai, 2008). This study concluded that there is no significant evidence to support the use of probiotics as an adjunct therapy of CDI or as a sole therapy.

Overall, several meta-analyses show efficacy of *L. rhamnosus* GG and *S. boulardii* in the prevention of AAD. However, only *S. boulardii* has efficacy in the treatment of recurrent antibiotic associated diarrhea (RCAAD). Many trials of probiotics are limited by small sample size, variable probiotics and doses, and variable combinations of probiotics. Although early trials of probiotics show promise, much more work needs to be done.

21.5 Safety of Probiotics

While the safety profile of probiotics is generally excellent, these are not without risk, especially in severely ill and immunocompromised patients. Most of the adverse events reported are case reports. There have been cases of *L. rhamnosus* GG-associated sepsis, liver abscess, and endocarditis (Land et al., 2005; Salminen et al., 2004) along with cases of *S. boulardii* fungemia, especially in patients with central lines and those who are immunocompromised (Muñoz et al., 2005; Niault et al., 1999; Riquelme et al., 2003). A case of *B. breve* meningitis was reported in an infant (Hata et al., 1988). It is probably wise to avoid any probiotic in severely ill patients and those who are immunocompromised and perhaps preterm infants. There are also theoretical risks that probiotics could persist in the GI tract, translocate, or acquire resistance genes. Further information on the safety of probiotics is provided in another chapter in this book.

21.6 Grading the Evidence for Probiotics

An evidence based medicine recommendation for grading levels used by experts is as follows (McDonald et al., 2004):

Grade A Recommendations

Level 1a: Evidence from large randomized clinical trials or systematic reviews

Level 1b: Evidence from at least one "all or none" high quality cohort study

Level 1c: Evidence from at least one moderate-sized randomized controlled trial or a meta-analysis of small trials

Level 1d: Evidence from at least one randomized controlled trial

Grade B Recommendations

Level 2: Evidence from at least one high quality study of non-randomized cohorts who did and did not receive new therapy

Level 3: Evidence from at least one high quality case control study

Level 4: Evidence from at least one high quality case series

Grade C Recommendation

Expert opinion

Using this scale, we can assign efficacy rates to the various probiotics (◉ *Tables 21.1*, ◉ *21.2*): for prevention of AAD, grade A Level 1 for *L. rhamnosus* GG and *S. boulardii*, and grade A Level 1 for treatment of RCDI with *Saccharomyces.* Other probiotics have lower levels of efficacy to date. Further larger and better studies are needed (Pham et al., 2008).

21.7 Conclusion

Antibiotics can cause mild diarrhea or serious colitis due to *C. difficile*. Many probiotics have been studied for prevention AAD. Of these, the best evidence is efficacy for *L. rhamnosus* GG and *S. boulardii* (*Saccharomyces cerevisiae*). Probiotics have no proven role in the treatment of *C. difficile*, either as sole therapy or as an adjunct one. One exception is recurrent *C. difficile* infection, where *S. boulardii* may be effective when combined with high dose of vancomycin. *L. plantarum* is another probiotic that shows promise for RCDI.

21.8 Summary

- Antibiotic-associated diarrhea is a frequent side effect of antibiotics, but there are no good ways to predict who will get diarrhea.
- Probiotics have good efficacy in the prevention of AAD, with best evidence for *L. rhamnosus* GG and *Saccharomyces boulardii* (*Saccharomyces cerevisiae*).
- *C. difficile* is a severe type of AAD, with a spectrum ranging from diarrhea to colitis, and to pseudomembranous colitis.
- Probiotics are no proven treatment of *C. difficile*, either as sole therapy or as adjunct therapy.

- Recurrent *C. difficile* infection is difficult to treat, with many treatment options and, none uniformly successful. The probiotic *S. boulardii* has shown some efficacy in the treatment of RCDI.
- There are preliminary studies suggesting probiotics may prevent *C. difficile* diarrhea but more studies are needed.
- In order for probiotics to be widely used, additional information is needed including:
  - Identification of high risk patients who would benefit
  - Identification of which probiotics, doses, activity, and duration of therapy should be used
  - Cost-benefit analyses
  - Safety studies

List of Abbreviations

AAD antibiotic-associated diarrhea
CDI clostridium difficile infection
RCDI recurrent clostridium difficile infection

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22 Probiotics for Infectious Diarrhea and Traveler's Diarrhea – What Do We Really Know?

Patricia L. Hibberd

22.1 Introduction

Worldwide, diarrhea is the sixth leading cause of premature death (Lopez et al., 2006), accounting for more than two million deaths each year. The majority of the burden is borne in lower and middle income countries, and in children under age 5 (Kosek et al., 2003). Even in the United States where there is easy access to "safe" food and water, there are an estimated 211–375 million episodes of acute diarrhea each year, resulting in 900,000 hospitalizations and 6,000 deaths (Herikstad et al., 2002; Mead et al., 1999). While mortality from diarrhea has decreased over the last 30 years, the incidence and morbidity associated with diarrhea has not improved (Kosek et al., 2003). During the same time period an ever increasing number of enteric pathogens as well as non-infectious conditions have been recognized as causes of acute diarrhea (Guerrant et al., 2001).

Since the majority of adults and children will experience at least one episode of diarrhea each year, there is great interest in its prevention and treatment. Practice guidelines for the diagnosis and management of infectious diarrhea have been developed, focusing on oral rehydration and appropriate use of antibiotics (Guerrant et al., 2001). However, there is little guidance on the safe and appropriate use of the estimated 400 over-the-counter products that are promoted in the United States for their anti-diarrheal properties (Thielman and Guerrant, 2004).

Probiotics, and more recently prebiotics, are increasingly being recommended in the medical and popular press to prevent and/or treat diarrheal diseases. In this chapter, the rationale and evidence for the use of the various different probiotics for the prevention and treatment of infectious diarrhea that is not associated with the administration of antibiotics or the diagnosis of

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Clostridium difficile is reviewed (please see chapter by Dr C Surawicz). Also, the challenges of making recommendations for the use of probiotics are addressed and new directions to developing recommendations for safe use of these agents are suggested.

Before discussing the evidence base for the use of probiotics for the treatment of diarrhea, several terms that will be used in this chapter need to be defined.

22.2 What Exactly is Diarrhea?

The definition of diarrhea is unexpectedly controversial. There is widespread agreement that diarrhea involves increased liquidity or decreased consistency of stools, usually associated with increased stool frequency. Since it is impractical to measure stool fluid, information about diarrhea mostly has to come from patient self-report, but self-report of *symptoms* of diarrhea does not correlate well with self-report of *episodes* of diarrhea (Talley et al., 1994). For epidemiologic purposes, diarrhea is usually defined by self-report or mother's report as a "decrease in consistency (i.e. soft or liquid) and an increase in frequency of bowel movements to $\geq$3 stools per day" (Guerrant et al., 2001). For clinical trials, diarrhea is often defined as passage of three or more loose stools or watery stools in a 24-h period (Ruiz-Palacios et al., 2006), but frequently the definition of diarrhea is inadequately specified.

Diarrhea is usually classified as either osmotic or secretory, either type resulting in fluid and electrolyte secretion in the small or large intestine or both (Schiller and Sellin, 2006). If substances in the lumen are responsible for fluid secretion, the diarrhea that results is considered osmotic. If endogenous substances are responsible for fluid secretion, the resulting diarrhea is considered secretory. These pathogenic mechanisms are not necessarily mutually exclusive because some diarrheal diseases cause both osmotic and secretory diarrhea. However, it may be important to consider the mechanism by which diarrhea occurs when evaluating the safety and efficacy of probiotics.

22.3 What is Acute Versus Chronic Versus Persistent Diarrhea?

Unlike the definition of diarrhea, there is extensive agreement with arbitrary time limits in which acute diarrhea lasts 14 days or less, persistent diarrhea lasts

15–30 days and chronic diarrhea lasts 30 days or more (Guerrant et al., 2001). In this chapter, the focus is mostly on acute diarrhea, because it accounts for the majority of infectious diarrhea worldwide.

22.4 How Common is Acute Diarrhea?

Population based estimates of the incidence of acute diarrhea are limited because of the lack of reliability of patient self-report of diarrheal illness. In addition, de Wit et al. estimated that only 22% of patients with gastroenteritis consulted a physician (de Wit et al., 2000), and Feldman et al. reported that only 5% submitted a stool sample (Feldman and Banatvala, 1994). Both of these observations would result in gross underestimates of the incidence of diarrhea based on physician or facility based data.

Despite these limitations, in children under age 5 years in the United States, there are about 25 million episodes of diarrhea (1–2 per child), 1.5 million outpatient visits, 200,000 hospitalizations and 300–400 deaths per year, costing more than one billion dollars (Santosham et al., 1997). By contrast, in developing countries, children under age 5 experience 6–7 episodes of diarrhea per year and worldwide, an estimated 2.5 million children die as a result of diarrheal illness – it is the number 3 killer of children under age 5 years, after perinatal causes and pneumonia (Kosek et al., 2003).

22.5 What Causes Acute Diarrhea?

In a European study, 65% of children with acute diarrhea had pathogens detected in stool samples – about half of the pathogens were rotavirus; *Escherichia coli* (*E. coli*), *Salmonella* and *Campylobacter* were the most common bacterial isolates (Guandalini et al., 2000). Similar overall rates of detection of pathogens have been reported in developing countries, although pathogenic *E. coli* and parasites are more frequently isolated in developing than developed countries (Kang, 2006; Kang et al., 2001).

There are six types of pathogenic *E. coli* that cause acute diarrhea. Enterotoxigenic *E. coli* (ETEC) is the most common pathogenic *E. coli* worldwide. Other types include diffusely adherent *E. coli* (DAEC) which is responsible for about 10% cases in developed countries; Enteroaggregative *E. coli* (EAggEC) and Enteropathogenic *E. coli* (EPEC) which are common in developing countries

but rare in developed countries; the foodborne Enteroinvasive *E. coli* (EIEC) which is endemic in developing countries and Enterohemorrhagic *E. coli* (EHEC) which causes rare foodborne epidemics in developed countries. EHEC differs from other pathogenic *E. coli* by production of one or more Shiga toxins. It is the Shiga toxins produced by the EHEC (also known as Shiga toxin-producing *E. coli* (STEC)) that are responsible for the disease processes caused by this type of *E. coli*. More recently recognized diarrheal pathogens include noroviruses that cause food and water-borne outbreaks (Tseng et al., 2007; O'Reilly et al., 2007), enterotoxigenic *Bacteroides fragilis* (Cohen et al., 2006), *Klebsiella oxytoca* (Hogenauer et al., 2006) and *Laribacter hongkongensis* (Ni et al., 2007).

Diarrheal disease in travelers is predominantly caused by bacteria (80–85%) and mostly transmitted by food or water (Black, 1990). Parasites account for about 10% and viruses for about 5% of traveler's diarrhea. The most common organism isolated is enterotoxigenic *E. coli* (ETEC), but the epidemiology of traveler's diarrhea varies by location.

No pathogen is detected in about 35% of stool samples from children with acute diarrhea. These cases may be caused by infections not detected by routine microbiology procedures, or other causes such as food allergies, drugs, poisons, intussusception, toxic megacolon and appendicitis. In adults, the differential for acute diarrhea includes infection, drugs, inflammatory bowel disease, irritable bowel syndrome, laxative abuse, partial obstruction, diabetes, Whipple's disease, diverticulosis, celiac sprue and ischemic colitis.

22.6 How is Acute Diarrhea Diagnosed and Treated?

The first step in diagnosis of acute diarrhea is obtaining a thorough history that includes clinical and epidemiologic data and a directed physical examination focusing on signs of extracellular volume depletion, fever and peritoneal signs, as described in the Infectious Diseases Society of America (IDSAs) "Practice Guidelines for Management of Infectious Diarrhea" (Guerrant et al., 2001). Fecal testing (culture or other testing for infectious pathogens) is recommended for community-acquired or traveler's diarrhea, when the diarrhea is accompanied by fever, bloody stools, signs of systemic illness including hypovolemia, recent antibiotics, attendance at daycare and recent or current hospitalization. Diagnostic work-ups are also recommended for elderly and immunocompromised patients.

Administration of oral rehydration solution (ORS) is the most important first step in the treatment of adults and children with acute diarrhea. The World

Health Organization (WHO) recommends either standard or reduced osmolarity ORS, although reduced osmolarity ORS was recently shown to be superior to standard ORS in the treatment of children with acute diarrhea not due to cholera (CHOICE Study Group, 2001).

The 2001 IDSA practice guidelines stress the need to carefully weigh the risks and benefits of antimicrobial therapy for the treatment of acute diarrhea (Guerrant et al., 2001). While antibiotics eliminate pathogens and limit their carriage and systemic effects, for most diarrheal illnesses they do not alter the course of disease significantly. Use of empiric antibiotics (pending culture results) are recommended for patients with moderate to severe traveler's diarrhea (more than four unformed stools daily, fever, blood, pus or mucus in the stool), patients with volume depletion or symptoms for more than one week, patients with signs and symptoms of bacterial diarrhea, patients being considered for hospitalization and patients who are immunocompromised.

For adults, empiric antibiotic therapy includes an oral fluoroquinolone for three to five days except for suspected EHEC (where antibiotics should be avoided) or in areas where fluoroquinolone-resistant infections are common (azithromycin for three days is recommended in these settings, Thielman and Guerrant, 2004). In children, empiric antibiotic therapy for moderate to severe traveler's diarrhea and community-acquired suspected invasive diarrhea is trimethoprim-sulfamethoxazole, although fluoroquinolones can also be considered for serious illness. Erythromycin or azithromycin should be considered for fluoroquinolone-resistant infections (Thielman and Guerrant, 2004). When microbiology results are available, treatment should be tailored to the specific pathogen and its resistance pattern.

The antimotility agent loperamide (Imodium) may be used for the symptomatic treatment of patients with acute diarrhea, when the patient does not have fever and the stools are not bloody. Diphenoxylate (Lomotil) is an alternative agent, but its safety and efficacy have not been studied in randomized controlled studies. Concern has been raised that both antimotility agents may facilitate the development of the hemolytic-uremic syndrome in patients infected with EHEC (Cimolai et al., 1990). Neither these agents nor other antimotility agents are recommended for children with acute diarrhea.

Recent advances in the management of diarrhea have focused on protecting infants against severe diarrhea and death from rotavirus infection. Two vaccines that provide protection against rotavirus are currently licensed and in widespread use in developed countries. However, most developing countries are unable to afford these vaccines. Before making recommendations for global use, the World

Health Organization (WHO) has requested that trials to assess safety and efficacy be conducted in Africa and Asia to address concerns about the varying performance of these oral rotavirus vaccines. Two new lower cost rotavirus vaccines are in development – one is the 116E human monovalent vaccine and the other is a multivalent vaccine that contains the four most common rotavirus strains as well as strains found in Asia and Africa. Vaccine candidates to protect against *Shigella* and enterotoxigenic *E. coli* infection are in development.

Based on several recent studies (Bhutta et al., 2000), WHO and UNICEF also recommend use of zinc supplementation for the treatment of acute diarrhea to reduce the duration and severity of the illness and mortality. However, given the burden of illness caused by acute diarrhea and the ever increasing problem of antimicrobial resistance in bacterial pathogens, there is increasing interest in other easily accessible and cost effective ways to prevent or to use as adjuncts to ORS to treat diarrheal disease.

22.7 What are Probiotics?

Probiotics are defined as "live microorganisms which when administered in adequate amounts confer a health benefit on the host" (Joint FAO/WHO Working Group, 2002). Many of the probiotic microorganisms are either isolated from humans or are derived from food sources, particularly cultured milk products. The microorganisms listed as probiotics is ever increasing and includes strains of lactic acid bacilli (e.g., *Lactobacillus* and *Bifidobacterium*), a nonpathogenic strain of *E. coli* (*E. coli* Nissle 1917), *S. thermophilus*, *Bacillus subtilis* and the yeast *Saccharomyces boulardii* (*Saccharomyces cerevisiae*). "Designer" probiotics (Paton et al., 2006) (strains of bacteria that have been genetically modified) are under development for the prevention of enteric infections, as are strategies to combine probiotics, prebiotics and antibiotics to manipulate the commensal microbial flora and restore the homeostasis of gut ecology (Jia et al., 2008).

22.8 Why Might Probiotics be Useful for the Prevention or Treatment of Infectious Diarrhea?

The mechanisms of action of probiotics are incompletely understood and increasingly are being recognized to vary from probiotic organism to probiotic organism. General effects include: suppression of growth or epithelial

binding/invasion by pathogenic bacteria, such as *Salmonella, Shigella*, enterotoxigenic *E. coli* and *Vibrio cholerae*; improvement of intestinal barrier function and modulation of the immune system.

Suggested mechanisms for the effects of probiotics on the gastrointestinal microbiota in relation to the prevention and treatment of diarrhea include direct effects, such as reduction of intestinal pH, production of organic acids and gut protective metabolites, and binding and metabolism of toxic metabolites. There is growing evidence that the host's systemic and mucosal immune system can be modulated by bacteria in the gut. Mechanisms may include modulation of the microbiota itself, improved barrier function with consequent reduction in immune exposure to microbiota and direct effects of bacteria on different epithelial and immune cell types. Advances in knowledge relating to host-microbe crosstalk are urgently needed to address the rationale for use of current and future probiotic based products in the prevention and treatment of diarrheal diseases.

22.9 Where Does the Evidence on the Use of Probiotics for Infectious Diarrhea Come From?

We used a search strategy similar to Sazawal et al. (2006) to retrieve all clinical trials relating to the prevention or treatment of infectious and traveler's diarrhea through August 2008. PubMed, Medline and the Cochrane Controlled Trials Registry were searched using the key words – probiotic, diarrhea, acute diarrhea, traveler's diarrhea, diarrhea prophylaxis, *Lactobacillus*, *Lactobacillus GG*, *Lactobacillus rhamnosus GG*, LGG, *Lactobacillus acidophilus*, *Bacillus subtilis, Saccharomyces*, *Saccharomyces boulardii* (*Saccharomyces cerevisiae*), *Bifidobacterium*, in various combinations. Data from all articles that were identified as clinical trials and checked references in review articles and meta-analyses were extracted, using standard criteria (Moher et al., 1995). No attempt was made to synthesize the data in a meta-analysis or systematic review, because of the wide variation in the settings and age groups studied, the definitions of diarrhea, either as an inclusion criterion or outcome, the use of microbiology to assess the etiology of diarrhea, the follow-up duration, the type, dose, duration of administration and manufacturer of the probiotic studied as well as the methodological rigor of the trial. Data are abstracted in *Tables 22.1*–*22.3* in this chapter. The purpose of these tables is to illustrate the information available in the clinical trials and the challenges of making evidence based recommendations regarding the use of probiotics for the prevention and treatment of diarrheal diseases.

Table 22.1

Details of trials using probiotics to prevent infectious diarrhea (*Cont'd* p. 854)

Reference	Population	Probiotic studied	# Randomized to probiotic/ # (%) completing trial	# Randomized to placebo/# (%) completing trial	Reasons for not completing trial	Randomi-zation described	Double blinded	Outcome - diarrhea	Result probiotic versus placebo group	Statistics	Adverse events
Pediatric studies, healthy children											
Pedone et al. (1999)	6–36 months healthy, not breast fed in daycare, France	Group fermented milk (YC) = *L. bulgaricus* + *S. thermophilus* + *L. casei* (strain DN-114 001); Group standard yogurt (Y) = *L. bulgaricus* + *S. thermophilus*, dose NS, CIRDC Pilot Laboratory, France × 12 weeks	Group Y NS/86–% not known; Group YC NS/92–% not known	NS/87–% not known	Moved away, extended holidays, severe disease, surgical intervention, refusal to consume the supplemented product	Y	N	≥3 loose or watery stools per 24 h; acute diarrhea = episode that lasts less than 2 weeks	Diarrhea: Group YC 23.3%; Group Y 28.3% versus Placebo 26.4%	P = 0.75	NS
Pedone et al. (2000)	6–24 months healthy not breastfed in daycare, France	Group supplemented yogurt (SY) = *L. bulgaricus* + *S. thermophilus* + *L. casei* (strain DN-114 001); Group standard yogurt (Y) = *L. bulgaricus* + *S. thermophilus*; dose NS, CIRDC Pilot Laboratory, France, 5 days/week × 12 weeks	Group SY - 463/384–83%; Group Y - 465/395–85%	N/A	Lost to follow-up or poor compliance	Y	N	Loose or watery stools for ≥2 consecutive days	Diarrhea: Group YS 61 (15.9%) versus Group Y 87 (22%)	P = 0.029	NS

Chouraqui et al. (2004)	<8 months healthy, not breastfed in residential nurseries or foster care, France	*S. thermophilus* + *L. helveticus* + *Bifidobacterium lactis* strain Bb 12 > 10^8 cfu/day, varied by amount ingested, Chr Hansen, Denmark, duration NS	46/46–100%	44/44–100%	N/A	Y	Y	>3 unusually loose stools per day x > 24 h or increment of >50% in daily number of stools if previously >2	Diarrhea: Probiotic 28.3% versus Placebo 38.7%	P = 0.3	None
Thibault et al. (2004)	4–6 m healthy, not breastfed in daycare or multiple sibs at home, France	*B. breve* C50 + *S. thermophilus* 065, dose NS, Calisma, Bledina SA, France × 5 months	484/464–96%	484/449–93%	Lost to follow-up, other sickness, parental decision, formula tolerance problem	Y	Y	≥2 liquid stools/day for at least 24 h	Diarrhea episodes: Probiotic 56.7% versus Placebo 55.9%	P-NS	NS
Weizman et al. (2005)	4–10 months healthy, not breastfed, in daycare, Israel	Group 1 *B. lactis* (Bb12) mean 1.2 × 10^9 cfu/day Chr Hansen, Denmark; Group 2 *L. reuteri* (ATCC 55730) mean 1.2 × 10^9 cfu/day, BioGaia, Sweden × 12 weeks	Group 1 73/71–97%; Group 2 68/65–96%	60/58–97%	Protocol violation, poor compliance	Y	Y	≥3 watery stools/day	Diarrhea Episodes: Probiotic Group 1 0.31 versus Probiotic Group 2 0.02 versus Placebo 0.31	P < 0.001	None

Table 22.1 (*Cont'd* p. 856)

Reference	Population	Probiotic studied	# Randomized to probiotic/ # (%) completing trial	# Randomized to placebo/# (%) completing trial	Reasons for not completing trial	Randomi-zation described	Double blinded	Outcome - diarrhea	Result probiotic versus placebo group	Statistics	Adverse events
Binns et al. (2007)	1–3 year in 29 daycare centers, Australia	CUPDAY milk formula: *B. lactis* (CNCM I-3446) dose NS + prebiotic 50% Raftilose P95 FOS, Orafti, Belgium, 50% Acacia gum CNI, Rouen, France × 5 months	248/248–100%	248/248–100%	Per protocol analysis also done excluding child who left center, disliked milk taste, left on advice of medical doctor, ill health of child, parent forgot to give milk every day, diary too time-consuming, went on holidays, too many family problems, no reason given	Y	Y	≥4 stools/day causing child to be absent from childcare	Absent from Daycare (=Days with >3 stools/ day): Synbiotic Group 1293 versus Placebo 1430	P = 0.003	NS

Chouraqui et al. (2008)	≤14 days not in daycare, healthy, not breastfed, France	Group 1 *B. longum* (BL999) + *L. rhamnosus* (LPR); Group 2 BL999 + LPR + GOS/FOS; Group 3 BL999 + *L. paracasei* ST11 + GOS/FOS; Groups 1&2 BL999 $7.7–9.7 \times 10^8$ cfu/day; Group 3 BL999 $1.5–1.9 \times 10^9$ cfu/day; LPR $3.9–4.8 \times 10^9$ cfu/day; ST11 $1.5–1.9 \times 10^9$ cfu/day, manufacturer NS, to age 16 weeks	Group 1 70/60–86%; Group 2 70/54 (77%); Group 3 74/60 (81%)	70/53–76%	Change of formula, adverse events, loss to follow-up	Y	Y	≥3 loose or watery stools in 24 h	Diarrhea: Group 1 BL999 + LPR 6%; Group 2 BL999 + LPR + Prebiotic 6%; Group 3 BL999 + LPR + ST11 + Prebiotic 4% versus Placebo 5%	P-NS	29 SAEs: gastroenteritis, milk allergy, diarrhea, gastroesophageal reflux disease, febrile infection, vomiting, surgery, pyrexia, rectal hemorrhage, pyelonephritis, bronchiolitis, cough, drug toxicity, inguinal hernia and 184 AEs - similar in 4 groups
Pediatric Studies – Children in Developing Countries											
Oberhelman et al. (1999)	6–24 months undernourished, free living, breastfed and non breastfed, Peru	LGG 3.7×10^{10} cfu/day, manufacturer NS × 15 months	99/99–100%	105/105–100%	N/A	Y	Y	1 day with at least 4 liquid stools	Diarrhea episodes/child/year: Probiotic 5.21 versus Placebo 6.02	P = 0.028	NS
Chandra (2002)	Newborns India	*L. sporogenes* (correct name *Bacillus coagulans*) 1×10^8 cfu/day, manufacturer NS × 12 months	55/55–100%	57/57–100%	N/A	Y	Y	Change in consistency of stools from "firm formed normal" to "loose," or an increase in frequency by two additional bowel movements from normal 1–3 per day to ≥3 per day	Diarrhea episodes: Probiotic 3.4 versus Placebo 8.6	P < 0.02	NS

Table 22.1

Reference	Population	Probiotic studied	# Randomized to probiotic/ # (%) completing trial	# Randomized to placebo/# (%) completing trial	Reasons for not completing trial	Randomi-zation described	Double blinded	Outcome - diarrhea	Result probiotic versus placebo group	Statistics	Adverse events
Pediatric Studies – Hospitalized Children											
Saavedra et al. (1994)	5–24 months long-term care for chronic illnesses, USA	*B. bifidum* mean 1.3–2.5 × 10^{10} cfu/day + *S. thermophilus* mean 1.0–1.9 × 10^{9} cfu/day, Chr Hansen, Denmark × duration of hospitalization	29/29–100%	26/26–100%	Note: readmissions re-randomized and five removed from trial because they received formula for <24 h	Y	Y	≥5 liquid stools/ day	Diarrhea episodes: Probiotics 7% versus Placebo 31%	P = 0.035	None
Szajewska et al. (2001)	1–36 months not hospitalized for diarrhea, Poland	LGG 1.2 × 10^{10} cfu/ day, Dicofarm SpA, Italy × duration of hospitalization	45/45–100%	36/36–100%	N/A	Y	Y	≥3 loose or watery stools/24 h	Diarrhea: Probiotics 6.7% versus Placebo 33.3%	P = 0.002	None
Adult Studies – Healthy Subjects											
Clements et al. (1981)	18–35 year healthy volunteers challenged with E coli 10^{8}–10^{10}, USA	*L. acidophilus* + *L. bulgaricus* (dried & viable) 6.4 × 10^{8}–2.7 × 10^{9} cfu/day, Hynson, Westcott, and Dunning, USA × 132 hours	23/23–100%	25/25–100%	N/A	N	Y	≥3 unformed stools or 2 unformed stools totaling at least 200 ml within 48 h, or a single voluminous liquid stool (≥300 ml)	Diarrhea: Probiotic 70% versus Placebo 68%	P-NS	NS
Pereg et al. (2005)	Mean 18.1 year healthy Israeli military recruits, Israel	*L. casei* DN-114 001 1×10^{10} cfu/day, Strauss Ltd, Israel × 8 weeks	275/254–92%	266/248–93%	Recruits transferred to other army units	N	Y	Passage of >2 loose stools in a 24-h period	Diarrhea: Probiotic 12.2% versus Placebo 16.1%	P = 0.207	None
Adult Studies – Hospitalized Patients											
Bleichner et al. (1997)	Probiotic Group 64.9±14.1 year; Placebo Group 61.6±12.3 year, ICU patients receiving tube feeds, France	*Saccharomyces boulardii* 2 g/day, manufacturer NS × 21 days or withdrawal of enteral nutrition	66/64–97%	65/64–98%	Exclusion criteria present	Y	Y	≥3 non-formed stools per day	Mean diarrhea days: Probiotic 14.2% versus Placebo 18.9%	p = 0.0069	None

Y yes; *N* no; *NS* not stated; *N/A* not applicable; *P-NS* not statistically significant

Table 22.2

Details of trials using probiotics to treat infectious diarrhea (*Cont'd* p. 858)

Reference	Population	Definition of diarrhea for enrollment	Diarrheal pathogens	Probiotic studied	Antibiotics to treat diarrhea	# Randomized to probiotic/# (%) completing trial	# Randomized to placebo/# (%) completing trial	Reasons for not completing trial	Randomization described	Double blinded	Definition of resolution of diarrhea	Result Probiotic versus Placebo Group	Statistics	Adverse Events
Pediatric Studies														
North America														
Pearce and Hamilton (1974)	<3 year admitted for acute diarrhea, Canada	NS	NS	3–8 × 10^8 cfu/day 50–60% *S. thermophilus, lactis* + 35–45% *L. acidophilus* + 5% *L. bulgaricus*, Rougier Laboratories, Canada, until discharge	N	53/53–100%	41/41–100%	N/A	N	Y	NS	Mean duration of diarrhea: Probiotic 8.0 eight-hour shifts versus Placebo 6.2 eight-hour shifts	P-NS	NS
Central/South America														
Cetina-Sauri and Sierra Basto (1994)	3 months–3 years admitted for acute diarrhea, Mexico	NS	ND	*Saccharomyces boulardii* 600 mg/day, manufacturer NS × 4 days	N	65/65–100%	65/65–100%	N/A	Y	Y	<4 stools in 24 h and absence of liquid stools	Mean number (SD) of stools on day 4: Probiotic 2.00 (1.94) versus Placebo 3.29 (2.19)	$p < 0.05$	None
Costa-Ribeiro et al. (2003)	1–24 m admitted for acute diarrhea, not severely malnourished, Brazil	≥3 watery or loose stools per 24 h during at least one 24-h period in the 72 h before admission	Rotavirus, *Salmonella, Shigella, Campylobacter, Yersinia*	LGG 1 × 10^{10} cfu/day, manufacturer NS, duration <7 days	N	61/61–100%	63/63–100%	N/A	Y	Y	Passage of 2 semi-formed or formed stools or no stool for 24 h	Duration of diarrhea: Probiotic 38.27 ± 3.78 h versus Placebo 39.09 ± 4.60 h	P = 0.59	NS

Table 22.2 (*Cont'd* p. 860)

Reference	Population	Definition of diarrhea for enrollment	Diarrheal pathogens	Probiotic studied	Antibiotics to treat diarrhea	# Randomized to probiotic/# (%) completing trial	# Randomized to placebo/# (%) completing trial	Reasons for not completing trial	Randomization described	Double blinded	Definition of resolution of diarrhea	Result Probiotic versus Placebo Group	Statistics	Adverse Events
Salazar-Lindo et al. (2004)	3–36 m admitted for acute diarrhea, slightly malnourished, Peru	≥3 watery stools/day for <48 h	*Salmonella, Shigella, E. coli, Vibrios, Campylobacter, Aeromonas*	LGG 6–8 × 10^{11} cfu/day, Valio, Finland × 5 days maximum or end of diarrhea	N	90/90–100%	89/89–100%	N/A	Y	Y	Passage of formed stool or passage of no stool for 12 consecutive h	Total stool output: Probiotic 195.0± 171.9 ml/kg versus Placebo 247.8± 180.2 ml/kg	P = 0.047	None
Lievin-Le et al. (2007)	10 m admitted for acute diarrhea, Ecuador	>3 liquid stools during last 24 h of suspected infectious origin, <72 h before inclusion	Rotavirus	*L. acidophilus* LB, heat-killed + culture medium 2 × 10^{10} cfu/day, Laboratoire du Lacteol, France × 72 h	N	42/42–100%	38/38–100%	N/A	N	Y	Passage of first "normal" stool	Duration of diarrhea: Probiotic 39.5±10.5 h versus Placebo 63.4± 14.9 h	p < 0.01	NS
Villarruel et al. (2007)	3–24 m outpatients, Argentina	≥3 loose/liquid stools in preceding 24 h but for <7 days	NS	*Saccharomyces boulardii* 250–500 mg/day, manufacturer NS × 6 days	Y per MD	50/44–88%	50/44–88%	Lack of compliance	Y	Y	<3 stools/day or improved stool consistency for at least 24 h	Mean duration of diarrhea: Probiotic 4.70 days versus Placebo 6.16 days	p < 0.05	NS
Europe														
Isolauri et al. (1991)	4–45 m admitted for acute diarrhea, Finland	>3 watery stools during previous 24 h, <7 days	Rotavirus, *Salmonella, Yersinia, Campylobacter, Shigella*	LGG 2 × 10^{10-11} cfu/day, Valio, Finland × 5 days	N	Milk: 24/24–100%; Powder: 23/23–100%	24/24–100%	N/A	N	Y	Last appearance of watery stools	Mean duration of diarrhea: Probiotic milk 1.4 (0.8) days versus Probiotic powder 1.4 (0.8) days versus Placebo 2.4 (1.1) days	P < 0.001	NS

Kaila et al. (1992)	7–37 m admitted for acute rotavirus diarrhea during a rotavirus epidemic, not breastfed, Finland	NS	Rotavirus	LGG $2 \times 10^{10-11}$ cfu/day, Valio, Finland × 5 days	N	NS/22–NS	NS/17–NS	Rotavirus negative	N	NS	Last appearance of watery stools	Mean duration of diarrhea: Probiotic 1.1 (0.6) days versus Placebo 2.5 (1.4) days	P = 0.001	NS
Isolauri et al. (1994)	5–28 m inpatient and outpatient with acute gastroenteritis, Finland	>3 watery stools during previous 24 h, less than 7 days	Rotavirus, *Salmonella*, *Shigella*, *Campylobacter*, *Yersinia*	LGG 2×10^{10} cfu/day, manufacturer NS × 5 days	N	21/21–100%	21/21–100%	N/A	N	NS	NS	Duration of diarrhea: Probiotic 1.5 days versus Placebo 2.3 days	P = 0.002	NS
Kaila et al. (1995)	1.3–38.4 m inpatient and outpatient with acute rotavirus gastroenteritis, Finland	NS	Rotavirus	LGG $2 \times 10^{10-11}$ cfu/day, Valio, Finland × 5 days	NS	20/13–65%	21/13–62%	Rotavirus negative	N	Y	NS	Duration of diarrhea: Probiotic 2.5 days versus Placebo 2.9 days	P = 0.53	NS
Majamaa et al. (1995)	6–35 m admitted with acute rotavirus gastroenteritis, Finland	≥3 watery stools in the last 24 h before admission	Rotavirus	Group 1 LGG 1.3×10^{10} cfu/day, Valio Finland; Group 2 *L. casei rhamnosus* (Lactophilus) 5.5×10^{8} cfu/day, Laboratoires Lyocentre, France; Group 3 *S. thermophilus* + *L. delbruckii bulgaricus* (Yalacta) 7.0×10^{9} cfu/day, Caen Cedex, France; all × 5 days	N	LGG: 16/16–100%; Lactophilus: 14/14–100%; Yalacta: 19/19–100%	None	N/A	N	N/A	NS	Mean duration of diarrhea: (1) LGG 1.8 (0.8) days; (2) Lactophilus 2.8 (1.2) days; (3) Yalacta 2.6 (1.4) days	P = 0.04	NS

Table 22.2 (*Cont'd* p. 862)

Reference	Population	Definition of diarrhea for enrollment	Diarrheal pathogens	Probiotic studied	Antibiotics to treat diarrhea	# Randomized to probiotic/# (%) completing trial	# Randomized to placebo/# (%) completing trial	Reasons for not completing trial	Randomization described	Double blinded	Definition of resolution of diarrhea	Result Probiotic versus Placebo Group	Statistics	Adverse Events
Canani et al. (1997)	Children, age NS, admitted with acute diarrhea, Italy	NS	Rotavirus	LGG, dose, manufacturer, and duration NS	N	31/31–100%	30/30–100%	N/A	N	NS	NS	Duration of diarrhea in rotavirus-positive patients: Probiotic 3.0±0.2 days versus Placebo 6.2±0.2 days	$p < 0.01$	NS
Guarino et al. (1997)	3–36 m outpatients with acute gastroenteritis, not breastfed, Italy	≥3 watery stools/day	Rotavirus	LGG 6×10^9 cfu/day, Dicofarm SpA, Italy × 5 days maximum	N	48/48–100%	52/52–100%	N/A	Y	N	Time since last loose or liquid stools	Duration of diarrhea: Probiotic ~3 days versus Placebo ~6 days	$P < 0.01$	NS
Shornikova et al. (1997c)	1–36 m admitted with acute diarrhea, Russia	≥1 watery stools during previous 24 h, <5 days	Rotavirus, *Salmonella*, *Shigella*	LGG 1×10^{10} cfu/day, Valio, Finland × 5 days	Y if Salmonella or Shigella	59/59–100%	64/64–100%	N/A	Y	Y	Last appearance of watery stools	Duration of diarrhea in hospital: Probiotic 2.7 days versus Placebo 3.8 days	$P = 0.02$	NS
Shornikova et al. (1997b)	6–36 m admitted receiving ORS, Finland	≥3 watery stools during the preceding 24 h, <7 days	Rotavirus	*L. reuteri* 1×10^{10} cfu/day or 1×10^7 cfu/day, BioGaia Biologics AB, Sweden × 5 days maximum	N	high dose: 21/21–100%; low dose: 20/20–100%	25/25–100%	N/A	N	Y	Absence of watery stools for a 24 h period	Duration of diarrhea after initiation of treatment: Probiotic high dose 1.5 days versus Probiotic low dose 1.9 days versus Placebo 2.5 days	$p = 0.01$	NS

Shornikova et al. (1997a)	6–36 m admitted for acute diarrhea, Finland	≥1 watery stools during preceding 24 h, <7 days	Rotavirus	*L. reuteri* SD 2112 $1 \times 10^{10-11}$ cfu/day, BioGaia Biologics, USA × 5 days maximum	N	19/19–100%	22/21–95%	Sib assigned to active group but patient in placebo group had probiotic in stool	N	Y	Last appearance of watery stools	Mean duration of watery diarrhea: Probiotic 1.7 (1.6) days versus Placebo 2.9 (2.3) days	P = 0.07	NS
Rautanen et al. (1998)	6–36 m admitted with acute diarrhea, Finland	NS	Rotavirus	Group 1 LGG 5×10^9 cfu once immediately; Group 2 LGG 1×10^{10} cfu/day during entire hospital stay; Group 3 placebo once immediately then LGG 1×10^{10} cfu/day throughout hospital stay, Valio, Finland	N	Group 1 28/28–100%; Group 2 33/33–100%; Group 3 32/32 - 100%	30/30–100%	N/A	N	Y	NS	Duration of diarrhea: Hypotonic ORS and early dose of LGG 17.7 h versus all other groups 29.5–30.4 h	P = 0.03	NS
Guandalini et al. (2000)	1 month–3 years admitted for acute diarrhea, 10 European countries	>4 movements/day of liquid or semi-liquid stools for 1–5 days	Rotavirus, Adenovirus, *Salmonella, Shigella, Campylobacter, Yersinia, Giardia, Cryptosporidium, Amoeba*	LGG 3.2×10^9–2×10^{11} cfu/day, Dicofarm SpA, Italy until end of diarrhea	N	NS/147–NS	NS/140–NS	Information incomplete or protocol violations	N	Y	Last fluid stool	Duration of diarrhea: Probiotic 58.3± 27.6 h versus Placebo 71.9± 35.8 h	P = 0.03	NS
Rosenfeldt et al. (2002a)	6–36 m inpatients and outpatients with acute diarrhea, Denmark	≥2 consecutive loose stools during 24 h, ≤7 days	Rotavirus (40), bacterial pathogen with rotaviral co-infection (6), Campylobacter jejuni (3), Salmonella typhimurium (1), no etiology (20)	*L. rhamnosus* 19,070–2 + *L. reuteri* DSM 12,246 2×10^{10} cfu/day, Chr Hansen A/S, Denmark × 5 days	N	37/30–81%	49/39–80%	Prescription of antibiotics; recovery within first day of intervention (did not pass stools within first 24 h after enrollment); noncompliance	N	Y	Time to appearance of the first normal stood (parent report)	Mean duration of diarrhea: Probiotic 81.5 h (37.3 SD) versus Placebo 101.1 h (47.6 SD)	P = 0.07	NS

Table 22.2 (*Cont'd* p. 864)

Reference	Population	Definition of diarrhea for enrollment	Diarrheal pathogens	Probiotic studied	Antibiotics to treat diarrhea	# Randomized to probiotic/# (%) completing trial	# Randomized to placebo/# (%) completing trial	Reasons for not completing trial	Randomization described	Double blinded	Definition of resolution of diarrhea	Result Probiotic versus Placebo Group	Statistics	Adverse Events
Rosenfeldt et al. (2002b)	9–44 m outpatients at daycare centers with acute diarrhea, Denmark	≥2 consecutive loose stools during 24 h, <7 days	Rotavirus, *Campylobacter jejuni, Clostridium difficile, Salmonella typhimurium*	*L. rhamnosus* 19,070–2 + *L. reuteri* DSM 12,246 2×10^{10} cfu/day, manufacturer NS × 5 days	N	27/24–89%	23/19–83%	Hospitalization for excessive vomiting and moderate dehydration	N	Y	NS	Mean duration of diarrhea: Probiotic 75.9 ± 39.7 h versus Placebo 115.7 ± 85.0 h	P = 0.05	None
Kurugol and Koturoglu (2005)	3 months–7 years inpatients and outpatients with acute diarrhea, Turkey	Liquid, mucous or bloody stools 2x + versus usual for 24 h–7 days before admission	Rotavirus, *Salmonellae, Shigella, Campylobacter jejuni, Vibrio cholera, E. coli, parasites*	*Saccharomyces boulardii* 250 mg/day, Sanofi, Turkey × 5 days	N	115/100–90%	117/100–85%	Prescription of antibiotics (23); noncompliance (9)	N	Y	NS	Duration of diarrhea: Probiotic 4.7 ± 2.5 days versus Placebo 5.5 ± 3.2 days	P = 0.03	None
Szymanski et al. (2006)	2 months–6 years inpatients and outpatients with acute diarrhea, not exclusively breastfed, Poland	≥3 bowel movements/day of stools looser than normal and may contain blood, pus or mucus, 1–5 days	Rotaviruses, adenoviruses, *Shigella, E. coli*	*L. rhamnosus* strains 573L/1, 573L/2, 573L/3, 2.4×10^{10} cfu/day, Biomed, Poland × 5 days	N	49/46–94%	44/41–94%	Incomplete and/or not compliant with protocol	Y	Y	First of no abnormal bowel movement for the last 12 h or time of second normal stool	Mean duration of diarrhea: Probiotic 84 ± 56 h versus Placebo 96 ± 72 h	P = 0.36	None

Canani et al. (2007)	3–36 m, outpatients with acute diarrhea, Italy	≥3 loose/liquid stools a day	NS	Group 1 LGG 1.2×10^{10} cfu/day, Dicoflor 60; Group 2 S boulardii 1×10^{10} cfu/day, Codex; Group 3 *Bacillus clausii* 2×10^{9} cfu/day, Entero-germina; Group 4 *L. delbrueckii* var *bulgaricus* 2×10^{9} cfu/day + *S. thermophilus* 2×10^{9} cfu/day + *L. acidophilus* 2×10^{9} cfu/day + *B. bifidum* 1×10^{9} cfu/day, Lactogermina; Group 5 *Enterococcus faecium* SF68 1.5×10^{8} cfu/day, Bioflorin; all × 5 days	N	Group 1 100/100–100%; Group 2 91/91–100%; Group 3 100/100–100%; Group 4 97/97–100%; Group 5 91/91–100%	92/92–100%	N/A	Y	N	Time to first normal stool output	Mean duration of diarrhea: various Probiotic Groups (1) 78.5 h; (2) 105.0 h; (3) 118.0 h; (4) 70.0 h; (5) 115.0 h versus Placebo 115.5 h	Group 1 < 0.001; Group 2 0.38; Group 3 0.76; Group 4 < 0.001; Group 5 0.61 (vs Placebo)	None
Henker et al. (2007)	2–47 m outpatients with acute diarrhea, Russia	>3 watery/loose stools in 24 h	*Salmonella, Campylobacter, Yersinia, E. coli* (ETEC, EPEC, EIEC, EHEC), *Shigella, Entamoeba histolytica, Cryptosporidium parvum,* rotaviruses adenovirus, noroviruses	*E. coli* Nissle 1917, dose by age: <1 year 1×10^{8} cfu/day; $1 \leq x \leq 3$ years 2×10^{8} cfu/day; $3 < x \leq 4$ years 3×10^{8} cfu/day; Ardeypharm, Germany × 10 days maximum	Y - culture positive	55/55–100%	58/58–100%	N/A	Y	Y	≤3 watery/loose stools over at least 2 consecutive days	Time to response: Probiotic 2.5 days versus Placebo 4.8 days	P = 0.0007	Probiotic group: rhinitis, abdominal pain; Placebo: acute otitis media

Table 22.2 (*Cont'd* p. 866)

Reference	Population	Definition of diarrhea for enrollment	Diarrheal pathogens	Probiotic studied	Antibiotics to treat diarrhea	# Randomized to probiotic/# (%) completing trial	# Randomized to placebo/# (%) completing trial	Reasons for not completing trial	Randomization described	Double blinded	Definition of resolution of diarrhea	Result Probiotic versus Placebo Group	Statistics	Adverse Events
Asia														
Raza et al. (1995)	1–24 months undernourished inpatients with acute bloody diarrhea, Pakistan	>3 watery stools during previous 24 h; <14 days	None	LGG $2 \times 10^{10-11}$ cfu/day, Scientific Hospital Supplies, UK × 2 days	N	21/19–90%	19/17–89%	Development of profuse diarrhea, vomiting and severe dehydration, pneumonia	N	Y	NS	Stool frequency on day 2: Probiotic 5.8 versus Placebo 7.0	P-NS	NS
Pant et al. (1996)	1–24 m inpatients with acute diarrhea, not exclusively breastfed, Thailand	>3 watery stools during previous 24 h; <14 days	Rotavirus. Astrovirus, *Cryptosporidium*	LGG $2 \times 10^{10-11}$ cfu/day, Scientific Hospital Supplies, UK × 2 days	Antibiotics before admission - probiotic group (9), placebo group (7)	20/20–100%	19/19–100%	Not clear if ITT analysis	N	Y	Time of last watery stool	Mean duration of diarrhea: Probiotic 1.9 (0.6) days versus Placebo 3.3 (2.3) days	P = 0.055	NS
Simakachorn et al. (2000)	3–24 m admitted with acute diarrhea, Thailand	Diarrhea present for ≤5 days	Rotavirus	*L. acidophilus* LB + fermented culture medium 2×10^{10} cfu/day, Laboratoire du Lacteol, France × 3 days	Y	37/37–100%	36/36–100%	N/A	Y	Y	2 consecutive well-formed stools following an unformed stool or no stool passed for 12 h	Mean duration of diarrhea: Probiotic 43.4 h versus Placebo 57.0 h	P = 0.012	None
Lee et al. (2001)	6–60 m admitted with acute diarrhea, Taiwan	>3 watery stools during previous 24 h and <5 days	NS	*L. acidophilus* + *B. infantis* 3×10^{9} cfu/day of each strain, Berna, Switzerland × 4 days	N	50/50–100%	50/50 - 100%	N/A	N	N	No more watery stool appearance	Duration of diarrhea in hospital: Probiotic 3.1 days versus Placebo 3.6 days	P < 0.01	NS

Hafeez et al. (2002)	3–60 m outpatients with acute diarrhea, Pakistan	NS	ND	*Saccharomyces boulardii* 500 mg/day, manufacturer NS × 6 days	N	59/51–86%	58/50–86%	NS	N	N	NS	Mean frequency of stools per day: Probiotic day 3 (4.39) and on day 6 (3.22) versus Placebo day 3 (5.73), day 6 (3.31)	Day 3: P = 0.021; day 6: P = 0.173	None
Khanna et al. (2005)	6 months–12 years inpatients and outpatients with acute diarrhea, India	NS	Rotavirus, Cholera	*L. acidophilus* 1.5×10^{10} cfu/day, Raptakos, India × 3 days	N	48/48–100%	50/50–100%	N/A	N	Y	Time to first of three consecutive semi-formed stools or no stools for 12 h	Mean duration of diarrhea: Probiotic 54.45 h versus Placebo 55.08 h	P-NS	NS
Sarker et al. (2005)	4–24 m admitted for acute non-bloody diarrhea, Bangladesh	<2 days	Rotavirus, *V. cholera, Salmonella, Shigella, Campylobacter*	*L. paracasei* ST11 1×10^{10} cfu/day, Nestle Research Centre, Switzerland × 5 days	N	115/115–100%	115/115–100%	N/A	Y	Y	Passage of the last watery or loose stool before passage of two consecutive soft or formed stools or no stool in >2 consecutive 8-h periods	Total duration of diarrhea: Probiotic 123.8±46.0 h versus Placebo 126.1±44.8 h	P = 0.71	NS
Billoo et al. (2006)	2 months–12 years patients with acute diarrhea, not severely malnourished, Pakistan	NS	Rotavirus	*Saccharomyces boulardii* 500 mg/day, Hilton Pharma (Pvt) Ltd., licensed by Biocodex, France x 5 days	N	50/50–100%	50/50–100%	N/A	N	N	NS	Mean number of stools: Probiotic day 3 (2.8) & day 6 (1.6) versus Placebo day 3 (4.4) & day 6 (3.3)	day 3 P = 0.01; day 6 P = 0.001	None
Agustina et al. (2007)	3–12 m admitted with acute diarrhea, not breastfed, Indonesia	>3 stools for more than 24 h and <7 days, moderate dehydration (WHO)	Rotavirus, Enteropathogenic *E. coli* (EPEC), *Salmonella* spp, *Shigella* spp, *Vibrio cholera*	*L. rhamnosus* LMG P-22,799 + inulin + dietary fiber + Zn & Fe, Probiotic up to 7×10^8 cfu/day, manufacturer NS × 7 days maximum	N	NS/30–NS	NS/28–NS	Some subjects considered pilot to evaluate study execution; secondary infection occurred; withdrawn from study by parents; no explanation	N	Y	<2 liquid/semi-liquid stools within 24 h	Duration of diarrhea in hospital: Probiotic 1.63±0.35 versus Placebo 2.45±0.18 days	P = 0.02	NS

Table 22.2

Reference	Population	Definition of diarrhea for enrollment	Diarrheal pathogens	Probiotic studied	Antibiotics to treat diarrhea	# Randomized to probiotic/# (%) completing trial	# Randomized to placebo/# (%) completing trial	Reasons for not completing trial	Randomization described	Double blinded	Definition of resolution of diarrhea	Result Probiotic versus Placebo Group	Statistics	Adverse Events
Basu et al. (2007a)	Mean 4.15 year admitted for persistent diarrhea, malnourished, India	Persistent diarrhea: diarrhea for ≥14 days, stool pH < 5.5, stool reducing substance >1%	*E. coli, Shigella spp., Clostridium difficile*	LGG 1.2×10^{11} cfu/day, manufacturer NS × 7 days or until diarrhea stopped	Y - culture positive	125/117–94%	128/118–92%	Developed septicemia, renal failure, withdrew consent	Y	Y	NS	Mean duration of diarrhea: Probiotic 5.3 days versus Placebo 9.2 days	$P < 0.05$	None
Basu et al. (2007b)	Mean 1.19 year inpatient and outpatient with acute non-bloody diarrhea, malnourished to well nourished, India	≥3 watery stools/day	Rotavirus	LGG 1.2×10^{11} cfu/day, manufacturer NS × 7 days or until diarrhea stopped	N	330/323–98%	332/323–97%	Electrolyte imbalance, septicemia, withdrew consent	Y	Y	2 consecutive soft or formed stools or no stool for 12 consecutive hours	Average duration of diarrhea: Probiotic 6.8±2.1 days versus Placebo 6.6 ±2.3 days	$p > 0.05$	Electrolyte imbalance and septicemia
Htwe et al. (2008)	3 months–10 years admitted with acute diarrhea, Myanmar	≥3 loose stools/day (loose stool takes shape of container); acute watery diarrhea w/ duration <7 days	*E. coli*	*Saccharomyces boulardii* 500 mg/day, manufacturer NS × 5 days	N	50/50–100%	50/50–100%	N/A	N	NS	<3 stools/day or stools with solid consistency only	Duration of diarrhea: Probiotic 3.08 days versus Placebo 4.68 days	$P < 0.05$	NS
Narayanappa (2008)	3 months–years admitted for acute rotavirus diarrhea, India	NS	Rotavirus	Strain, dose, and manufacturer NS, x14 days or recovery from diarrhea	N	40/40–100%	40/40–100%	N/A	N	Y	NS	Duration of diarrhea: Probiotic 4.35 days (1.25) versus Placebo 5.45 days (1.69)	$P = 0.001$	None

Henker et al. (2008)	1–47 m outpatients with acute diarrhea, Ukraine	>3 watery or loose non-bloody stools in 24 h of a diarrheal episode persisting for >4 but ≤14 days	Bacteria, viruses	*E. coli* Nissle 1917, dose by age: <1 year 1×10^8 cfu/day; $1 \leq x \leq 3$ year 2×10^8 cfu/day; $3 < x < 4$ year 3×10^8 cfu/day; Ardeypharm, Germany × 21 days	N	75/75–100%	76/76–100%	N/A	Y	Y	Reduction of daily stool frequency to ≤3 watery or loose stools over ≥4 consecutive days	Time to response: Probiotic 2.4 days versus Placebo 5.7 days	$p < 0.0001$	11 total - Probiotic - limb injury, traumatic hematoma, hypersensitivity; placebo - influenza, vomiting, abdominal pain, cough, rhinorrhea, dermatitis, viral upper respiratory tract infection, nasopharyngitis
Adult Studies														
Wunderlich et al. (1989)	Mean 33 year inpatients with acute diarrhea, Switzerland	≥2 liquid or semi-liquid stools/day	NS	*Enterococcus* SF 68 > 2.3×10^8 cfu/day, Giuliani SA, Switzerland × 7 days	N	40/36–90%	38/33–87%	"Dropout unrelated to medication"	N	Y	NS	Persistence of diarrhea by day 7: Probiotic 0.6% versus Placebo 8.7%	$P < 0.01$	None
Buydens and Debeuckelaere (1996)	18–75 year inpatient and outpatients with acute diarrhea and no chronic causes of diarrhea, Belgium	≥3 watery or loose stools in 24 h before admission	*Salmonella, Campylobacter jejuni,* Yersinia	*Enterococcus* SF 68 2.3×10^8 cfu/day, Giuliani SA, Switzerland × 5 days minimum	N	105/93–89%	106/92–88%	Violation of protocol, noncompliance, lost to follow-up before visit 3	Y	Y	Return to normal stool frequency (<3) and normal stool consistency (semisolid or solid stool) without associated symptoms	Proportion with diarrhea on day 2 of treatment: Probiotic 61% versus Placebo 96%	$P < 0.01$	None
Margreiter et al. (2006)	18–75 year outpatients with acute diarrhea, no chronic causes of diarrhea, Austria	≥3 watery or loose stools in 24 h before admission	ND	Group 1 *L. gasseri* $6 \times 10^{7-8}$ cfu/day + *B. longum* $6 \times 10^{7-8}$ cfu/day, Omniflora; Group 2 *Enterococcus faecium* SF 68 strain Cernelle 68 2.3×10^8 cfu/day, Bioflorin; x 6 days maximum	N	169/166–98% (2 groups: combination, NS; monobacterium: NS)	N/A	3 lost to follow-up	NS	Y	"Normal bowel function"	Mean duration of diarrhea: Probiotic 2.70 days versus Placebo 2.67 days	$P = 0.59$	None

Table 22.3

Details of trials using probiotics to prevent traveler's diarrhea

Reference	Population	Probiotic studied	# randomized to probiotic/ # (%) completing trial	# randomized to placebo/# (%) completing trial	Reasons for not completing trial	Randomization described	Double Blinded	Outcome – diarrhea	Result Probiotic versus Placebo Group	Statistics	Adverse Events
de dios Pozo-Olano et al. (1978)	>18 year healthy US travelers to Mexico	*L. acidophilus* + *L. bulgaricus* 3.6–7.2 × 10^9 cfu/day, Hynson, Westcott & Dunning, Inc., USA × 8 days	26/26–100%	24/24–100%	N/A	N	Y	≥3 liquid stools passed in 1 day or liquid stools of any number passed in 2 or more consecutive days	Diarrhea: Probiotic 35% versus Placebo 29%	P-NS	NS
Black et al. (1989)	Mean 51 year Danish travelers to Egypt	*L. acidophilus* + *B. bifidum* + *L. bulgaricus* + *S. thermophilus* 9 × 10^9 cfu/day, manufacturer NS × 16 days,	47/40–85%	47/41–87%	Use of antacids or not stated	N	Y	≥3 watery stools/24 h or 1 watery stool with 1 + of (fever, nausea/, vomiting, or abdominal cramps)	Diarrhea: Probiotic 43% versus Placebo 71%	P = 0.019	None
Oksanen et al. (1990)	10–80 year health status NS, Finnish travelers to Turkey	LGG 2 × 10^9 cfu/day, Valio, Finland, 9 days or 16 days	402/NS–% not known	418/NS–% not known	Incomplete information or form not returned, used other probiotics, ill at start of travel, forgot to take medication on trip, stopped taking preparation due to AEs	N	Y	≥3 unformed stools lasting more than 24 h or 1–2 unformed watery stools in less than 24 h	Diarrhea: Probiotic 41% versus Placebo 46.5%	P = 0.06	None in completers but excluded two subjects excluded from analysis due to AE/non-compliance (nausea - 1; dizziness - group unknown)

Katelaris et al. (1995)	Adult British military traveling to Belize	Group 1 *L. fermentum* strain KLD (LF-KLD) 2×10^{11} cfu/day; Group 2 *L. acidophilus* (LA) 2×10^{11} cfu/day; manufacturer NS × 3 weeks or until diarrhea	Group 1 NS/80–% not known; Group 2 NS/101–% not known	NS/101–% not known	N/A	N	Y	≥3 unformed stools in 24 h or any number of loose stools with blood, fever, abdominal pain, or vomiting	Diarrhea: Probiotic LF-KLD 23.8% versus Probiotic LA 25.7% versus Placebo 23.8%	P-NS	None
Hilton et al. (1997)	>18 year healthy US travelers to developing countries	LGG 2×10^{9} bacteria/day, manufacturer NS, 2 days prior to departure until end of travel	200/126–63%	200/119–60%	Never took study medication (114); took partial course (28); trip cancelled (13)	N	Y	≥3 loose bowel movements per day associated with abdominal cramping	"Risk of having diarrhea on any day": Probiotic 3.9% versus Placebo 7.4%	P = 0.05	"Side effects attributable to *Lactobacillus* GG were few" abdominal cramping (2)
(Briand et al., 2006)	≥18 year healthy French travelers to areas with intermediate or high risk of diarrheal illness	*L. acidophilus* (non viable) + fermented culture medium 2×10^{10} cfu/day, manufacturer NS, 1 d pre-travel through 3 days post- travel	174/123–71%	174/122–71%	Cancelled travel, did not obtain study medication, were not eligible, did not follow-up	Y	Y	≥3 unformed stools in 24 h	Episodes: Probiotic 61.4/100 person-months versus Placebo 43.4/100 person-months	P = 0.14	125 events, 64 in probiotic group, 61 in placebo group, 34% gastrointestinal, headache (14%); ear, nose, and throat symptoms (15%); or cutaneous eruption (13%); no difference between groups

22.10 What is the Evidence for the Use of Probiotics for the Prevention of Infectious Diarrhea?

Table 22.1 includes details of seven trials of various probiotics to prevent diarrheal disease (various definitions) in healthy infants and children under age 3 years in developed countries. Most of the studies included non-breastfed infants in daycare. Three of the seven trials showed benefit of the probiotic studied, while four did not show benefit. All studies had at least one methodological issue, mostly lack of intent to treat analyses or lack of blinding of study treatment. *Table 22.1* also includes details of two studies showing benefit of the studied probiotic – *Lactobacillus* GG or *Bacillus coagulans* – based on the number of diarrheal disease episodes (not intent to treat analysis), although in one of the studies, the benefit was mainly observed in non-breastfed infants. By comparison, in two studies of healthy adults, there was no benefit of the studied probiotics (*L acidophilus* and *L bulgaricus* in one study, *L casei* DN-114 001 in the other) for the prevention of diarrheal disease. One study not included in the table was Hatakka's study of Finnish children aged 1–7 years in daycare who received *Lactobacillus* GG or placebo for 7 months (Hatakka et al., 2001). The outcome of the study was absence from the daycare center due to respiratory infection. Time without diarrhea, a secondary outcome, was similar in the two groups.

There are two studies of hospitalized children under age 3 years in which the studied probiotic (*Bifidobacterium bifidum* and *S. thermophilus* in one study, *Lactobacillus* GG in the other) reduced diarrheal episodes or diarrhea during the hospital stay. One study of critically ill adults receiving enteral nutrition showed that those receiving *Saccharomyces boulardii* (*Saccharomyces cerevisiae*) had fewer days of diarrhea than those receiving placebo.

In 4 of the 11 pediatric studies, subgroup analyses were done to evaluate the effect of the studied probiotic on prevention of rotavirus diarrhea. In all four studies (investigating different probiotics), the authors conclude that the studied probiotic reduces either rotavirus shedding or the incidence and duration of rotavirus diarrhea. There are no subgroup analyses showing benefit of probiotics in the prevention of bacterial diarrhea, probably because all studies would be underpowered to detect differences in the prevention of the less common bacterial versus viral diarrhea.

Adverse events were not addressed in seven of the studies in *Table 22.1*. In six studies the authors stated that there were no adverse events in the probiotic or comparison groups, and in one study of various probiotic and prebiotic combinations, adverse events were similar in the various study groups.

Over the last eight years there has been one meta-analysis (Sazawal et al., 2006) and one systematic review (Szajewska and Mrukowicz, 2001) evaluating the efficacy of probiotics in the prevention of diarrhea, both of which conclude that there are insufficient data to recommend the use of probiotics for the prevention of acute childhood diarrhea unrelated to administration of antibiotics.

On balance, while the use of probiotics for the prevention of infectious diarrhea is promising, particularly for non-breastfed infants in daycare, there is insufficient evidence to recommend any specific probiotic or probiotics in general for the prevention of infectious diarrhea. Given that this promising efficacy in the studies reported in *Table 22.1* may be due to prevention of rotavirus diarrhea – for which two vaccines are now available, at least in North and South America and Europe – the value of probiotics is further brought into question. In the studies reviewed in *Table 22.1*, there do not appear to be any safety concerns relating to the use of probiotics to prevent diarrhea, although this topic is further addressed in Section 12 below.

22.11 What is the Evidence for the Use of Probiotics for the Treatment of Infectious Diarrhea not Related to the Use of Antibiotics?

Table 22.2 includes details of 38 trials of various probiotics to treat infectious diarrheal disease (variable definitions) in children – 1 in North America, 5 in Central/South America, 18 in Europe and 14 in Asia. There is a great deal of heterogeneity in the studies due to these variable definitions of diarrhea at enrollment as well as the definition of resolution of the diarrheal episode. The etiology of diarrhea also varied widely (some studies included children with only rotavirus diarrhea, others included children with dysentery, some on any type of diarrhea and 7 of the 38 did not determine whether any pathogen was present).

In 17 of the 38 studies (45%), the probiotic tested was *Lactobacillus* GG (also known as LGG, *Lactobacillus rhamnosus* GG, *Lactobacillus case* GG), but the dose and duration of even this one probiotic varied across the studies. Twelve of the 17 Lactobacillus GG studies showed benefit of the probiotic on diarrhea (mostly duration of diarrhea), while five did not.

The next most common probiotic studied was *Saccharomyces boulardii* in 7 of the 38 studies (one study included five groups – LGG, *Saccharomyces, Bacillus clausii,* a combination of probiotic organisms, *Enterococcus faecium* SF68 and placebo (Canani et al., 2007)). The *Saccharomyces* studies also had variations in

definitions of diarrhea at study entry and exit, as well as dose and duration of administration of the probiotic. All but one of the *Saccharomyces* studies reported benefit of the probiotic.

The remaining 15 studies involved testing of a wide range of probiotic organisms again with the same issues relating to definitions of diarrhea. Twelve of the 15 showed benefit of the probiotic studied.

Although rotavirus diarrhea was the most commonly studied pathogen in the 38 studies, probiotics did not always reduce the duration of rotaviral diarrhea. Based on the information provided in the trials, it is difficult to evaluate whether any specific probiotic was efficacious in the treatment of any pathogen other than rotavirus.

As with the studies of prevention of infectious diarrhea in Section 9 of this chapter, all studies had at least one methodological issue, mostly due to unclear definitions of diarrhea at study entry and exit, lack of intent to treat analyses or lack of blinding of study treatment.

There are three small studies of treatment of infectious diarrhea in adults, two of which showed benefit of *Enterococcus faecium* SF 68 and in the third, a combination product was not effective. These studies have the same methodological issues as those raised for the pediatric studies above.

Adverse events were not addressed in 24 of the 38 pediatric and in three adult studies in *Table 22.1*. In fourteen studies the authors stated that there were no adverse events in the probiotic or comparison groups, and in three studies of various probiotic and prebiotic combinations, adverse events were similar in the various study groups.

Over the last six years there has been one systematic review from the Cochrane group (Allen et al., 2004) (last updated in 2002) evaluating the efficacy of probiotics in the treatment of infectious diarrhea. This review concluded that probiotics appear to be a useful adjunct to rehydration therapy for both adults and children, but that more research is needed to address particular regimens for specific patient populations. There is one meta-analysis on the use of *Saccharomyces boulardii* that concluded that there was a moderate clinical benefit to use of this probiotic for infectious diarrhea in otherwise healthy infants and children, but also cautioned about the methodological limitations of the five studies included in the meta-analysis (Szajewska et al., 2007a). In a similar meta-analysis involving eight trials on the use of *Lactobacillus* GG, use of *Lactobacillus* GG was associated with moderate clinical benefits for the treatment of infectious diarrhea, again with caution regarding the methodological limitations, but much of the benefit of *Lactobacillus rhamnosus* GG may be for children with rotavirus diarrhea (Szajewska et al., 2007b).

In the studies reviewed in *Table 22.2*, there do not appear to be any safety concerns relating to the use of probiotics to treat infectious diarrhea, although a full risk-benefit assessment is difficult because details of several of the adverse effects are limited. This topic is further addressed in Section 12 below.

On balance, certain probiotics appear to reduce the duration of acute infectious diarrhea, particularly for diarrhea caused by rotavirus. Not all probiotics are equally efficacious, and based on the heterogeneity of available data it is difficult to make specific recommendations for the use of particular probiotics and specific doses and durations of treatment. As above, another concern relating to use of probiotics for infectious diarrhea is the likely changing epidemiology of the pathogens causing diarrhea as a result of increasing uptake of the rotavirus vaccines that are widely available in North and South America and Europe. Since there may be delay before the rotavirus vaccine is available in Africa and Asia, specific probiotics, such as *Lactobacillus* GG, may be useful as treatment adjuncts to oral rehydration solution in these locations, although benefit may be to reduce the duration of diarrhea by about 24 h. It may not be possible to generalize the benefit for the treatment of rotavirus diarrhea that was seen in the European rotavirus studies to Africa and Asia since rotavirus genotypes vary by geographic location. Another concern is the potential delay in initiating treatment if children are not brought as promptly to medical care in Africa and Asia. At least one of the studies in *Table 22.2* demonstrated that early administration of probiotics was beneficial while later administration was not (Rautanen et al., 1998).

22.12 What is the Evidence for the Use of Probiotics for the Prevention of Traveler's Diarrhea?

Table 22.3 includes details of six trials of various probiotics to prevent traveler's disease (again, various definitions) in adults. Two studies reported benefit of the studied probiotic while four did not, and in one study, there was a trend towards benefit in the probiotic group (Oksanen et al., 1990). Again, all studies had at least one methodological issue, mostly lack of intent to treat analyses and lack of information on the randomization process. Adverse events were not addressed in one of the studies in *Table 22.3*, in two studies trial "completers" had no adverse events and in two studies, adverse events were similar in probiotic and placebo group.

Although it is appealing to imagine that probiotics are effective in the prevention of traveler's diarrhea, on balance, there is insufficient evidence in support of use of any specific probiotic or probiotics in general. Part of the

difficulty in preventing traveler's diarrhea is the range of potential pathogens to which travelers visiting multiple different locations are exposed. It may be unrealistic to expect a single probiotic to protect against multiple pathogens with different mechanisms of action in the gastrointestinal tract. In the studies reviewed in *Table 22.3*, there do not appear to be any safety concerns relating to the use of probiotics to prevent diarrhea, although this topic is further addressed in Section 12 below.

22.13 What do we know About Safety of Probiotics for Prevention and Treatment of Infectious Diarrhea?

Since the precise mechanisms by which most probiotics exert their effects are not known, concerns about safety arise from the desirable characteristics of probiotics, such as ability to survive in food products in known quantities over a predefined "shelf life," as well as ability to survive in and transit through the gastrointestinal tract. The main areas of concern relate to the potential for bacteria and fungi to translocate, crossing the gastrointestinal barrier and resulting in invasive infection, and the possibility for antibiotic resistance to be transferred from some probiotics to potentially pathogenic bacteria in the gastrointestinal tract (Salyers et al., 2004; Senok et al., 2005). Other concerns relate to possible immune stimulation (Henriksson et al., 2005; Ishibashi and Yamazaki, 2001; Saarela et al., 2000; Senok et al., 2005) and inadequate product quality, such as products that do not contain the probiotic on the label or that contain contaminants not listed on the label.

Despite a biologic basis for translocation of probiotic organisms, invasive infection due to administration of probiotics appears rare. Data are available from published clinical trials, case reports of invasive infection and epidemiologic studies.

22.13.1 Safety Information from Clinical Trials

From 1970 to August 2008 no cases of bacteremia, fungemia or invasive infection due to probiotics have been reported in clinical trials. In February 2008 Besselink et al. reported the results of a randomized clinical trial in which a 16% mortality rate was observed in patients with acute pancreatitis who were treated with a

multispecies probiotic preparation consisting of four *Lactobacillus* species and two *Bifidobacteria* species, versus 6% mortality in those receiving placebo (Besselink et al., 2008). Nine cases of bowel ischemia were reported in the probiotic treated patients – eight of whom died – versus no cases in the placebo group. The mechanism by which the study probiotics might have increased the risk of bowel ischemia is not known, but is not likely to be related to bacterial translocation as there was no difference in the rates of invasive infection due to probiotic organisms or other bacteria in the two groups. The reason for the excess deaths is still under investigation, but questions (still unanswered as of September 1, 2008) have been raised about the safety of probiotic administration to severely ill hospitalized patients with pancreatitis. No specific issues have been raised relating to the use of probiotics to prevent or treat infectious diarrhea.

22.13.2 Safety Information from Case Reports

The number of case reports of bacteremia or fungemia attributed to probiotic consumption (versus infection from naturally occurring organisms) is small. *Table 22.4* lists the case reports of invasive infection associated with the use of bacterial probiotics and *Table 22.5* lists the case reports associated with use of fungal probiotics. It has been recognized that cases of probiotic associated bacteremia or fungemia are likely to be under-reported due to challenges in isolation and characterization of clinical isolates. These reports are difficult to retrieve from the literature due to changing names of probiotics and lack of use of standard keywords. However, the biggest challenge to assessing whether consumed probiotics result in invasive infection is the absence of confirmation that the clinical isolate matches the consumed strain in many of the reported cases. Acceptable molecular methods that are used to determine whether the invasive isolate matched the consumed probiotic include polymerase chain reaction (PCR) and pulsed field gel electrophoresis (PFGE). Use of the API 50 CHI identification system is not adequate (Boyd et al., 2005). This capability, long available for bacterial isolates, is now also available for fungal isolates (Posteraro et al., 2005).

In *Table 22.4*, all fourteen patients with invasive disease attributed to probiotic bacteria had underlying medical conditions or were immunosuppressed. Of note, molecular methods to confirm that the invasive isolate matched the consumed isolate were done in only 6 of the 14 cases. Any published reports of invasive infection with *Bifidobacterium* spp was not found, but culture of this

Table 22.4

Case reports of invasive infection associated with use of bacterial probiotics (*Cont'd* p. 878)

Reference	Patient		Risk factors				Probiotic use		Invasive infection	
	Age	Gender	Underlying condition	Immunocompromise	ICU	Central line	Consumed	Indication	Positive cultures and molecular methods (MM)	Treatment and outcome
Lactobacillus GG										
Rautio et al. (1999)	74 year	F	Diabetes mellitus	Diabetes	NS	NS	LGG 0.5L dairy products/day, brand, manufacturer, and duration NS	Self administered for abdominal discomfort	Liver abscess PCR and PFGE	Pen G, Pip-Tazo, Cipro, Clinda - Patient survived
MacKay et al. (1999)	67 year	M	Mitral valve regurgitation dental extraction (amoxicillin prophylaxis)	NS	NS	NS	L. *rhamnosus* 3 × 10^9 cfu/day, L. *acidophilus* dose NS, *S. faecalis*, dose and brand NS	Self administered	Blood - No MM	Amp, Gent - Patient survived
Kunz et al. (2004)	3 m	M	Short gut syndrome	Born at 36 weeks	NS	NS	LGG (Culturelle) 1 capsule/day, dose NS, ConAgra Foods, Omaha, Nebraska	Not clear, possibly to treat cholestasis	Blood - No MM done	Amp - Outcome not stated
	<1 m	M	Short gut syndrome, gastrochisis	Born at 34 weeks	NS	NS	LGG 1 capsule/day, dose and brand NS	Prevent small bowel bacterial overgrowth	Blood - PFGE	Ceftr, amp - outcome not stated

Land et al. (2005)	4 m	M	Cardiac surgery, multiple post-operative complication	NS	NS	Y	LGG (Culturelle) 10×10^9 cfu/day ConAgra Foods, Omaha, Nebraska	Treat diarrhea	Blood, catheter infected - PCR	Catheter removed, Pen G, Gent - Patient survived
	6 year	F	Cerebral palsy, jejunostomy feeding, microcephaly, seizure disorder	NS	NS	Y	LGG (Culturelle) 10×10^9 cfu/day, ConAgra Foods, Omaha, Nebraska	Treat diarrhea	Blood, catheter infected - PCR	Amp - Patient survived
De Groote et al. (2005)	11 m	M	Necrotizing Enterocolitis, Short gut syndrome, parenteral nutrition	Previously Premature (26 weeks)	NS	Y	LGG, 1/8 capsule 2x/day, dose and brand NS	Treat rotavirus diarrhea	Blood obtained via catheter - PFGE	Catheter removed, vanco, ceftaz - Outcome not stated
Lactobacillus rhamnosus										
Ledoux et al. (2006)	38 year	M	AIDS, stage IV Hodgkin's disease	AIDS, chemotherapy	No	Y	*L. acidophilus,* dose and manufacturer NS	NS	Blood - No MM	Clinda and gent - Patient survived
Zein et al. (2008)	54 year	F	Diabetes	Diabetes	NS	NS	*Bifidobacterium bifidus* + *L. acidophilus* + *L. bulgaricus* + *L. casei* + *B. longum* + *L. rhamnosus* + *S. thermophilus*, dose and manufacturer NS	Self administered for dyspepsia	Blood - L rhamnosus - No MM	Amoxicillin - Patient outcome NS

Table 22.4

Reference	Patient		Risk factors				Probiotic use		Invasive infection	
	Age	Gender	Underlying condition	Immunocompromise	ICU	Central line	Consumed	Indication	Positive cultures and molecular methods (MM)	Treatment and outcome
Bacillus subtilis										
Richard et al. (1988)	47 year	M	Head trauma	NS	NS	NS	*Bacillus subtilis* 8×10^9 spores/day, Merell-Dow, USA, distributed by Wellcome	Prevention of diarrhea due to enteric feeding	Blood - No MM	Cefoperazone - Patient died of neurological complications
	25 year	M	Head trauma	NS	NS	NS			Blood - No MM	Erythromycin - Patient survived
	63 year	F	Endometrial carcinoma	Cancer	NS	NS			Blood - No MM	Erythromycin - Patient survived
	79 year	F	Stroke	NS	NS	NS			Blood - No MM	Pen G - Patient died of pulmonary infection
Oggioni et al. (1998)	73 year	M	Chronic lymphocytic leukemia	Leukemia	NS	N	*Bacillus subtilis* 10^9 spores/day, manufacturer NS	NS	Blood - PCR	Imipenem; recurrence - ceftazidime, amikacin, vanco - Patient died

M Male; *F* Female; *NS* Not stated; *Y* yes; *Pen G* Penicillin G; *Pip-Tazo* Pipericillin-Tazobactam; *Cipro* Ciprofloxaxin; *Clinda* Clindamycin; *Amp* Ampicllin; *Gent* Gentamicin; *Ceftr* Ceftriaxone; *Vanco* Vancomycin; *Ceftaz* Ceftazidine; *PCR* Polymerase chain reaction; *PFGE* Pulsed field gel electrophoresis; *Cfu* Colony forming units

Table 22.5

Case Reports of Invasive Infection Associated with Use of Fungal Probiotics (*Cont'd* p. 880)

	Patient		Risk factors				Probiotic use		Invasive infection	
Reference	Age	Gender	Underlying condition	Immunocompromise	ICU	Central line	Consumed	Indication	Positive cultures and molecular methods	Treatment and outcome
Zunic et al. (1991)	33 year	M	Ulcerative colitis, colectomy	Steroids	NS	Y	*Saccharomyces boulardii* (Ultra-levure) 1.5 g/day, manufacturer NS	Control GI transit	Blood - No MM	Amphotericin B, fluconazole - Patient survived
Viggiano et al. (1995)	14 year	M	Severe burn	Burn	NS	NS	*Saccharomyces boulardii* (Ultralevure) dose NS, Laboratoires Biocodex, France	Improve tolerance to enteral nutrition	Blood - No MM	Amphotericin B + 5FC - Patient survived
Pletincx et al. (1995)	1 year	F	Diarrhea	Malnutrition	NS	Y	*Saccharomyces boulardii* (Perenterol) 0.6 g/day, manufacturer NS	Prevent bacterial overgrowth	Blood and via CVC - No MM	Fluconazole - Patient survived
Bassetti et al. (1998)	51 year	F	Polyarteritis nodosa	Steroids, cyclophosphamide	NS	Y	*Saccharomyces boulardii* (Perenterol) 1 g/day, manufacturer NS	Relapsed C difficile diarrhea	Blood - PFGE	Amphotericin - Patient survived

Table 22.5 (*Cont'd* p. 882)

	Patient		Risk factors				Probiotic use		Invasive infection	
Reference	Age	Gender	Underlying condition	Immunocompromise	ICU	Central line	Consumed	Indication	Positive cultures and molecular methods	Treatment and outcome
Fredenucci et al. (1998)	49 year	M	Pneumonia	NS	NS	NS	*Saccharomyces boulardii* (Ultralevure) dose NS, Laboratoires Biocodex, France	While taking enteral nutrition	Blood - PFGE	Fluconazole - Outcome not stated
Niault et al. (1999)	78 year	F	COPD, diarrhea	N	Y	Y	*Saccharomyces boulardii* (Ultra-levure) 1.5 g/day, Biocodex, Montrouge, France	Treat diarrhea	Blood - No MM	Fluconazole - Patient survived
Cesaro et al. (2000)	8 months	NS	AML	Neutropenia	NS	Y	*Saccharomyces boulardii* (Codex) dose NS, Smith Kline Beecham, Milan, Italy	Preserve normal GI flora	Blood via CVC - No MM	Catheter removed, Amphotericin B - Patient survived

Hennequin et al. (2000)	2 year	M	Ileal atresia, GI surgery, CF	NS	NS	Y	*Saccharomyces boulardii* 0.75 g/day, brand and manufacturer NS	Improve tolerance to enteral nutrition	Blood and via CVC - PFGE	Catheter removed, Amphotericin B - Patient survived
	36 year	M	AIDS, CNS lymphoma, chemotherapy	HIV, cancer, chemotherapy	NS	Y	*Saccharomyces boulardii* 1.5 g/day, brand and manufacturer NS	Persistent diarrhea	Blood via CVC - PFGE	Fluconazole - Patient survived
	47 year	M	Esophageal cancer, esophagectomy	Cancer	NS	Y	*Saccharomyces boulardii* 2 g/day, brand and manufacturer NS	Diarrhea	Blood and via CVC - PFGE	Catheter removed, Fluconazole - Patient survived
	78 year	F	COPD, ARDS,	Chronic renal failure	Y	Y	*Saccharomyces boulardii* 1.5 g/day, brand and manufacturer NS	"With enteral nutrition"	Blood - PFGE	No antifungal therapy - Patient survived
Perapoch et al. (2000)	3.5 months	M	Cardiac surgery	NS	NS	Y	*Saccharomyces boulardii* (Ultra-Levura) dose and manufacturer NS	NS	Blood - PFGE	Catheter removed, Amphotericin B - Patient survived
	Infant	F	Surgery for intestinal atresia, bowel resection	NS	NS	Y	N	In cot adjacent to above patient	Blood - PFGE	Catheter removed, liposomal Amphotericin B - Patient survived

Table 22.5 (*Cont'd* p. 884)

	Patient		Risk factors				Probiotic use		Invasive infection	
Reference	Age	Gender	Underlying condition	Immunocompromise	ICU	Central line	Consumed	Indication	Positive cultures and molecular methods	Treatment and outcome
Rijnders et al. (2000)	74 year	M	Neurosurgery for subarachnoid hemorrhage	No	Y	Y	*Saccharomyces boulardii* (Perenterol) 0.6 g/day, Biodiphar, Brussels	Treat diarrhea (not C difficile)	Blood - No MM	Fluconazole - Patient died 4 weeks later
Lherm et al. (2002)	50 year	M	Cardiac arrest	NS	Y	Y	*Saccharomyces boulardii* 1.5 g/day, manufacturer NS	NS	Blood - PFGE	None - Patient died
	51 year	F	Aortic surgery	Cachexia	Y	Y	*Saccharomyces boulardii* 1 g/day, manufacturer NS	NS	Blood - PFGE	Fluconazole - Patient died
	50 year	M	ARDS/gastric ulcers	NS	Y	Y	N	In ICU with S boulardii use	Blood via CVC - PFGE	Fluconazole - Patient survived
	82 year	F	Acute respiratory failure	NS	Y	Y	*Saccharomyces boulardii* 1.5 g/day, manufacturer NS		Blood - PFGE	None - Patient survived

	75 year	M	Acute respiratory failure	Not clear	Y	Y	*Saccharomyces boulardii* 2 g/day, manufacturer NS	NS	Blood - PFGE	None - Patient survived
	77 year	M	Peritoniti, duodenal ulcers	Not clear	Y	Y	*Saccharomyces boulardii* 3 g/day, manufacturer NS	NS	Blood - PFGE	Amphotericin B - Patient died
	71 year	F	Hemorrhagic CVS	Not clear	Y	Y	*Saccharomyces boulardii* 3 g/day, manufacturer NS	NS	Blood - PFGE	None - Patient survived
Cassone et al. (2003)	34 year	F	Head and thoracic trauma, hypoxia	NS	Y	Y	N	S boulardii, Codex, Italy, in use in ICU	Blood - PFGE	Catheter removed, Fluconazole - Patient outcome NS
	48 year	M	Ruptured CNS aneurysm	NS	Y	Y	N		Blood - PFGE	Catheter removed, Fluconazole - Patient outcome NS
	75 year	F	AMI	NS	Y	Y	N		Blood via CVC - PFGE	Catheter removed, Fluconazole - Patient outcome NS
	35 year	F	Multiple trauma	NS	Y	NS	Unknown	Unknown	Blood - PFGE	Unknown - Patient survived
Lestin et al. (2003)	48 year	M	Diabetes mellitus, peripheral arterial disease	NS	Y	NS	*Saccharomyces boulardii* (Perenterol) dose and manufacturer NS	Treat C difficile diarrhea	Blood - no MM	NS - Patient died from multi-organ failure

Table 22.5 (*Cont'd* p. 886)

	Patient		Risk factors				Probiotic use		Invasive infection	
Reference	Age	Gender	Underlying condition	Immunocompromise	ICU	Central line	Consumed	Indication	Positive cultures and molecular methods	Treatment and outcome
Riquelme et al. (2003)	42 year	F	Renal-pancreas transplant	Cyclosporine, mycophenolate mofetil, prednisone	NS	NS	*Saccharomyces boulardii* (Perenteryl) 1 g/day, Merck, Nunoa-Santiago, Chile	Treat diarrhea	Blood - PFGE	Fluconazole - Patient survived
	41 year	M	TB, syphilis, HIV	HIV untreated	NS	NS	*Saccharomyces boulardii* (Perenteryl) 0.75 g/day, Merck, Nunoa-Santiago, Chile	Treat persistent diarrhea	Blood - PFGE	Amphotericin B - Patient survived
Lungarotti et al. (2003)	1 month	M	Prematurity, IUGR	NS	NS	Y	Codex DNB 2.5×10^9 cfu/day, manufacturer NS	Prevent bacterial overgrowth	Blood - No MM	Amphotericin B - Patient outcome NS
Cherifi et al. (2004)	89 year	F	Colitis, anorexia nervosa	NS	N	No	*Saccharomyces boulardii* (Perenterol) 0.3 g/day, Biodiphar, Brussels	Treat C difficile diarrhea relapse with vancomycin	Blood - No MM	Fluconazole - Patient died

Henry et al. (2004)	65 year	M	Head and neck cancer	Chemotherapy, external beam radiotherapy	Y	N	*Saccharomyces boulardii* (Perenterol) dose and manufacturer NS	Treat diarrhea	Blood - No MM	Amphotericin B - Patient survived
Burkhardt et al. (2005)	19 year	M	Brain abscesses, spastic tetraparesis	NS	Y	NS	*Saccharomyces boulardii* (Perenterol) 150 mg o.p.d., manufacturer NS	NS	Blood - No MM	Fluconazole (failed) Voriconazole - Patient outcome NS
Munoz et al. (2005)	76 year	F	Diabetes mellitus heart surgery	NS	Y	NS	*Saccharomyces boulardii* (Ultralevura) dose NS, Bristol-Myers Squibb	Treat C difficile diarrhea	Blood - PCR	Fluconazole - Patient died after stroke
	72 year	F	Heart surgery	NS	Y	NS	*Saccharomyces boulardii* (Ultralevura) dose NS, Bristol-Myers Squibb		Blood - PCR	No antifungal therapy provided - Patient died unexpectedly
	74 year	F	RA heart surgery	NS	Y	Y	*Saccharomyces boulardii* (Ultralevura) dose NS, Bristol-Myers Squibb		Blood - PCR	Fluconazole - Patient died of Enterococcus faecium bacteremia
Belet et al. (2005)	45 days	F	Esophageal atresia and surgeries	N	NS	Y	N	N/A	Blood via CVC - No MM	CVC removed, Amphotericin B - Patient outcome NS

Table 22.5

	Patient		Risk factors				Probiotic use		Invasive infection	
Reference	Age	Gender	Underlying condition	Immunocompromise	ICU	Central line	Consumed	Indication	Positive cultures and molecular methods	Treatment and outcome
Graf and Gavazzi (2007)	66 year	F	Gastric adenocarcinoma, extensive GI surgery	Steroids, chemotherapy	N	Y	N	N/A	Blood and through CVC - No MM	CVC not removed patient refused, Fluconazole then voriconazole, but fungemia persisted - Patient died
Piechno et al. (2007)	61 year	NS	Cancer of pyriform sinus	Cancer	NS	Y	*Saccharomyces boulardii* (Ultra-Levure) 0.45–0.6 g/day, manufacturer NS	Pseudomembranous colitis (C difficile negative)	Blood via CVC - No MM	Voriconazole - Patient survived
Lolis et al. (2008)	56 year	M	Sepsis	NS	Y	Y	*Saccharomyces boulardii* (Ultra-Levure) 2 g/day, Biocodex, Beauvais, France	Treat diarrhea	Blood - MM but NS	Caspofungin - Patient survived

bacterium is technically challenging. Similarly, in Table 22.5, all 39 patients with fungemia attributed to consumption of *Saccharomyces boulardii* had underlying medical conditions, were in intensive care units (ICUs), had central venous catheters or were immunosuppressed. Molecular methods to confirm that the invasive isolate matched the consumed isolate were done in 24 of the 39 patients. These case reports highlight potential at-risk patients in whom probiotics may be contraindicated.

22.13.3 Safety Information from Epidemiologic Studies

Since case reports can be reported in a biased fashion, it is important to evaluate epidemiologic data, particularly trends in bacteremia and fungemia, in locations where probiotics are in widespread use. The most extensive data are available in relation to safety of the probiotic *Lactobacillus* GG.

Lactobacillus GG is used in almost 40 countries. An estimated three million kilograms of *Lactobacillus* GG-containing products were safely consumed by a minimum of 40,000 persons in Finland alone in 1992 (Salminen et al., 2002). Salminen et al. recently evaluated the possible effects of increased use of *Lactobacillus* GG in Finland since 1990 by studying *Lactobacillus* bacteremia at the Helsinki University. Lactobacillus were isolated in 0.02% of all blood cultures with positive results in Helsinki University Central Hospital and in Finland as a whole. No trends were seen that suggested an increase in *Lactobacillus* bacteremia. The average incidence was 0.3 cases/100,000 inhabitants/year in 1995–2000 in Finland. Identification to the species level was done for 66 cases of *Lactobacillus* bacteremia, and 48 isolates were confirmed to be *Lactobacillus* strains. Twenty-six of these strains were *L. rhamnosus*, and eleven isolates were identical to *L. rhamnosus* GG. The results indicate that increased probiotic use of *L. rhamnosus* GG has not led to an increase in *Lactobacillus* bacteremia (Salminen et al., 2002). The same authors recently reviewed the 89 cases with *Lactobacillus* bacteremia reported between 1990 and 2000 to study the risk factors and outcomes in these patients (Salminen et al., 2004). Of the 89 cases, the blood isolate was not confirmed to be *Lactobacillus* in 42, was a non-*rhamnosus Lactobacillus* in 22, was *L. rhamnosus* but not GG in 14, and was *L. rhamnosus* GG in 11. Patient charts were reviewed for predictors of mortality. *Lactobacillus* GG use by these patients was not studied. Mortality after *L. rhamnosus* bacteremia (*Lactobacillus* GG and non *Lactobacillus* GG combined) was associated with severe or fatal co-morbidities. In a recent Finnish study, three of these "*Lactobacillus* GG-like"

blood isolates were examined closely for phenotypic characteristics, and while they may not be distinguishable from *Lactobacillus* GG by pulsed-field gel electropheresis, they were found to differ from LGG in other characteristics such as adhesion properties, resistance to serum-mediated killing and induction of respiratory burst (Ouwehand et al., 2004).

22.13.4 Safety Information Relating to Transfer of Antimicrobial Resistance

Regarding the potential for transfer of antimicrobial resistance genes from probiotics to gastrointestinal bacteria, most of the attention has focused on transfer of vancomycin resistance to and from probiotic bacteria. Many strains of *lactobacilli* are naturally resistant to vancomycin (chromosomally based) and there would be no selective advantage to acquiring additional vancomycin resistance plasmids. These vancomycin resistance genes are not easily transferable to other genera (Klein et al., 2000; Tynkkynen et al., 1998). However, Mater et al. demonstrated transfer of vancomycin resistance (VanA cluster) from *Enterococcus* to a commercial strain of *Lactobacillus acidophilus*, both *in vitro* and in the gut of mice (Mater et al., 2005, 2008). Since the mice were colonized with human microbiota and this transfer occurred in the absence of selective pressure from antibiotics, further investigation of the potential is urgently needed.

22.13.5 Safety Information Relating to Product Quality

There are numerous articles raising concerns about the quality of probiotic products. Temmerman et al. studied 55 European products – 30 dried food supplements and 25 dairy products (Temmerman et al., 2003b). No viable strains were isolated from 11 of the 30 (37%) dried food supplements, 15 (50%) either had more probiotic species isolated than were listed on the product label or contained species that were not listed. In only 4 (13%) did the probiotic product label match the contents. A similar trend was observed with the dairy products. Masco et al. have also drawn attention to the need to use culture-independent analysis of probiotics, based on a recent study of 58 products claiming to contain *Bifidobacterium* strains (Masco et al., 2005). In their study, 71% of products contained culturable bifidobacteria, while bifidobacteria could be detected in 97% of these products based on nested PCR of bacterial DNA, Denaturing

Gradient Gel Electrophoresis and identification of species from the band patterns. These results focus attention on the urgent need for product quality standards and methods of measuring product quality (Hibberd and Davidson, 2008; Hoffman et al., 2008; Masco et al., 2005, 2007; Sutton, 2008; Tamayo, 2008; Temmerman et al., 2003a, c). Specifically, manufacturers of probiotics should improve the quality of available products to ensure that the probiotic organism is viable during recommended shelf life and that probiotic products contain only the organisms identified on the product label.

22.13.6 Synthesis of Safety Information in Relating to Prevention and Treatment of Infectious Diarrhea

Based on comparison of the large number of people who have consumed probiotics to the small number of people in whom serious adverse events have been reported, probiotics appear to be safe. However, since probiotics can cause invasive infection, probiotics should be used with caution in individuals who have an abnormal gastrointestinal mucosal barrier and should be avoided in children with short gut syndrome. Probiotics should also be avoided or used with care in patients with central venous catheters, particularly when lyophilized formulations are being handled prior to administration. Doctors also recommend against use of probiotics in severely immunocompromised patients and critically ill patients in intensive care units. Similarly, patients with comorbid conditions that place them at increased risk of invasive infection should avoid probiotics, although it is not clear whether this recommendation should extend to severely malnourished patients.

22.14 Summary

- Inadequate quality of data reported in many clinical trials investigating the safety and efficacy of probiotics limits recommendations that can be made about their use in the prevention and treatment of infectious diarrhea.
- Probiotics for the prevention of diarrhea is promising, particularly for non-breastfed infants in daycare. There is insufficient evidence to recommend any specific probiotic or probiotics in general for the prevention of infectious diarrhea.

- Certain probiotics appear to reduce the duration of acute infectious diarrhea, particularly for diarrhea caused by rotavirus. Not all probiotics are equally efficacious, and based on the heterogeneity of available data it is difficult to make specific recommendations for the use of particular probiotics and specific doses and durations of treatment.
- Use of probiotics to prevent or treat infectious diarrhea due to rotavirus may be less useful in the future due to availability of rotavirus vaccines. Since there may be delay before rotavirus vaccines are available in Africa and Asia, specific probiotics may be useful adjuncts to oral rehydration solution.
- There is insufficient evidence to support the use of any specific probiotic or probiotics in general for the prevention of traveler's diarrhea.
- Based on comparison of the large number of people who have consumed probiotics to the small number of people in whom serious adverse events have been reported, probiotics appear to be safe for the prevention and treatment of infectious diarrhea.
- Probiotics should be avoided in children with short gut syndrome, in patients with central venous catheters, severely immunocompromised patients and critically ill patients in intensive care units. Caution should be used in patients with comorbid conditions that increase the risk of invasive infection as a result of probiotic use.
- Future recommendations for the use of probiotics to prevent and/or treat infectious diarrhea should be specific for probiotic, strain, manufacturer, dose and duration of therapy.

List of Abbreviations

DAEC	diffuse adhering E. coli
E. coli	Escherichia coli
EAggEC	Enteroaggregative E. coli
EHEC	Enterohemorrhagic E. coli
EIEC	Enteroinvasive E. coli
EPEC	Enteropathogenic E. coli
ETEC	Enterotoxigenic E. coli
FAO	Food and Agriculture Organization of the United Nations
FOS	fructo-oligosaccharide
GOS	galacto-oligosaccharide
ICU	intensive care unit
IDSA	Infectious Diseases Society of America

Lactobacillus	LGG, Lactobacillus GG
ORS	Oral rehydration solution
STEC	Shiga toxin-producing E. coli
UNICEF	United Nations International Children's Emergency Fund
WHO	World Health Organization

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23 Immunological Effects of Probiotics and their Significance to Human Health

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23.1 Introduction

Probiotics are defined as live microorganisms which when administered in adequate amounts confer a health benefit upon the host (FAO/WHO, 2001). Lactic acid bacteria, particularly *Lactobacillus* and *Bifidobacterium* species are commonly used as probiotics. Other less commonly used probiotics include the yeast *Sacchromyces cerevisiae* and some non-pathogenic *Escherichia coli* and *Bacillus* species. Studies over the past 20 years have demonstrated that probiotic intake is able to confer a range of health benefits including modulation of the immune system, protection against gastrointestinal and respiratory tract infections, lowering of blood cholesterol levels, attenuation of overt immuno-inflammatory disorders (such as inflammatory bowel disease, allergies) and anti-cancer effects. However, the strongest clinical evidence for probiotics relates to their effectiveness in improving gut health and modulating (via stimulation or regulation) the host immune system. This chapter provides an overview of the current status of our knowledge regarding the immunostimulatory and immunoregulatory effects of probiotics on the immune system and their significance to human health.

23.2 Gut Microbiota

The human gastrointestinal tract (GIT) is home to a complex and dynamic community of microbes representing over 500 species. Colonization of the GIT begins immediately after birth and evolves towards the normal adult flora over the first 24 months of life. In breast-fed babies, the microbiota is dominated by

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bifidobacteria whereas *Bacteroides* species are found in abundance in formula-fed babies. It is estimated that there are ten times more bacteria than cells in the human body and that the combined genome of intestinal microbiota is 50 times greater than the human genome. Most of the microbes present in the gut are beneficial and confer a range of health benefits. Recent studies have shown that the human gut microbiome encodes a larger proportion of metabolic pathways that are important for human life than the human genome itself (Sekirov and Finlay, 2006). In a healthy state, a balance between different groups (health-enhancing and potential pathogens) of bacteria ensures intestinal homeostasis. Dearrangements in the intestinal microbial ecosystem perturb intestinal homeostasis and enhance susceptibility to disease. There is overwhelming evidence that probiotics could be used to restore intestinal microbial balance and optimize health.

23.3 The Immune System

The immune system is a highly adaptable defense system that has evolved to preserve the integrity of an organism by eliminating all elements perceived as foreign. The protective function is mediated by a complex network of cells and molecules that are capable of specifically recognizing and eliminating a large variety of pathogenic organisms. Following recognition of a pathogen/foreign agent, the immune system recruits appropriate effector cells and molecules to eliminate or neutralize the threat. This also leads to the induction and expansion of memory cells that ensure more prompt and augmented immune response (both humoral and cellular) to subsequent challenges by the same pathogen.

The immune system consists of innate and adaptive components. The innate (non-specific) immune responses constitute the first line of host defense and comprise a set of resistance mechanisms that are non-adaptive and non-specific to a given pathogen. The major effectors of innate immune system include phagocytic cells (neutrophils/polymorphonuclear cells (PMNs), monocytes/macrophages and dendritic cells) and natural killer cells (NK cells). Failure of the innate system to contain an infection, results in the activation of adaptive immune responses.

Unlike the innate immune system, the adaptive (specific) immunity exhibits a high degree of specificity and memory. It consists of both cellular and humoral components. The key cellular constituents of the adaptive system include thymus-derived T helper lymphocytes (Th) and cytotoxic T lymphocytes (Tc),

bone-marrow-derived B lymphocytes and accessory cells such as dendritic cells and macrophages. The Th cells can be further divided into two subtypes; Th1 and Th2, each carrying out distinct and opposing functions. A proper balance between Th1 and Th2 immune responses is critical for immune homeostasis. T cells influence the activities of other immunocompetent cells by producing a wide array of cytokines. The adaptive humoral immunity is mediated by antibodies produced by plasma cells (mature B lymphocytes). Central to the activation and regulation of immune responses is the production of cytokines (such as interferons, interleukins, colony-stimulating factors).

The innate and adaptive immune systems are highly integrated and interdependent. In addition to being a pre- requisite for adaptive immunity, the innate immune response is responsible for the detection and elimination of pathogens (Haddad et al., 2005).

23.4 Intestinal Microbiota and Immune Development

At birth, the immune system is immunologically naive and functionally immature (Kelly and Coutts, 2000). The commensal flora acquired during early life plays an important role in the development and maturation of the host immune system. This is best illustrated by studies with gnotobiotic animal models that exhibit enhanced susceptibility to infectious diseases (Roach and Tannock, 1980; Yamazaki et al., 1982). All aspects of the intestinal immune system (e.g., IgA plasma cells, CD4 T cells, dendritic cells, intraepithelial lymphocytes, levels of immunoglobulins) are underdeveloped in germfree mice (Crabbe et al., 1999; Crabbe, 1968; Gordon and Bruckner-Kardoss, 1961; Gordon and Pesti, 1971; Mazmanian et al., 2005; William et al., 2006) but are rapidly restored upon the introduction of even single bacterial species or when reared in a conventional environment (Bauer et al., 1965; Crabbe et al., 1999).

It has also been demonstrated that intestinal microbiota is pivotal for the induction and maintenance of oral tolerance. Germfree mice fail to develop oral tolerance. However, the reconstitution of gut microbiota at the neonatal stage, but not any later, results in the development of normal tolerance (Sudo et al., 1997). Defective immunoregulation resulting from reduced or aberrant exposure to microbes during early life has also been associated with an increased incidence of atopic and autoimmune disorders in western societies (Rook et al., 2006). The microbial species and their components that are critical for driving the development of the gut mucosal immune system during early life are not known.

However, differences in the intestinal microbiota and humoral immune responses of vaginally versus caesarean delivered infants whose mothers received prophylactic antibiotics (Gronlund et al., 2000), and enteric flora of atopic versus non-atopic infants (Bjorksten et al., 2001) suggests that the signals imparted by diverse species of microbes during early life are critical for educating and shaping the developing immune system.

23.5 Probiotics and Stimulation of the Immune System

Several *in vitro* and *in vivo* studies have shown that specific strains of probiotics are able to modulate the functioning of the immune system; stimulate the immune function to protect against infectious diseases and cancers (*Table 23.1*), and regulate over expressed immune responses associated with immunoinflammatory disorders such as allergy and IBD (Gill & Guarner, 2004). It has also been shown that probiotic strains exhibit large variation in their capacity to modulate the immune system and the degree of response is dose-dependent. The viability of

Table 23.1

Examples of immunostimulatory effects of probiotics observed in healthy human subjects

Function	Effects
Cellular immunity	↑ Phagocytic capacity of PMN and monocytes
	↑ Expression of phagocytosis receptors (CR1 and CR3 in PMN)
	↑ NK cell activity
	↑ $CD3^+$, $CD4^+$, $CD25^+$ and $CD56^+$ (NK cells) cells
	↑ Oxidative burst activity
Humoral immunity	↑ Serum and mucosal IgA levels
	↑ Serum and/or mucosal antibody responses (IgG, IgA or IgM) to oral/systemic immunizations (such as rotavirus, *S. typhi*, polio and Hib vaccine)
	↑ IgG, IgM, IgA immunoglobulin-secreting cells
Production of cytokines	↑ IFN-γ levels in blood
	↑ 2–5A-synthetase activity in blood mononuclear cells
	↑ IFN-α in serum
	↑ *In vitro* and *ex vivo* production of pro- and anti-inflammatory cytokines following stimulation with mitogens

probiotics has also been found to be critical for maximal effect (Gill & Rutherfurd, 2001a). The following sections are mainly focused on the immunomodulatory effects of probiotics in human subjects and readers can consult reviews by Gill (1998, 2003), Cross (2002), and Erickson and Hubbard (2000) for additional information on immunomodulatory effects in animal models.

23.6 Effect on Innate (Non-specific) Immune Responses

23.6.1 Phagocytic Cell Function

The effect of probiotic supplementation on phagocytic cell function and NK cell activity has been the subject of many human studies. Schiffrin et al. (1995) and Donnet-Hughes et al. (1999) reported an augmentation of phagocytic capacity of peripheral blood leucocytes (neutrophils and monocytes) in healthy adults given fermented milk containing *L. johnsonii* La1 or *B. lactis* Bb12. These improvements in phagocytic activity were found to be dose-dependent (Donnet-Hughes et al., 1999) and were maintained for several weeks after cessation of probiotic consumption (Gill et al., 2001a, b; Schiffrin et al., 1995). Furthermore, supplementation with probiotics or standard yoghurt has also been shown to counteract the decrease in phagocytic cell function caused by dietary deprivation of fermented foods in healthy adult human volunteers (Olivares et al., 2006a).

Neutrophils comprise about 60% of blood leukocytes and are the main phagocytic cells in blood. Monocytes constitute only 3–7% of blood leukocytes and therefore contribute relatively less to the overall phagocytic capacity of blood leukocytes. In studies with probiotics, PMNs have been found to exhibit significantly greater enhancement in phagocytic activity compared with monocytes (Schiffrin et al., 1995).

It has also been shown that probiotics mediate disparate effects on phagocytic function depending upon the health status of recipient subjects. *Lactobacillus rhamnosus* GG (*Lactobacillus* GG) supplementation increased the expression of phagocytosis receptors (CR1, CR3, FcγRI and FcαR) on neutrophils in healthy subjects but had an opposite effect in milk-hypersensitive subjects (Pelto et al., 1998). Similarly, observations in healthy volunteers and patients with atopic dermatitis have been made by Roessler et al. (2008). Probiotic supplementation led to a significant increase in phagocytic activity of monocytes and granulocytes in healthy volunteers, but had no effect in patients with atopic dermatitis.

In addition to enhancing phagocytic activity, probiotics have also been shown to enhance oxidative burst or microbicidal capacity of PMN cells in subjects fed probiotics (Arunachalam et al., 2000; Mikes et al., 1995; Parra et al., 2004; Roessler et al., 2008).

Ageing is associated with a decline in immune competence including phagocytic capacity of neutrophils and macrophages. It has been reported that probiotic administration is able to correct the age-related deficit in phagocytic cell function (Gill et al., 2001b, c). Healthy elderly subjects given milk containing *L. rhamnosus* (HN001) or *B. lactis* (HN019) for 3–6 weeks showed significantly higher phagocytic activity than subjects given milk without probiotics (Arunachalam et al., 2000; Gill et al., 2001a, b; Gill and Rutherfurd, 2001b; Sheih et al., 2001). Notably, subjects with relatively poor pre-intervention immunity status consistently showed greater improvement in phagocytic cell function compared with subjects with adequate pre-intervention immune status (Gill et al., 2001c). Furthermore, enhancement in phagocytic capacity was also age-related; subjects older than 70 years exhibited significantly greater improvements in phagocytic cell function than those under 70 years of age (Gill et al., 2001a, b; Gill and Rutherfurd, 2001a, b). A trend towards an increase in blood phagocytic activity in hospitalized, enterally-fed elderly whose initial level was low, following consumption of fermented milk containing *L. johnsonii* La1, has also been reported by Fukushima et al. (2007).

Neutrophil dysfunction is associated with increased susceptibility of patients with alcoholic liver disease to infectious diseases. Treatment with *L. casei* Shirota for 4 weeks was reported to be effective in significantly increasing the phagocytic capacity of neutrophils in patients with alcoholic cirrhosis (Stadlbauer et al., 2008).

However, contrary to these results, several studies have reported little or no effect of probiotic intake, even at high doses, on the phagocytic activity of blood leukocytes when compared to the placebo (Christensen et al., 2006). Failure of probiotics (*L. acidophilus*) to influence early innate immune responses in infants at high risk of developing allergic disease has also been reported by Taylor et al. (2006).

23.6.2 NK Cell Activity

Low NK cell activity is associated with enhanced susceptibility to infectious diseases and cancers. Elderly people with low NK cell activity exhibit a higher

mortality rate due to infection compared to their counterparts with adequate NK cell activity. Populations with low NK cell activity have also been found to exhibit a significantly higher risk of cancer than populations with intermediate or high NK cell activity.

Supplementation with probiotics has been shown to augment NK cell activity (*ex vivo*) in healthy adults and elderly subjects. For example, Nagao et al. (2000) and Takeda and Okumura (2007) reported significant enhancement of NK activity in middle-aged volunteers given fermented milk containing *L. casei* for 3 weeks. The effect was more pronounced in subjects with low NK activity. Similar observations have been made by others (Chiang et al., 2000; Gill et al., 2001a; Nagao et al., 2000; Olivares et al., 2006a, b; Sheih et al., 2001). The NK activity remained elevated for 3 weeks post cessation of probiotic intake (Nagao et al., 2000).

Ageing, smoking and viral infections (such as T-cell lymphotropic viruses) are all associated with decline in NK cell function (Matsuzaki et al., 2005; Morimoto et al., 2005; Ogata et al., 1997). Supplementation with specific strains of probiotics has been shown to be effective in correcting these deficits in NK cell function in all these population groups (Gill et al., 2001a; Matsuzaki et al., 2005; Morimoto et al., 2005). As with phagocytic activity, improvements in NK cell function in the elderly subjects following intake of probiotics were significantly correlated with age (Gill et al., 2001c). It has been suggested that the enhancement of NK cell activity is mediated by probiotic-induced IL-12 (Takeda et al., 2006). Similar observations regarding enhancement of phagocytic and NK cell function have been made in animals fed probiotics (Cross, 2002; Gill, 1998). Differences in the efficacy of live versus dead probiotics have also been reported (Gill and Rutherfurd, 2001a, b).

Probiotic administration has also been shown to affect the relative proportions/numbers of NK cells. In a randomized, double-blind, placebo-controlled study, intake of milk fermented with *L. casei* DN114001 for 6 months during the puerperium stage was found to result in significant increase in NK cells (Ortiz-Andrellucchi et al., 2008). A significant increase in the absolute numbers/proportions of NK cells in healthy volunteers (Roessler et al., 2008), students under examination-related stress (Marcos et al., 2004) and adults immunized with influenza vaccine following probiotic administration have also been observed (Olivares et al., 2007).

It is important to note however that several studies have found no effect of probiotic intake on natural immune function (Spanhaak et al., 1998). Whether this has been due to the poor immunostimulatory capacity of the probiotic

strains used, sub-optimal dosage, probiotic viability or some other reason is not known. Strain- and dose-dependent differences in the ability of probiotics to modulate immune function are well documented (Donnet-Hughes et al., 1999; Gill, 1998).

Studies have also shown that commensal bacteria may reinforce innate immunity by stimulating mucosal IgA production, without the activation of T cells, and that this may be instrumental in reducing mucosal penetration by pathogenic microbes (McPherson and Uhr, 2004). Enhanced levels of fecal sIgA observed in infants fed probiotic-enriched formula suggests that, in addition to other mechanisms, probiotics may strengthen host defenses by stimulating production of mucosal IgA (Bakker-Zierikzee et al., 2006).

23.7 Effect on Adaptive (Specific) Immune Responses

As previously highlighted, the failure of the innate immune system to contain or eliminate a given pathogen results in the activation of adaptive immunity. The adaptive immunity is mediated by antibody and cell-mediated processes and is characterized by its specificity and memory. Antibodies produced by mature B lymphocytes (plasma cells) are effective at neutralizing or eliminating extracellular pathogens and antigens. Specific classes of antibodies perform distinct functions. For example, IgA is the predominant immunoglobulin produced at mucosal surfaces and is effective in preventing adherence of pathogens to the gastrointestinal mucosa. IgG and IgM are involved in systemic neutralization of bacterial toxins and promote phagocytosis by monocytes/macrophages. On the other hand, cell-mediated immunity plays a central role in protection against intracellular pathogens (including viral infections) and cancers.

Numerous studies using different probiotic strains have been conducted to determine the effect of probiotics on adaptive immune responses. These studies have provided evidence that specific strains of probiotics are effective in augmenting specific antibody responses to parenteral and oral vaccines and to some infectious agents. For example, administration of fermented milk containing *L acidophilus* La1 and bifidobacteria for 3 weeks was found to be effective in enhancing the efficacy of an orally delivered attenuated *Salmonella typhi* Ty21a vaccine in healthy human subjects; the increase in specific serum IgA titer to *S. typhi* Ty21a was more than fourfold and significantly higher ($P = 0.04$) in the probiotic group than the control group. A trend towards increased anti-*Salmonella* IgA levels in subjects receiving *Lactobacillus* GG and oral *Salmonella*

vaccine has also been reported (Fang et al., 2000). In a randomized, double-blind, placebo-controlled study, de Vrese et al. (2005) found a significantly higher virus-neutralizing antibody response (mainly IgA) following vaccination with live attenuated polioviruses in subjects given yoghurt containing *L. rhamnosus* and *L. paracasei* compared with subjects given placebo (chemically acidified milk). The levels of polio-specific serum IgG and IgA antibodies in subjects receiving yoghurt were also significantly increased. Enhancement of anti-influenza plasma IgA antibody responses following vaccination in adults given *L. fermentum* CECT5716 has also been reported (Olivares et al., 2007).

Similar observations on the beneficial effects of probiotics in children have also been made. Isolauri et al. (1995) reported enhanced efficacy of a live rotavirus vaccine in 2–5 years old children who received *Lactobacillus* GG concomitantly with rotavirus vaccination; children given *Lactobacillus* GG had significantly more IgA- and IgM-secreting cells compared with infants given vaccine only. In another study, infants given a formula containing bifidobacteria and immunized against poliovirus, several months prior to enrolment in the study, were found to exhibit higher total fecal IgA and anti-poliovirus fecal IgA responses (Fukushima et al., 1998). Furthermore, enhanced anti-poliovirus IgA antibody responses and a positive correlation between antibody titers and bifidobacteria counts in infants fed a fermented milk formula has also been reported (Mullie et al., 2004).

Probiotic supplementation has also been shown to improve responses to Hib immunization (higher frequency of protective Hib antibody titers and a trend towards higher anti-Hib IgG levels) in allergy-prone infants (Kukkonen et al., 2006). However, antibody responses to diphtheria and tetanus were not affected by probiotic intake.

Recently, West et al. (2008) determined the impact of *Lactobacillus* F19 (LF19) during weaning periods in infants on gastro-intestinal infections and IgG antibody responses to routine vaccines in a double-blind, placebo-controlled randomized intervention trial. It was concluded that feeding LF19 did not prevent infections, but increased the capacity to raise immune responses to protein antigens, with a pronounced effect in breast-fed infants (<6 months of age). The outcomes of these studies once again suggest that orally-ingested lactic acid bacteria have an adjuvant-like effect on the humoral responses.

The ability of probiotics to augment specific antibody responses following rotavirus infection has also been demonstrated. In a randomized, placebo-controlled study involving children with roravirus gastroenteritis, Kaila et al. (1992) found significantly higher levels of specific mucosal and serum antibody

responses in children given *Lactobacillus* GG fermented milk compared with children receiving a placebo. It has also been reported that viable probiotics are superior at stimulating rotavirus-specific immune responses than non-viable probiotics; the proportion of subjects exhibiting rotavirus-specific response at the convalescent stage was higher in subjects given live probiotics (10 out of 12 children) compared with the group given dead bacteria (2 out of 13) (Kaila et al., 1995; Majamaa et al., 1995).

To date, relatively few studies have examined the effect of probiotics on acquired cell-mediated immune responses in human subjects. However, there is some evidence that probiotic intake may influence the relative as well as absolute numbers or proportions of T and B lymphocytes. For example, Marcos et al. (2004) and Ortiz-Andrellucchi et al. (2008) reported increases in CD3, CD19 and CD8 T cell counts in subjects given probiotics.

The precise mechanisms by which probiotics mediate these adjuvant effects are still unclear. It appears likely, however, that probiotics mediate these effects by improving antigen presentation function (Heyman, 2001) and/or by stimulating an increase in the numbers of B cells (De Simone et al., 1991).

23.8 Cytokine Production

Cytokines are pleiotropic group of small signaling molecules that play a central role in the selection, initiation, maintenance and resolution of both innate and acquired immune responses.

Several studies have reported increased levels of IFN-γ (as well as 2′,5′-adnylate synthatase activity), IFN-α and IL-2 in healthy subjects following ingestion of probiotics (Aattouri and Lemonnier, 1997; Arunachalam et al., 2000; De Simone et al., 1986; Halpern et al., 1991; Kishi et al., 1996; Solis Pereyra and Lemonnier, 1991; Wheeler et al., 1997). Long-term consumption of conventional yogurt has also been shown to enhance production of IL1β, IL-6, IL-10, IFN-γ and TNF-α (Aattouri and Lemonnier, 1997; Halpern et al., 1991; Miettinen et al., 1996; Solis Pereyra and Lemonnier, 1993). Probiotic-induced secretion of cytokines such as IFN-γ, IL-1, TNF-α, IL-10, IL-12, IL-18 and TGF-β by mononuclear cells and DCs *in vitro* is also well documented (Lammers et al., 2003; Miettinen et al., 1998; Niers et al., 2005; Solis Pereyra et al., 1997). It has also been reported that the nature of cytokines induced by probiotic are strain-dependent.

The ability of probiotics to induce broad and disparate cytokine responses by immune cells may explain how they are able to exert diverse effects on immune

function. For example, IL-12 and IL-18 induce IFN-γ production by T, B and NK cells whilst IFN-γ enhances phagocyte-capacity of macrophages, induces MHC1 and MHCII expression on a variety of immunocompetent cells, augments anti-tumor cytotoxicity, promotes T helper cell function and enhances immunogenicity of vaccines (Nussler and Thomson, 1992). TNF-α, together with IFN-γ, potentiates the microbicidal capacity of macrophages and exerts cytotoxic effect against tumors. IFN-α plays an important role in early stages of host protection against viral infections and cancers. IL-1 stimulates proliferation of T and B cells, IL-6 induces differentiation to antibody-secreting plasma cells, IL-2 stimulates proliferation and differentiation of B and NK cells and plays a role in the induction and regulation of T cell-mediated immune responses (Gill, 2003). IL-10 and TGF-β are pivotal for regulating polarized Th1 and Th2 responses.

23.9 Immunostimulation and Protection Against Infectious Diseases

An optimally functioning immune system is critical for protection against infections diseases. Specific populations groups with less than adequate immune competence, such as immunocompromised individuals, children and the elderly are known to exhibit increased susceptibility to gastrointestinal and respiratory tract infections. Current disease prevention and management strategies rely on the use of vaccines, antibiotics and other anti-microbials. However, the currently available vaccines are not fully effective and also there is a widespread occurrence of microbes that are resistant to commonly used antibiotics. Several well-designed studies have provided evidence that specific strains of probiotics may be useful in the prevention and control of gastrointestinal, respiratory and urogenital tract infections.

23.9.1 Gastrointestinal Infections

The effectiveness of probiotics in the management and treatment of diarrheal disease has been the subject of numerous randomized, double-blind, placebo-controlled studies and several meta-analyses (Johnston et al., 2006; McFarland, 2006, 2007; Sazawal et al., 2006; Szajewska and Mrukowicz, 2001; Van Neil et al., 2002). Despite significant heterogeneity between studies, these meta-analyses have concluded that probiotics are effective in reducing the duration of acute

diarrhea, incidence of antibiotic-associated diarrhea and the risk of traveler's diarrhea. For example, a meta-analysis of data from 34 masked, randomized, placebo-controlled trials showed that probiotics significantly reduced antibiotic-associated diarrhea by 52%, reduced the risk of travelers' diarrhea by 8% and that of acute diarrhea due to diverse causes by 34%. Furthermore, probiotic intervention was more effective in children (risk ratio: 0.43) compared with adults (risk ratio: 0.74). Similarly, the pooled results from 23 controlled trials showed that probiotics reduced the mean duration of diarrhea by 30 h, and were more effective in the treatment of acute diarrhea if given early during the course of illness (Allen et al., 2007).

Several mechanisms by which probiotics mediate their protective effects have been suggested (Gill, 2003). However, the relative significance of various mechanisms remains unclear. Results of several animal studies suggest that an augmentation of host immunity by probiotics may play an important role in host defense. For example, a positive relationship between resistance to challenge infection with *Salmonella* and *E. coli* and enhanced natural (phagocytic cell function, NK cell activity) and acquired immune responses (specific mucosal and serum antibody responses) in mice fed probiotics was reported by Gill et al. (2001d), Shu and Gill (2002), and Shu et al. (2000). Similar observations have also been made by others (reviewed by Cross, 2002).

In humans, however, only a few studies have reported concomitant measurement of immune responses (*Table 23.2*). It has been reported that a reduction in the duration of diarrhea in children with acute rotavirus gastroenteritis, following administration of probiotics, was associated with enhanced specific (frequency of cells producing rotavirus-specific IgA antibody and anti-rotavirus serum IgA levels) and non-specific immune responses (frequency of circulating IgG-, IgM- and IgA-secreting cells), (Majamaa et al.,1995; Kaila et al., 1995). Similarly, supplementation with *B. breve* in infants attending a residential institution was shown to reduce the frequency of rotavirus shedding and enhance titers of anti-rotavirus IgA in stool samples. Furthermore, Kaila et al. (1995) demonstrated that the protective effect of viable versus inactivated probiotics was due to their superior immunostimulatory capacity; infants receiving viable *Lactobacillus* GG exhibited higher anti-rotavirus serum IgA response and higher frequency of rotavirus-specific IgA-secreting cell responses compared with subjects given inactivated probiotics.

Since challenge infection studies are not possible in humans, several studies have examined immune responses to attenuated or non-virulent pathogens. These studies have indicated that probiotic supplementation is effective in

Table 23.2

Probiotic-induced immunostimulation and the prevention and/or treatment of diarrhea: examples

Author	Study design and population	Probiotic treatment/ Intervention	Health effect	Immune correlate
Kaila et al. (1992)	RDBPC Children with acute rotavirus diarrhea	*Lactobacillus* GG fermented milk or a placebo, following oral rehydration	↓ Duration of diarrhea (1.1 vs. 2.5 days)	↑ IgG, IgM and IgA-secreting cell numbers during the acute phase ↑ % of subjects with rotavirus-specific IgA-secreting cell response at convalescence stage (90% vs. 46%)
Majamaa et al. (1995)	RDBPC Children with rotavirus gastroenteritis	*Lactobacillus GG, L. casei* subsp. *rhamnosus* (Lactophilus) or a combination of *S. thermophilus* + *L. delbruckii* (Yalacta)	↓ Reduction in duration of diarrhea in LGG group (1.8 days vs. 2.8 days in Lactophilus and 2.6 days in Yalacta groups)	Enhancement of rotavirus specific IgA-secreting cell numbers and serum IgA antibody levels at convalescent stage in LGG group
Kaila et al. (1995)	Infants with acute rotavirus diarrhea	Viable or inactivated *Lactobacillus* GG, following oral rehydration	↓ Duration of diarrhea in subjects given viable compared with inactivated *Lactobacillus* GG	Higher anti-rotavirus serum IgA response and higher frequency of subjects with rotavirus-specific IgA-secreting cell response in subjects given viable vs. inactivated *Lactobacillus GG* (10/12 vs. 2/13)
Araki et al. (1999)	Infants attending a residential institution	*B. breve* (strain YIT 4064) for 28 days	↓ Rotavirus shedding	Tendency toward increased rotavirus-specific IgA in the stools
Phuapradit et al. (1999)	RDBPC Children (6–36 months age) Prevention of rotavirus diarrhea	*Bifidobacterium* Bb12 alone or with *S. thermophilus*	Prevention of symptomatic rotavirus infection	No significant increase in antibody levels in treatment group indicating no infection (30% of control group showed sub-clinical infection)

augmenting immune responses to live rotavirus (Isolauri et al., 2000), *Salmonella* (Link-Amster et al., 1994) and polio vaccines (de Vrese et al., 2005).

23.9.2 Extra-intestinal Infections

27.9.2.1 Respiratory Tract Infections

Perhaps the best evidence for a pivotal role of immunological defense mechanisms in host protection comes from studies demonstrating the effectiveness of probiotics against pathogenic organisms at extra-intestinal sites (Lenoir-Wijnkoop et al., 2008), particularly the respiratory tract (de Vrese et al., 2006; Habermann et al., 2001; Hatakka et al., 2001; Turchet et.al., 2003). Many of these studies have measured changes in immune function and health effects concomitantly and found that probiotic-mediated protection is accompanied by stimulation of host immunity (*Table 23.3*). For example, de Vrese et al. (2006) and Winkler et al. (2005) reported that significant reduction in total symptom score, the duration of common cold episodes and days with fever during an episode in healthy adults following probiotics supplementation during winter/spring period was accompanied by significantly higher cytotoxic/suppressor T cell and helper T cell numbers. In a randomized, double-blind, placebo-controlled study, a reduction in the duration of infections and the frequency of respiratory tract symptoms in probiotic-fed subjects was associated with enhanced phagocytic activity of blood leukocytes (Fukushima et al., 2007). Probiotic therapy also modulated inflammatory responses as indicated by a reduction in the levels of blood IFN-α. Normalization of perturbed intestinal flora in children with acute viral and bacterial respiratory tract infections and enhancement in aspects of T and B cell function, and NK cell activity in children with acute viral and bacterial respiratory tract infections following administration with probiotics, has also been reported (Lykova et al., 2000).

Furthermore, respiratory tract infections impair the host's capacity to produce interferons, a vital component of cell-mediated host defense mechanism. Supplementation with probiotics was found to restore the interferon production capacity of children suffering from acute respiratory tract infections (Lykova et al., 2001). As highlighted earlier, interferons (alpha and gamma) play an important role in host protection, especially against intracellular/viral infections.

Activation of Th1 type immune responses is pivotal for effective eradication of intracellular pathogens, including viruses. Infants that produce high levels of

Table 23.3

Efficacy of probiotics in the prevention of respiratory tract infections: some examples (*Cont'd* p. 916)

Author	Study design/population	Intervention	Health effect	Immune correlate
Fukushima et al. (2007)	RDBPC Enterally-fed in-patients (n = 24) aged over 70 years	Fermented milk containing *L. johnsonii* La1 or placebo	Significant reduction in the percentage of days with infection and lower frequency of respiratory symptoms in the probiotic group	↓TNF-α levels in blood ↑ Phagocytic activity of blood leukocytes in subjects whose initial levels were low in the La1 group
Cobo et al. (2006)	RDBPC Children (3–12 years old, n = 251)	Given *L. casei* (DN-114001) fermented milk (n = 142) or a placebo (n = 109) for 20 weeks to determine effect on infectious disorders	Lower incidence of lower respiratory tract infections in the probiotic group (32% vs. 49%, $P < 0.05$)	ND
de Vrese et al. (2006)	RDBPC Healthy adults (n = 479)	*L. gasseri* PA 16/8, *B. longum* SP 07/3 and *B. bifidum* MF 20/5 + vitamins and minerals or placebo	Reduction ($P < 0.06$) in total symptom score, duration of common cold ($P = 0.05$) and number of days with fever ($P = 0.02$) in the probiotic group	Significant increase in $CD8^+$ and $CD4^+$ cells in the probiotic treated group
Winkler et al. (2005)	RDBPC Health adults (n = 477)	Probiotics (*Lactobacillus* and *Bifidobacterium spp*) plus vitamins and minerals or placebo	Reduction ($P < 0.07$) in the incidence of respiratory tract infections, total symptom score $P = 0.12$), and number of days with fever ($P = 0.03$) in the probiotic group	Significant increase in T-lymphocytes (including $CD4^+$ and $CD8^+$ cells) and monocytes

Table 23.3

Author	Study design/population	Intervention	Health effect	Immune correlate
Tubelius et al. (2005)	RDBPC Healthy adults (n = 262)	*L. reuteri* or placebo	Fewer subjects in the *L. reuteri* group reported sick-leave compared with the placebo group (10.6% vs. 26.4%, $P < 0.01$; frequency of sick leave 0.4% vs. 0.9%, $P < 0.01$). Amongst the shift workers, 33% reported sick leave compared with 0% in the probiotic group, $P < 0.005$)	ND
Gluck and Gebbers (2003)	ORPC Healthy adults (n = 209)	Fermented milk drink containing *Lactobacillus* GG (ATCC 53103), *Bifidobacterium* sp B420, *L. acidophilus* 145, and *S. thermophilus*; or standard yogurt	Significant reduction ($P < 0.001$) in the number of potential pathogenic bacteria in nasal cavity in probiotic group	ND
Turchet et al. (2003)	ORC Free-living elderly subjects (n = 360)	*L. casei* (DN-114001) fermented milk or a placebo for 3 weeks	No effect on the incidence of infections Reduction (20%, $P < 0.05$) in the duration of infection in probiotic group	ND

Lykova et al. (2001)	Hospitalized children (n = 46); 33 with complicated forms of acute respiratory virus infection and 13 with vegetov – ascular dystonia (comparison group)	Bifidobacterin forte	Not reported	Normalization of an impaired interferon status of children with respiratory tract infections
Hatakka et al. (2001)	RDBPC Day-care children (1–6 years, n + 571) in 18 day care centers	Milk with or without *Lactobacillus* GG (n = 289) or standard milk	Children in LGG group had – 17% fewer respiratory tract infections, fewer days of absence from day care (16% ↓, $P < 0.05$) and longer time without respiratory symptoms (5 vs. 4 days, $p < 0.05$) and 19% less antibiotic usage ($P < 0.05$)	ND
Lykova et al. (2000)	Children with acute viral and bacterial infections of the respiratory tract (n = 129)	Bifidobacterin forte	Normalization of intestinal microbiota	Improvements in the indices of T and B cell immunity, NK cell activity and interferon producing capacity of blood leukocytes

ORC open label randomized controlled trial, *RDBPC* randomized double-blind placebo-controlled, ND not done, ORPC open, randomised, placebo-controlled

Th1 cytokines in response to respiratory viral infections (Sin et al., 2001) and mice that are genetically engineered to overproduce Th1 cytokines exhibit less severe disease following infection (Pinto et al., 2006). A negative relationship between PHA (phytohaemagglutinin)-induced secretion of IFN-γ by cord blood mononuclear cells and the incidence of viral illnesses during the first year of life has also been reported (Copenhaver et al., 2004).

23.9.2.2 Urogenital Infections

Vaginal flora in healthy women is dominated by *Lactobacillus* species. The depletion of lactobacilli is associated with increased occurrence of urinary and vaginal infections. As a result, the use of probiotics to maintain and/or restore vaginal microbiota and prevent and/or treat urogenital infections has been the focus of active investigation. Whilst a small number of well-designed studies have provided evidence for the therapeutic and prophylactic efficacy of specific probiotic strains, others have found little or no therapeutic benefit (Barrons and Tassone, 2008; Reid, 2008). Whether this has been simply due to differences in strains, dosages or frequency of treatment used by various studies or some other reason is unclear. The mechanisms by which lactobacilli/probiotics mediate their protective effects are also not fully known, but thought to include competitive exclusion, competition for nutrients, production of antimicrobial compounds (such as hydrogen peroxide, bacteriocins, organic acids) and biosurfactants and immune stimulation. However, direct evidence supporting a role for immune mechanisms is limited and requires further research. It is quite likely, however, that being a part of the common mucosal system, sensitized immune cells induced in the gastrointestinal tract may relocate to distant mucosal sites such as urogenital tract. This is supported by the effectiveness of orally-delivered probiotics against urogenital infections and the fact that most of these infections are caused by pathogens ascending from the rectal area.

23.10 Immunostimulation and Protection Against Cancer

Cancer, a major cause of morbidity and mortality, is one of the most serious health problems afflicting human population worldwide. In addition to genetic factors, many lifestyle factors (environmental and behavioral) have been implicated in the

development of cancer. Despite significant advances in science and technology, including sequencing of the human genome, progress in developing preventive and therapeutic strategies for cancer has remained slow. Emerging evidence from animal and human studies suggest that specific strains of probiotic may have a protective effect against colon and bladder cancers.

23.10.1 Colorectal Cancer

Colorectal cancer (CRC) is a major cause of death from cancer in the developed world (Saikali et al., 2004). It has been suggested that environmental factors (especially diet) and colonic microbiota play an important role in colorectal carcinogenesis. As a result, there has been an increasing interest in exploring the protective effects of fermented milks and probiotics against CRC. Results of animal studies have shown that administration of probiotics is effective in reducing the incidence of CRC/or of precursor lesions (Capurso et al., 2006). The protective effect was found to be more pronounced when probiotics were administered before but not after the carcinogenesis. *In vitro* studies have also yielded similar results. There is also evidence from epidemiological and case-control studies that consumption of fermented products may have a protective effect against colorectal cancer. However, there is relatively little direct evidence of the anti-cancer efficacy of probiotics or fermented products in human subjects. Rafter et al. (2007) reported reduction in several colorectal cancer biomarkers in a randomized, double-blind, placebo controlled trial involving polypectomized and colon cancer patients following synbiotic (a combination of prebiotic SYN1 and probiotics *Lactobacillus* GG and *Bifidobacterium lactis* BB12) intervention. Patients receiving synbiotics exhibited significant reduction in colorectal proliferation and the capacity of fecal water to induce necrosis in colonic cells, and an improved epithelial barrier function. Additionally, synbiotic consumption prevented an increased secretion of IL-2 by peripheral blood mononuclear cells in polypectomized patients and increased the production of IFN-γ in cancer patients. These favorable changes in CRC biomarkers were also accompanied by significant changes in fecal flora: populations of *Bifidobacterium* and *Lactobacillus* increased and *Clostridium perfringens* decreased. A reduced rate of tumors in cancer patients following probiotic intervention has also been observed in another large randomized clinical study (Ishikawa et al., 2005). The occurrence rate of tumors with a moderate or severe atypia and tumors larger than 4 mm was significantly lower in subjects receiving probiotics. The ability of specific strains

of probiotics to reduce the concentration of bacterial enzymes that convert procarcinogens to carcinogens has also been demonstrated in several human studies (Rafter, 2002).

The mechanisms by which probiotics mediate these protective effects are not clear, however, an association between enhanced immune function and tumor suppression suggest an important role of probiotic-induced immunostimulation in host protection. Perdigon et al. (1998) and Feghali et al. (1997) reported that an inhibition of carcinoma development in mice fed yoghurt was accompanied by a significant increase in the number of IgA secreting cells and $CD4^+$ T lymphocytes in the lamina propria of the large intestine together with a decrease in the number of IgG^+ and $CD8^+$ cells. Similarly, mice given cytoplasmic fractions of *L. casei* and *B. longum* were found to exhibit significant anti-Tumor immunity and enhanced counts of CD8 T cells, total T cells, NK cells and $MHCII^+$ cells (Lee et al., 2004). In another study, Takagi et al. (2001) showed that NK cells were pivotal for probiotic-mediated anti-cancer immunity. Delayed tumor onset and reduced tumor incidence in normal mice administered *L. casei* were associated with enhanced NK cell activity and numbers of NK cells. However, *L. casei* failed to exert any protective effect in beige mice that are genetically deficient in NK cells. In patients with Dukes A stage colorectal cancer, administration of *L.casei* Shirota was found to increase the percentage of T helper cells and NK cells and decrease the proportion of T suppressor cells (Sawamura et al., 1994). *In vitro*, probiotics have been shown to mediate anti-cancer effects by promoting apoptosis through enhancing mitogen activated protein kinase (MAPK) activities including C-Jun N-terminal Kinase and P38 MAPK, and down regulating Nuclear Factor-KB (NF-kB) dependent gene products that mediate cell proliferation (COX-2, cyclin D1) and survival (Bcl-2, Bcl-XL) (Iyer et al., 2008). The ability of probiotic-derived components to induce macrophage activation and significantly increase production of TNF-α and NO that exhibit cytotoxic effects against tumor cells (Caco-2, HT-29, and SW480) has also been observed by Lee et al. (2008a).

23.10.2 Bladder Cancer

Probiotic administration has also been shown to reduce the recurrence rate of superficial bladder cancer after transurethral resection (Aso et al., 1992, 1995). A similar protective effect of fermented milk containing *L. casei* Shirota against bladder cancer was reported by Ohashi et al. (2002). Stimulation of the immune

system, as indicated by increases in the percentage of T-helper cells and NK cells and enhancement of NK cell cytotoxic activity have been suggested to play an important role in the prevention of tumor development.

23.11 Probiotics and Attenuation of Immuno-Inflammatory Disorders

A balance between Th1 and Th2 responses is essential for immune homeostasis. Polarization of immune responses towards either the Th1-type or Th2-type results in an increased occurrence of immunoinflammatory disorders. For example, diabetes mellitus type-1 (DM) and rheumatoid arthritis (RA) are associated with an over expression of Th1 responses, whereas allergies are associated with Th2 cell predominance. Of the various factors suggested to be responsible for the increased incidence of immunoinflammatory disorders, a lack of or inappropriate exposure to microbes early in life (due to improved hygiene, vaccination and antimicrobial medication) that are essential for the development of a balanced immune system (establishment of regulatory networks) has received wide acceptance. Although, drug based therapy is available for controlling these disorders, it invariably suffers from adverse side effects and exorbitant cost beyond the reach of the low income population groups.

There is overwhelming evidence that specific strains of probiotics are endowed with unique immunoregulatory properties and therefore, may provide a safe and effective alternative to drug therapy for the prevention or management of immunoinflammatory disorders. The possible mechanisms by which probiotics attenuate various inflammatory disorders are discussed in the following section.

23.11.1 Allergies

Allergic disorders are the consequence of aberrant Th2 type immune responses to innocuous environmental antigens in genetically susceptible individuals and are characterized by increased IgE synthesis and recruitment and activation of eosinophils. At birth, the immune system of the newborn is biased towards Th2 profile. In healthy infants, the polarized Th2 profile is down-regulated by the activation of Th1 cells, whereas the Th2 profile remains augmented in atopic infants due to the absence of counter-regulatory processes. An alarming increase

in the incidence of allergic diseases in children and adults observed in recent years has been attributed to lack of exposure to microbial antigens during early life. Signals transmitted by microbes have been suggested to be pivotal for the maturation of the immune system and the development of regulatory networks. The role of indigenous flora in shaping the development of the immune system is also highlighted by qualitative and quantitative differences in the composition of microbiota of atopic versus non-atopic children (Bjorksten et al., 2001); children who develop allergy were found to have fewer bifidobacteria and enterococci than non-allergic children.

As specific probiotic strains exhibit potent immunoregulatory properties, several studies have examined their effectiveness in the treatment or prevention of allergic disorders, especially atopic dermatitis (*Table 23.4*). The results of these studies have been mix.

In an open label study, Majamaa and Isolauri (1997) reported improvement in SCORAD (SCOring Atopic Dermatitis) and reduction in inflammatory markers (fecal α1-antitrypsin and TNF-α) in children with AD following 1 month supplementation with probiotics. Similar observations on the therapeutic effects of *Lactobacillus* GG in children weaned onto a probiotic-supplemented hydrolyzed formula were made by Isolauri et al. (2000). However, in subsequent studies, improvements in eczema were found to be limited only to subjects with allergic sensitization (Rosenfeldt et al., 2003; Sistek et al., 2006; Viljanen et al., 2005). On the other hand, several studies have found no effect of LGG (Folster-Holst et al., 2006; Gruber et al., 2007) or other probiotics on treatment of atopic dermatitis in infancy (Betsi et al., 2008). Furthermore, a number of studies have also demonstrated the protective effect of probiotics in high-risk infants. Kalliomaki et al. (2001, 2003) demonstrated that probiotic supplementation prenataly and postnataly to mothers with a history of atopic disease and their newborns suppresses the development of atopic dermatitis in high-risk children in later life (at least up to the age of 4 years). Similar observations regarding the protective effects of *L. rhamnosus* HN001 given during pregnancy and lactation have also been reported (Wickens et al., 2008). It has been shown that probiotics may mediate their protective effect by modulating maternal and fetal immune responses and through the induction of immunoregulatory factors in breast milk (Prescott et al., 2008). Contrary to these observations, however, Kopp et al. (2008) reported that supplementation with *Lactobacillus* GG during pregnancy and early infancy neither reduced the incidence nor the severity of atopic dermatitis in affected children but was associated with an increased rate of recurrent episodes of wheezing bronchitis. Taylor et al. (2006) noted that supplementation with *L. acidophilus* was associated

Table 23.4

Efficacy of probiotics in the prevention and treatment of atopic dermatitis: some examples (*Cont'd* p. 924)

Reference	Study design and population	Probiotic used	Outcome	Immune effect
Wickens et al. (2008) and Prescott et al. (2008)	RDBPC	*L. rhamnosus* HN001, *Bifidobacterium lactis* H019 or placebo daily from 35 weeks gestation until 6 months if breast-feeding, and their infants (same treatment as mothers) from birth to 2 years	Infants receiving *L. rhamnosus* had a significantly lower risk of eczema	Mothers receiving *L. rhamnosus* had significantly higher levels of cord blood IFN-γ and higher proportion had detectable blood IFN-γ compared with placebo
	Mothers and infants with family history of allergic disease		No significant effect of probiotics on atopy	Higher levels of TGF-β1 and IgA in breast milk mother given probiotics Prescott et al., 2008)
Abrahamsson et al. (2008)	RDBPC	*L. reuteri* ATCC55730 daily to mothers for 4 weeks before delivery and mother and baby daily for 12 months after delivery	No effect on eczema	Significant reduction in skin test reactivity in probiotic group
	Mothers and infants with family history of allergic disease		Infants receiving probiotics had less IgE-associated eczema at 2 years of age	
Kopp et al. (2008)	RDBPC	*Lactobacillus* GG or placebo starting 4–6 weeks before expected delivery, followed by postnatal supplementation for 6 months	No effect on the incidence or severity of atopic dermatitis	No difference in total immunoglobulin concentrations or number of specific sensitization to inhaled allergens
	Mothers and infants with family history of atopic disease			
Hol et al. (2008)	RDBPC	*L. casei* CRL 431 + *B. lactis* Bb12 or placebo	No effect on tolerance to cows milk	Higher percentage of pan T cells and helper T cells in placebo group than seen in probiotic treated infants
	Infants with cows milk allergy			

Table 23.4 (*Cont'd* p. 926)

Reference	Study design and population	Probiotic used	Outcome	Immune effect
Taylor et al. (2006)	RDBPC Infants with family history of atopic disease	*L. acidophilus* LAVRI-A1 or placebo daily for the first 6 months of life	No effect on risk of atopic dermatitis Higher percentage of children with skin-prick test and atopic dermatitis in the probiotic group	Higher rate of sensitization in placebo group
Kukkonen et al. (2007)	RDBPC Mother and infant pairs (up to 6 months). Prevention of food allergy, eczema, asthma and allergic rhinitis	Probiotics + galacto-oligosaccharide	Reduction in the incidence of atopic diseases ($p < 0.052$), eczema ($p < 0.035$) and atopic eczema ($p < 0.025$)	Not recorded
Morita et al. (2006)	Controlled Trial Subjects (n=15) with perennial allergic rhinitis/showing high serum IgE levels and allergic symptoms	*L. gasseri* TMC0356	Not reported	Reduction in serum total IgE levels ($p < 0.05$). Significant increase in the proportion of Th1 cells on days 14 ($p < 0.01$) and 28 ($p < 0.05$)
Xiao et al. (2006)	RDBPC Subjects with history of Japanese cedar pollinosis (JCPsis)	*B. longum* BB536	Significant improvements in eye symptoms in the probiotic group ($p = 0.0057$). Also, reduction in rhinorrhea and nasal blockage	Decrease in JCP-specific IgE levels
Weston et al. (2005)	RDBPC Children (6–18 months) with moderate to severe Atopic dermatitis (AD)	*L. fermentum* VRI-033	Significant reduction in the SCORAD index over time ($p < 0.03$)	Reduction in IgE level (35.7 ($\pm$6.0) in placebo group vs. 31.8 ($\pm$4.3) in probiotic group

Prescott et al. (2005)	RDBPC Young children with moderate-to-severe atopic dermatitis (AD)	*L. fermentum* PCC trade mark	Improvement in AD severity	Significant increase in IFN-γ production following stimulation with PHA and SEB at the end of the supplementation period (week 8: P = 0.004 and 0.046) as well as 8 weeks after cessation of supplementation (week 16: P = 0.005 and 0.021)
Ciprandi et al. (2005)	Controlled trial Adult subjects (mean age 22.3 years, n=10) with allergic rhinitis	Enterogermania (containing *Bacillus clausii*)	Symptoms not reported	Significant decrease in IL4 levels (P = 0.004); significant increase in IFN-γ (P = 0.038), TGF-β (P = 0.039), and IL10 (P = 0.009) levels
Viljanen et al. (2005)	RDBPC Infants with atopic eczema/dermatitis syndrome (AEDS) and food allergy	*Lactobacillus* GG (LGG), a mixture of four probiotic strains (MIX)	Reduction in SCORAD in IgE sensitized infants. Reduction in AD in the LGG group, but not in other treatment groups	Increase in IgA levels in the probiotic group compared with the placebo group (LGG vs. placebo, P = 0.064; MIX vs. placebo, P = 0.064), after challenge, in subjects with IgE-associated CMA infants, increase in fecal IgA (P = 0.014), and decrease in TNF-α compared to placebo
Ciprandi et al. (2004)	Controlled trial involving children attending the nursery school Allergic children (mean age 4.4 yr, n=10) with recurrent respiratory infections	Enterogermania (containing *Bacillus clausii*)	Symptoms not reported	Significant reduction in IL-4 levels ($P < 0.01$) and a significant increase in IFN-γ ($P < 0.05$), IL-12 ($P < 0.001$), TGF-β ($P < 0.05$), and IL-10 ($P < 0.05$) levels

Table 23.4

Reference	Study design and population	Probiotic used	Outcome	Immune effect
Rosenfeldt et al. (2003)	Double-blind, placebo-controlled, crossover study Children (1–13 year old) with atopic dermatitis	*L. rhamnosus* 19070–2 and *L. reuteri* DSM 122460)	Improvement in SCORAD	Reduction in serum eosinophil cationic protein levels (P = 0.03) in the probiotic group
Rautava et al. (2002)	RDBPC Mother-infant pairs with history of atopic diseases	Probiotics	Significant reduction in the risk of developing atopic eczema in probiotic group compared to placebo (15% and 47%, respectively; $P = 0.0098$)	Significant increase in TGF-β 2 level in human milk in probiotic group (2,885 pg/mL) vs. placebo (1,340 pg/mL) placebo; $P = 0.018$)
Kalliomaki et al. (2001) and Kalliomaki et al. (2003)	RDBPC Mother and infant pairs with history of atopic eczema	*Lactobacillus* GG prenataly to mothers and postnataly whilst breast feeding up to 6 months and to babies if not breast feeding until 6 months of age	Significant reduction in the incidence of atopic eczema up to 4 years of age	No effect on IgE levels/skin-prick test reactivity
Isolauri et al. (2000)	RDBPC Infants (mean age 4.6 months) with history of atopic eczema	*B. lactis* Bb-12 or *Lactobacillus* GG (ATCC 53103)	Reduction in SCORAD in the *B. lactis* Bb-12 group to 0 (0–3.8), and in the *Lactobacillus* GG group to 1 (0.1–8.7), vs. unsupplemented to 13.4 (4.5–18.2)	Reduction in the concentration of soluble CD4 in serum and eosinophilic protein X in urine
Pelto et al. (1998)	Double-blind, cross-over study Milk hyper sensitive and healthy adult subjects	*Lactobacillus* GG (ATCC 53103)	Down-regulation of immunoinflammatory response in milk-hypersensitive subjects	Significant reduction in the expression of CR1, Fc-γRI and Fc-αR in neutrophils and CR1, CR3 and Fc-αR in monocytes

RDBPC randomized double-blind placebo-controlled, *CMA* cows milk allergy Adapted from Gill and Prasad (2008)

with increased incidence of allergen sensitization in infants. The inability of probiotics to prevent the development of atopic dermatitis/eczema has been reported by others as well (Abrahamsson et al., 2008; Taylor et al., 2006). Whether this was simply due to difference in the strains used, probiotic dose, host or environmental factors is not clear. *Lactobacillus* GG, *B. lactis and L. rhamnosus* are known to possess potent immunomodulatory properties (reviewed earlier). Strain dependent differences in allergy-preventing efficacy of probiotics in humans have been demonstrated recently (Wickens et al., 2008).

It is important to note that probiotics administration has been found to be ineffective in influencing allergic manifestations in young adults and teenagers (Helin et al., 2002; Ishida et al., 2005). This suggests that the protective effects of probiotics are restricted to infancy when the immune system is still undergoing the learning/maturation process. Evidence from recent studies suggests that probiotics mediate their prophylactic or therapeutic anti-allergy effects through induction and activation of immunoregulatory and/or counter-regulatory immune responses. Ability of specific strains of probiotics to induce regulatory cells that produce IL-10 and TGF-β and inhibit proliferation and cytokine secretion by immune cells such as T cells has recently been demonstrated (Smits et al., 2005). Increased production of IL-10 and IFN-γ by PBMC *in vitro* and increased levels in breast-milk and serum following ingestion of probiotics has also been reported (Lammers et al., 2003; Pessi et al., 2000; Pohjavuori et al., 2004). Furthermore, induction of regulatory T cells in animal models (Di Giacinto et al., 2005) and increased *in vitro* production of regulatory cytokines (IL-10, TGF β after intake of probiotics) has also been demonstrated (Lammers et al., 2003). Involvement of other mechanisms such as reduction in the immunogenicity of potential allergens and strengthening of the mucosal barrier function have also been suggested to play a protective role.

Overall, the results of the clinical studies reported to date, although promising, are inconclusive. Further well-designed, large-scale, long-term studies are required to further evaluate the therapeutic and/or prophylactic effect of promising probiotic strains in defined population groups with specific endpoints. The precise mechanism involved in protection and the microbial factors responsible for inducing these responses also need to be elucidated.

23.11.2 Inflammatory Bowel Disease

Inflammatory Bowel disease (IBD) encompasses two distinct diseases; Crohn's disease (CD) and ulcerative colitis (UC). Both diseases are chronic in nature but

have quite distinct pathogeneses, underlying inflammatory profile, symptoms and treatment strategies (Geier et al., 2007). UC is largely restricted to the colon and/or rectum and is characterized by inflammation and superficial ulceration of the colonic mucosa. On the other hand, CD occurs as skip lesions in any region of the intestinal tract and is characterized by transmural granulomatous inflammation. Pouchitis is another related disorder that results from complicated ileal pouch-anal anastmosis (IPAA) surgery for UC. It is important to note that UC is a Th-2 immune response whereas CD is predominantly a Th-1 driven immune response.

The exact aetiology of IBD is unknown. Current evidence suggests that it results from a dysregulated immune response to certain enteric microbiota in genetically susceptible individuals. Although a range of microorganisms and their products have been identified in inflamed tissues of IBD patients, no specific microbe has been proven to cause IBD. There is overwhelming evidence, however, that the intestinal microbiota plays a central role in the initiation and perpetuation of the disease (Bengmark, 2007). This recently acquired knowledge has stimulated interest in exploring the effectiveness of new strategies aimed at manipulating intestinal microbiota and restoring immune system homeostasis.

Several studies have examined the potential of probiotic administration in the prevention and/or treatment of various inflammatory bowel disorders. There is a strong evidence for the effectiveness of specific probiotics (especially the commercial product VSL#3 containing a mixture of probiotic strains) in preventing the initial attack of pouchitis, and in maintaining antibiotic-induced remission in patients with recurrent or refractory pouchitis (Vanderpool et al., 2008). Promising results for the effectiveness of probiotics in UC have also been reported (Vanderpool et al., 2008). For example, *E. coli* Nissle has been found to be as effective as mesalazine in maintaining remission of UC. Protective effects of *Bifidobacterium*-fermented milk and VSL#3 in UC have also been demonstrated. Contrary to the above observations however, there is little evidence to support the effectiveness of probiotics in CD.

The primary mode of action of probiotics appears to be through restoration of gut microbial balance and mucosal barrier function, as well as up-regulation of immunoregulatory pathways. The ability of specific strains of probiotics to suppress production of pro-inflammatory cytokines and induce regulatory T cells is well documented (Lee et al., 2008b; Sheil et al., 2004; Vanderpool et al., 2008). For example, Cui et al. (2004) reported an association between increased expression of IL-10 and the prevention of flare-ups of chronic UC (Cui et al., 2004) and Lammers et al. (2005) found a reduction in pro-inflammatory cytokines in tissues obtained from subjects with pouchitis following treatment with probiotics. IL-10 and TGF-β have also been demonstrated to ameliorate inflammation

in *Helicobacter hepaticus*-induced IBD in an IL-10 deficient mouse model (Pena et al., 2005). A recent report has further shown that VSL#3 improves pouchitis disease activity index by increasing the number of mucosal regulatory T cells (Pronio et al., 2008).

Interestingly, in addition to live probiotics, some isolated components of probiotic bacteria have been demonstrated to exert immunomodulatory effects on the mucosal immune system. Even bacterial DNA has been demonstrated to have anti-inflammatory and immunomodulatory properties when administered subcutaneously in a number of animal models of colitis (Rachmilewitz et al., 2002). Genomic DNA isolated from VSL#3 inhibited TNF-α induced IL-8 secretion, mitogen-activated protein kinase activation and NFkB activation in HT-29 cells (Jijon et al., 2004). However, significant differences exist in the ability of various probiotics to induce anti-inflammatory versus pro-inflammatory cytokines and this may explain differences in the efficacy of various probiotic strains.

23.11.3 Diabetes Mellitus

Diabetes mellitus (DM) is a major cause of morbidity and mortality across the world. Type 1 Diabetes, which accounts for 1–10% of cases, results from autoimmune destruction of pancreatic β cells. Over-production of pro-inflammatory cytokines (such as IL-1β, TNFα and IFNγ) has been proposed as the possible mechanism for this (Rabinovitch and Suarez-Pinzon, 1998). Up-regulation of IL-10 has been shown to have a protective effect against this destruction of β cells (Goudy et al., 2003). The far more prevalent (90–95% of cases) Type 2 Diabetes involves a resistance of tissues to insulin, resulting in hyperglycaemia. An increased release of TNF-α, MCP-1 and additional products of macrophages and other cells that populate adipose tissue are thought to play a role in the development of this resistance (Wellen and Hotamisligil, 2005; Yang et al., 2002).

To date, only a small number of animal studies have explored the anti-diabetic effects of probiotics. Matsuzaki et al. (1997) reported that oral administration of *L. casei* in KK-A^{y} mice significantly decreased plasma glucose levels and inhibited the production of β cell specific CD4^{+} T cells and cytokines (INFγ and IL-2) associated with the induction of autoimmune diabetes. They further reported that diets containing *L. casei* strongly inhibited destruction of β cells and nitric oxide production. Later, Calcinaro et al. (2005) reported that VSL#3 prevented autoimmune diabetes by reducing insulitis in non-obese diabetic (NOD) mice. This preventative effect was associated with an increased production of IL-10 by Peyer's patches and splenocytes and a decreased expression of IL-1-mRNA in the

pancreas. IL-10 is generally considered to be an anti-inflammatory cytokine acting primarily on antigen-presenting cells to inhibit antigen presentation and the production of inflammatory cytokines. The effectiveness of IL-10 in preventing the development of autoimmune diabetes in NOD mice has also been demonstrated previously (Goudy et al., 2001). In another study, Matsuzaki et al. (2007) investigated the effects of *L. casei* strain Shirota (LcS) in autoimmune disease models using NOD mice. From the age of 4 weeks, female NOD mice were fed a diet containing *L. casei* and the onset of diabetes was recorded thereafter. The incidence of diabetes in the control group was significantly higher than that of the *L. casei*-treated group, and pathological analysis of the *L. casei* -treated group revealed a strong inhibition of pancreatic β-cell destruction. Moreover, the proportion of $CD8^+$ T cells amongst splenocytes was decreased in the *L. casei*-treated group, suggesting an inhibition of autoreactive T cells.

Since the anti-diabetic effect of probiotics is highly strain specific, more extensive work with a large number of strains is required to establish the immunomodulating/immunoregulatory potential of probiotics as oral therapy against DM. A study examining the protective effects of probiotics against β-cell autoimmunity in children at genetic risk of type-1 diabetes (the PRODIA study) is currently being conducted in Finland (Ljungberg et al., 2006). In the near future, there is a strong possibility of developing a probiotics-based therapy for combating this massive public health burden.

23.11.4 Rheumatoid Arthritis

Rheumatoid arthritis (RA), another important autoimmune disease, is characterized by chronic synovitis and causes stiffness, pain, loss of mobility and progressive erosion (deterioration) of the joints. It usually affects multiple joints symmetrically; the hand and wrists most commonly, but also the elbows, neck, shoulders, hips, knee, and feet. Extra-articular manifestations of RA can include development of nodules under the skin (especially at the elbows), lymphadenopathy, vasculitis and even peripheral neuropathy. Although many questions concerning the aetiology of RA remain unanswered, cumulative evidence suggests that $CD4^+$ T cells, which exhibit a predominantly Th1 pattern of cytokine expression, play an important role in the pathogenesis of the disease. Blockade of IL-12 and/or TNF-α has been shown to reduce progression of collagen-induced arthritis in mice (Butler et al., 2008) and leads to significant clinical improvement in subjects with RA (van Oosterhout et al., 2005).

Studies in animal models have shown that probiotics possess the ability to regulate over-expressed Th1-type responses and exert a beneficial effect in experimentally-induced arthritis. Oral administration of *L. casei* to DBA/1 mice prevented the onset of type II collagen (CII)-induced arthritis (CIA), reduced anti-collagen type II IgG2a and IgG2b serum antibodies and suppressed the CII-induced secretion of IFN-γ from splenocytes (Kato et al., 1998). In another study involving IL-10 knockout mice, Sheil et al. (2004) reported that even subcutaneous administration of *L. salivarius* 118 was effective in attenuating the development of collagen-induced arthritis and that the probiotic effect was associated with reduced production of proinflammatory (Th 1) cytokines and maintained production of anti-TGF-β. Recently, So et al. (2008) reported that probiotics mediate this effect by down-regulating Th1 effector functions. *L. casei* administration reduced type II collagen (CII)-reactive proinflammatory molecules (IL-1 β, IL-2, IL-6, IL-12, IL-17, IFN-γ, TNF-α and Cox-2) by $CD4^+$ T cells. *L. casei* administration also reduced translocation of NF-kB into the nucleus and CII-reactive Th1-type IgG isotypes IgG2a and IgG2b, while up-regulating immunoregulatory IL-10 levels. Preventative and curative effects of both live and heat-killed *Lactobacillus* GG in experimentally induced arthritis (Baharav et al., 2004) and the ability of *Enterococcus faecium* to improve the anti-inflammatory and anti-arthritic effects of methotrexate in adjuvant-induced arthritis in rats have also been demonstrated (Rovensky et al., 2005).

However, there is relatively little evidence regarding the effectiveness of probiotics against arthritis from human studies. In a randomized, double-blind placebo-controlled study, Hatakka et al. (2003) reported a beneficial effect of probiotic administration in RA patients. Although there were no statistically significant differences in the activity of RA, more subjects given *Lactobacillus* GG over a 12 month period reported subjective wellbeing compared with the placebo.

23.12 Mechanisms by Which Probiotics Modulate Immune Function

23.12.1 Recognition of Probiotics by the Immune System

The gastrointestinal tract is the largest body surface area permanently exposed to the external environment. It is continuously bombarded with a wide array of antigens derived from food, resident microbiota and the environment. Thus, the

challenge for the gut is to allow absorption of nutrients and exhibit tolerance towards indigenous flora whilst at the same time mount an effective immune response against potential pathogens. To perform this function optimally, the gastrointestinal tract harbors the largest number of immunocompetent cells (known as gut-associated lymphoid tissue, GALT) in the body.

Probiotics and probiotic-derived products in the gut are recognized by M cells (specialized epithelial cells overlying Peyer's patches), epithelial cells and dendritic cells (DCs) distributed in the intraepithelial and submucosal layers. Epithelial cells respond to bacterial components by releasing a variety of chemokines and cytokines (Haller et al., 2000) that recruit immune cells such as DCs to the mucosa. Antigens (probiotics and their products) taken up by M cells are delivered to antigen presenting cells (APCs), mainly macrophages and (DCs) located in the dome region. Antigen-loaded DCs travel to mesenteric lymph nodes and present antigens to T and B cells. Similarly, antigens/microbes phagocytosed by macrophages are either destroyed or presented to T and B cells. Active transport of live commensals by DCs from the mouse gut lumen to intestinal mesenteric lymph nodes has recently been reported by McPherson and Uhr (2004). Sensitized T and B cells circulate through the lymph and bloodstream and traffic back to populate mucosa remote from the inductive sites. APCs recognize conserved microbial signature molecules called pathogen-associated molecular patterns (PAMPS) through pattern-recognition receptors (Toll-like receptors (TLRs), C-type lectin receptors and Nod-like receptors) that are secreted or expressed on the surface of immune cells. These receptors are specific for various microbial components such as lipopolysaccharides, peptidoglycan and bacterial DNA. On recognition of PAMPS, TLRs activate a cascade of signaling pathways that induce antimicrobial effector responses and inflammation. The ability of probiotics to influence the expression level of TLRs and other TLR-like receptors on mucosal and systemic cells (Vanderpool et al., 2008) and to induce cytokine secretion from DCs and macrophages through the TLR-mediated signaling pathways is well documented (Shida and Nanno, 2008). Furthermore, activation of TLRs expressed on APCs play a central role in the initiation of acquired immunity; antigen recognition by APCs is followed by the secretion of cytokines and expression of co-stimulatory molecules. The nature of cytokines in the milieu, type and dose of antigen, phenotype and state of activation of APCs determine whether naive T lymphocytes differentiate into T helper 1 (Th1), T-helper 2 (Th2) or T regulatory (Treg) cells. For example, Th1 differentiation is dependent on IFN-γ and IL-12, and the Th2 differentiation relies on the presence of IL-4. Subsequent activation of Th1 cells results in the production of

IFN-γ, IL-2 and TNF-α and is associated with the development of cell-mediated and cytotoxic immunity. Activated Th2 cells produce IL-4, IL-5 and IL-13 and these promote antibody production and are associated with atopy. Treg cells secrete IL-10 and TGF-β and down-regulate the activities of both Th1 and Th2 cells.

23.12.2 Effect on Epithelial Cells

The epithelium (enterocytes, IECs) lining the gastrointestinal tract constitutes the first line of host defense and plays an important role in maintaining intestinal homeostasis. IECs express several antigen-presenting and costimulatory molecules and function as immunoregulatory cells. IECs recognize (MAMPS through TLRs and NOD) and respond to different bacteria and bacterial products in a discriminatory manner. IECs secrete a wide array of proinflammatory cytokines and chemokines, including IL-8 and TNF-α, in response to stimulation by pathogenic bacteria (Kagnoff and Eckmann, 1997), but show little or no response to resident microbiota. Through the release of cytokines and chemokines, IECs alert the host immune system and direct the development and deployment of effector (innate and/or acquired) immune responses to sites where the mucosal barrier has been breached. It has been demonstrated that in contrast to pathogens, specific probiotics are able to attenuate inflammatory pathways in epithelial cells through a variety of mechanisms (Tlaskalova-Hogenova et al., 2005). The ability of probiotics to attenuate inflammation and down-regulate over expressed immune responses in animal models of colitis and in subjects with milk allergy and IBD is well documented.

23.12.3 Regulation of Skewed Th1 and Th2 Responses and Attenuation of Immunoinflammatory Disorders

As mentioned earlier, T cells can be classified as Th1, Th2 or Treg/Th3 cells based upon their cytokine profiles. Treg cells produce IL-10 and TGF-β and are able to down-regulate skewed Th1/Th2 immune responses. A balance between Th1-Th2 is pivotal for immunological homeostasis, and polarization of immune responses towards Th1 or Th2 underlies the development of various immunoinflammatory

disorders. For example, allergies are driven by over-activation of Th2 immune responses and IBD and autoimmune disorders such as Type 1 diabetes are predominantly driven by Th1 type immune responses. Evidence from recent studies suggests that defective Treg cell activity may be the central cause for the concurrent rise in Th1 and Th2-mediated diseases observed over the recent decades; patients with Type 1 Diabetes, Multiple Sclerosis and individuals with a predisposition to allergy development are known to exhibit deficient Treg cell function (Guarner et al., 2006).

Results of *in vitro* and *in vivo* studies also suggest that the primary mechanism of probiotic action in inflammatory diseases is likely to be through the induction of regulatory T cells. Increased levels of TGF-β in breast milk (Rautava et al., 2002) and elevated levels of IL-10 and TGF-β in atopic children following administration of probiotics have been reported in several studies (Isolauri et al., 2000; Pessi et al., 2000). An association between increased IL-10 expression and the prevention of flare-ups of chronic UC (Cui et al., 2004) as well as a reduction in pro-inflammatory cytokines in tissue obtained from subjects with pouchitis following treatment with probiotics have also been demonstrated (Lammers et al., 2005).

Further support for the role of Treg cells in probiotic-mediated protection comes from studies that have shown an increased number of T reg cells following probiotic administration in both animals and humans. Di Giacinto et al. (2005) found an increased number of Treg cells bearing surface TGF-β, following administration of probiotics, in an animal model of colitis. These cells conferred protection against colitis in a cell-transfer system. Notably, the protective effect was dependent upon TGF-β and IL-10 and was abolished by appropriate neutralizing antibodies. An association between increased number of mucosal regulatory T cells and protection against puchitis has also been recently reported (Pronio et al., 2008). Chapat et al. (2004) showed that the ability of *L. casei* to reduce skin inflammation due to contact sensitivity was also Treg cell-dependent. In another recent study, probiotic administration was found to induce IL-10 production and prevent spontaneous autoimmune diabetes in the nonobese diabetic mouse (Calcinaro et al., 2005).

It has also been suggested that an interaction between DCs and probiotics is a critical step determining the development of various forms of immunity or tolerance. Maturation of DCs towards functionally distinct DC1, DC2 or DCreg subsets selectively directs the polarization of naive T cells towards Th1, Th2 or Treg phenotypes (Kapsenberg, 2003). Different probiotic strains induce distinct and even opposing DC responses (expression of cytokines and

maturation surface markers) with respect to their Th1/Th2/Treg-driving capacity (Christensen et al., 2002). The ability of certain probiotics to induce regulatory DCs has been reported by Hart et al. (2004) and Drakes et al. (2004).

Cumulatively, these observations suggest that mechanisms by which probiotics mediate their protective effects are not limited to the gut and are most likely mediated by Treg cells. Once induced, Treg cells are able to travel to other tissues in the body (Rook and Brunet, 2005). The effectiveness of orally or subcutaneously administered probiotics and bacterial DNA in attenuating colitis and arthritis in mice further supports this view (McCarthy et al., 2003; Rachmilewitz et al., 2004; Sheil et al., 2004).

Thus, probiotics have been demonstrated to confer health benefits by influencing the composition of gut flora and restoring intestinal homeostasis.

23.13 Conclusion

Of the many health benefits associated with the intake of probiotics, modulation of the immune system has received most attention. It is well documented that specific strains of probiotics are endowed with unique immunomodulatory properties. In healthy individuals, they have been shown to enhance phagocytic and microbicidal capacity of PMNs and monocytes, the tumor cell-killing capacity of NK cells, the immunogenicity of bacterial and viral vaccines (oral and systemically-delivered) and specific antibody responses to enteric pathogens. Emerging evidence also suggests that potentiation of innate and acquired immune responsiveness by probiotics may play an important role in protection against infectious diseases (both intestinal and extra-intestinal infections) and cancers. However, further studies, with concomitant measurement of immune responses and health outcomes, are needed to confirm these findings.

In subjects with immunoinflammatory disorders (such as IBD and allergies), probiotic administration has been shown to reduce the incidence or relieve the symptoms of various disorders by down-regulating aberrant Th1 or Th2 responses. Although the exact underlying mechanisms have not been elucidated, the available data suggests that probiotics mediate their effect through the induction of regulatory T cells that produce IL-10 and TGF-beta. Recent studies, mainly in experimental animals, also highlight the potential for using probiotics for the prevention/management of other inflammatory disorders such as RA and DM (especially type 1 DM). However, these observations still remain to be confirmed in human subjects.

Despite these major advances, significant deficits still remain in our knowledge regarding the effectiveness of different probiotics against various conditions, as well as the mechanisms by which probiotics promote health and protect against disease. It is important to note in this context, that most of the health benefits ascribed to probiotics have been proven only for a limited number of probiotic strains - in many cases only for a single strain. Thus, large scale, well-designed trials in relevant population groups are needed to unequivocally prove the clinical efficacy of probiotics. Effective/optimum dose rate and frequency of treatment also remains to be established for various probiotics for different population groups and health conditions.

List of Abbreviations

AD	atopic dermatitis
AEDS	atopic eczema/dermatitis syndrome
APC	antigen presenting cells
CD	Crohn's disease
CRC	colorectal cancer
DCs	dendritic cells
DM	diabetes mellitus
GALT	gut-associated lymphoid tissue
GIT	gastrointestinal tract
IBD	inflammatory bowel disease
IEC	intraepithelial cells
ND	not done
NK cells	natural killer cells
ORC	open-label controlled trial
ORPC	open randomised placebo-controlled
PAMP	pathogen-associated molecular patterns
PMN	polymorphonuclear cells
RA	rheumatoid arthritis
RDBPC	randomised, double-blind, placebo-controlled
SCORAD	SCOring atopic dermatitis
Tc	T cytotoxic cells
Th	T helper cells
TLRs	Toll-like receptors
UC	ulcerative colitis

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24 Probiotics and Chronic Gastrointestinal Disease

Francisco Guarner

24.1 Introduction

Human beings are associated in a symbiotic relationship with a huge population of microorganisms. During millennia, a considerable number of microbes have evolved and adapted to live and grow in the human intestine. The intestinal habitat of an individual contains billions of microorganisms including bacteria, protozoa, archaea, fungi, and viruses (Guarner and Malagelada, 2003; Ley et al., 2006), and the number of microbial cells within the gut lumen appears to be ten times larger than the number of eukaryotic cells of the human body. Some of these bacteria are potential pathogens and can be a source of infection and sepsis under some circumstances, for instance when the integrity of the bowel barrier is physically or functionally breached. However, growing evidence suggests that important health benefits to the human host derive from the constant interaction with its microbial guests. Recognition of these benefits in recent years is drawing particular attention to the functional implications of the gut microbial communities in host physiology.

The relationship between gut microbial communities and host can be optimized by pharmacological or nutritional intervention on the intestinal ecosystem using probiotics or prebiotics. Administration of exogenous bacteria with known beneficial properties may improve specific functions of the gut microbiota or prevent dysfunctions that are associated with disease. These bacteria are called *probiotics*, a term that refers to "live microorganisms which when administered in adequate amounts confer a health benefit on the host," as proposed by the Joint FAO/WHO Expert Consultation. The term *prebiotic* refers to "a selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microbiota that confers benefits upon host well being and health" (Gibson et al., 2004). A prebiotic should not be hydrolyzed by human intestinal enzymes, it should be selectively fermented by beneficial

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bacteria, and this selective fermentation should result in a beneficial effect on health or well-being of the host. The combination of probiotics and prebiotics is termed *synbiotic*, and is an exciting concept aimed at optimizing the impact of probiotics on the gut microbial ecosystem. These definitions were adopted by the International Scientific Association for Probiotics and Prebiotics (Reid et al., 2003).

This chapter reviews the impact of probiotics in the prevention and control of chronic gastrointestinal disorders. The first section focuses on the relationships between microbial communities and host, pointing out the disorders or dysfunctions of the gastrointestinal tract that may be secondary to alterations of the gut microbial ecosystem. The second section reviews the use of probiotics in human medicine with particular focus on chronic gastrointestinal diseases where the use of probiotics has proven some therapeutic success.

24.2 Section 1: The Intestinal Ecosystem

The gut microbiota is a diverse and dynamic ecosystem of microbial communities, which have adapted to live on the intestinal mucosal surface or within the gut lumen (Guarner and Malagelada, 2003; Ley et al., 2006). Gut microorganisms include native species that colonize permanently the tract, and a variable set of living microorganisms that transit temporarily through the tract. Native species are mainly acquired at birth and during the first years of life, whereas transient microbes are being ingested from the environment (food, drinks, etc.). The stomach and the small intestine contain only a few species of bacteria adhering to the epithelia and some other bacteria in transit. The paucity of bacteria in the upper tract appears to be due to the composition of the luminal medium (acid, bile, pancreatic secretion) that kills the vast majority of ingested microorganisms, and to the phasic propulsive motor activity towards the ileal end that impede a stable colonization of the lumen. In the upper gut, transit is rapid and bacterial density is low, but the impact on immune function is thought to be important because of the presence of a large number of organized lymphoid structures in the small intestinal mucosa (Peyer's patches). These structures have a specialized epithelium for uptake and sampling of antigens and contain lymphoid germinal centers for the induction of adaptive immune responses (Cummings et al., 2004).

In the colon, transit time is slow and microorganisms have the opportunity to proliferate by fermenting available substrates derived from either the diet or endogenous secretions. Thus, the large intestine harbors a complex and dynamic

microbial ecosystem with high densities of living bacteria that achieve concentrations up to 10^{11} or 10^{12} cells per gram of luminal contents. These concentrations are similar to those found in colonies growing under optimal conditions over the surface of a laboratory plate. Bacteria represent a large proportion of the fecal mass, around 60% of fecal solids.

Our current knowledge about the microbial composition of the intestinal ecosystem in health and disease is still very limited. Studies using classical techniques of microbiological culture can only recover a minor fraction of fecal bacteria. Over 50% of bacteria cells that are observed through microscopic examination of fecal specimens cannot be grown in culture (Eckburg et al., 2005). Molecular biological techniques based on the sequence diversity of the bacterial genome are being used to characterize non-cultivable bacteria (Frank and Pace, 2008). Molecular studies on the fecal microbiota have highlighted that only seven of the 55 known divisions or superkingdoms of the domain "bacteria" are detected in the human gut ecosystem, and of these, 2 bacterial divisions dominate, i.e., Bacteroidetes and Firmicutes (*Figure 24.1*). However, at species and strain level, microbial diversity between individuals is highly remarkable up to the point that each individual harbors his or her own distinctive pattern of bacterial composition (Eckburg et al., 2005). This pattern appears to be determined by the host genotype (Zoetendal et al., 2001) and by the initial colonization at birth by vertical transmission (Ley et al., 2006). In healthy adults, the fecal composition is stable over time, but temporal fluctuations due to environmental factors can be detected and may involve up to 20% of the dominant groups (Zoetendal et al., 2001). Bacterial composition in the lumen varies from cecum to rectum, and fecal samples do not reflect luminal contents at proximal segments. However, the community of mucosa-associated bacteria is highly stable from terminal ileum to the large bowel in a given individual (Lepage et al., 2005).

24.2.1 Host-Microbe Interactions in the Gut

Some of the bacteria in the gut are pathogens or potential pathogens when the integrity of the mucosal barrier is functionally breached. However, the normal interaction between gut bacteria and their host is a symbiotic relationship, defined as mutually beneficial for both partners. The host provides a nutrient-rich habitat and the bacteria can infer important benefits on host's health (Hooper et al., 2002). Comparison of animals bred under germ-free conditions with their conventionally raised counterparts (conventional microbiota) has

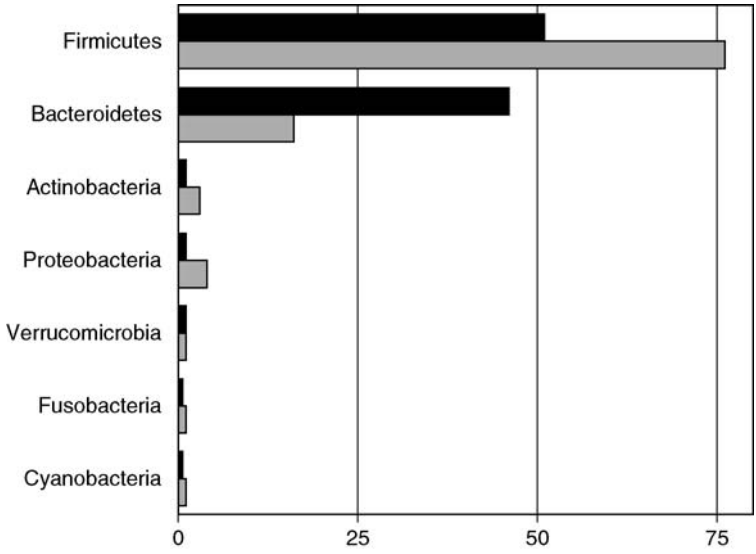

Figure 24.1
Molecular studies on the human fecal microbiota have highlighted that only 7 of the 55 known divisions or superkingdoms of the domain "bacteria" are detected in the human gut, and of these, 2 bacterial divisions dominate, i.e., Bacteroidetes and Firmicutes. Black bars represent relative abundance of bacteria 16sRNA sequences belonging to each division as percentage of the total number of sequences obtained from 3 healthy subjects. Sequences sharing 99% similarity were grouped into phylotypes (as indicator of species diversity) and relative abundance of phylotypes is represented by the gray bars. Data were published by Eckburg et al. (2005) as supporting online material.

revealed a series of anatomic characteristics and physiological functions that are associated with the presence of the microbiota (Falk et al., 1998). Organ weights (heart, lung, and liver), cardiac output, intestinal wall thickness, intestinal motor activity, serum gamma-globulin levels, lymph nodes, among other characteristics, are all reduced or atrophic in germ-free animals, suggesting that gut bacteria have important and specific functions on the host (*Figure 24.2*).

24.2.2 Primary Functions of the Gut Microbiota

Evidence obtained through studies in germ-free or mono-colonized animals demonstrates specific functions of the microbiota that have been ascribed into three main categories, i.e., metabolic, protective, and trophic functions (Guarner and Malagelada, 2003). *Metabolic functions* consist of the fermentation

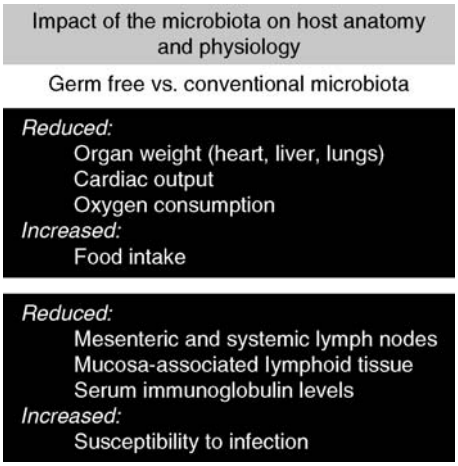

Figure 24.2
Comparison of animals bred under germ-free conditions with their conventionally raised counterparts (conventional microbiota) has revealed a series of anatomic characteristics and physiological functions that are associated with the presence of the microbiota. Organ weights (heart, lung, and liver), cardiac output, and oxygen consumption are reduced in germ-free animals, whereas the intake of food is increased. This observation suggests that microbial colonization plays a role in body growth and development, as well as in energy harvesting from food. On the other hand, mucosal lymphoid tissues, lymph nodes, and serum gamma-globulin levels are reduced in germ-free animals, whereas susceptibility to infection is highly increased. This indicates that colonization is needed for a normal development of the immune system.

of non-digestible dietary substrates and endogenous mucus. Gene diversity among the microbial community provides a variety of enzymes and biochemical pathways that are distinct from the host's own constitutive resources. Fermentation of carbohydrates is a major source of energy in the colon for bacterial growth and produces short chain fatty acids that can be absorbed by the host. This results in salvage of dietary energy, and favors the absorption of ions (Ca, Mg, Fe) in the cecum. *Protective functions* of gut microbiota include the barrier effect that prevents invasion by pathogens. The resident bacteria represent a crucial line of resistance to colonization by exogenous microbes or opportunistic bacteria that are present in the gut, but their growth is restricted. The equilibrium between species of resident bacteria provides stability in the microbial population, but use of antibiotics can disrupt the balance (for instance, overgrowth of toxigenic *Clostridium difficile*). *Trophic functions* include the control of epithelial cell proliferation and differentiation as well as the homeostatic regulation of the

immune system. Cell differentiation is highly influenced by the interaction with resident microorganisms as shown by the expression of a variety of genes in germ-free animals mono-associated with specific bacterial strains (Hooper et al., 2001). Bacteria also play an essential role in the development of a healthy immune system. Animals bred in a germ-free environment show low densities of lymphoid cells in the gut mucosa and low levels of serum immunoglobulins. Exposure to commensal microbes, expands the number of mucosal lymphocytes rapidly and increases the size of germinal centers in lymphoid follicles (Yamanaka et al., 2003). Subsequently, immunoglobulin producing cells appear in the *lamina propria* and there is a significant increase in serum immunoglobulin levels. Most interestingly, recent researches suggest that some commensals play a major role in the induction of regulatory T cells in gut lymphoid follicles. Regulatory pathways mediated by regulatory T cells are essential homeostatic mechanisms by which the host can tolerate the massive burden of innocuous antigens within the gut or on other body surfaces without responding through inflammation (Guarner et al., 2006).

24.2.3 Dysfunction of the Gut Microbiota?

Several disease states or disorders have been associated with changes in the composition or function of the enteric microbiota (Othman et al., 2008). For instance, acute diarrhea is usually caused by pathogens that proliferate, invade, or produce toxins. Antibiotic-associated diarrhea is due to imbalance in the composition and structure of the gut microbiota with overgrowth of pathogenic species, such as certain *Clostridium difficile* strains that produce toxins and may cause colitis of variable severity. It is believed that gut bacteria play a role in the pathogenesis of the irritable bowel syndrome (IBS) (Othman et al., 2008). Symptoms of abdominal pain, bloating, and flatulence may be related with excessive production of gas by fermentations taking place in the colon. Likewise, putrefaction of proteins by bacteria within the gut lumen is associated with the pathogenesis of hepatic encephalopathy in patients with chronic or acute liver failure.

Translocation of viable or dead bacteria in minute amounts constitutes a physiologically important boost to the immune system. However, dysfunction of the gut mucosal barrier may result in translocation of a conspicuous quantity of viable microorganisms, usually belonging to gram-negative aerobic genera. After crossing the epithelial barrier, bacteria may travel via the lymph to

extraintestinal sites, such as the mesenteric lymph nodes, liver, and spleen. Subsequently, enteric bacteria may disseminate throughout the body producing sepsis, shock, multisystem organ failure, or death of the host (Clark and Coopersmith, 2007). Bacterial translocation may occur in hemorrhagic shock, burn injury, trauma, intestinal ischemia, intestinal obstruction, major abdominal surgery, severe pancreatitis, acute liver failure, and cirrhosis (Tandon et al., 2008). As described later in this chapter, there is evidence implicating the gut microbiota as an essential factor in driving the inflammatory process in human inflammatory bowel diseases (IBD) (Sartor, 2008). Studies in Crohn's disease or ulcerative colitis have shown that abnormal activation of the mucosal immune system against enteric bacteria is the key event triggering inflammatory mechanisms that lead to intestinal injury. Patients show an increased mucosal secretion of IgG antibodies against commensal bacteria, and mucosal T-lymphocytes are hyper reactive against antigens of the common microbiota, suggesting that the local tolerance mechanisms are abrogated. Several factors may contribute to the pathogenesis of the aberrant immune response towards the indigenous microbiota, including genetic susceptibility, a defect in mucosal barrier function, and a microbial imbalance. Several studies suggest that gut bacteria populations in patients with chronic IBD differ from that in healthy subjects (Guarner, 2005).

In experimental models, it has been shown that intestinal bacteria may play a role in the initiation of colon cancer through production of carcinogens, co-carcinogens, or pro-carcinogens. The molecular genetic mechanisms of human colorectal cancer are well established, but epidemiological evidence suggests that environmental factors such as diet play a major role in the development of sporadic colon cancer. Dietary fat and high consumption of red meat, particularly processed meat, are associated with high risk in case-control studies. In contrast, a high intake of fruits and vegetables, whole grain cereals, fish, and calcium has been associated with reduced risk. Dietary factors and genetic factors interact in part via events taking place in the lumen of the large bowel (Rafter et al., 2004). The influence of diet on the carcinogenic process may be mediated by changes in metabolic activity and composition of the colonic microbiota.

24.3 Section 2: Therapeutic Use of Probiotics

Human and experimental studies with probiotics have targeted specific health benefits associated with the three functional areas of the gut microbiota (metabolic effects, protective effects, and trophic effects), and worldwide research on

the topic of use of probiotics in human and animal health has accelerated in recent years. The scientific bases of probiosis have been reviewed in depth by a task force of the American Academy of Microbiology (Walker et al., Probiotic microbes: the scientific basis. American Academy of Microbiology. http://www.asm.org/Academy/index.asp). In most human studies published so far, probiotics were administered alive either by oral route (as a food component or in the form of specific preparations of viable microorganisms) or in topical preparations (skin, nasal, or vaginal applications). The use of probiotics in human medicine is gradually growing. In July 2008, the Cochrane Central Register of Controlled Trials listed 435 human studies that had tested probiotic efficacy. The Cochrane Database of Systematic Reviews included 12 complete reviews on the use of probiotics in medicine.

24.3.1 Probiotics in Gastroenterology

A major area for probiotic applications has been the prevention or treatment of gastroenterological diseases. There is ample evidence to support the efficacy of some probiotic strains in several acute gastroenterological disorders. Many of these indications are already accepted in clinical practice. Recently, the World Gastroenterology Organization published a guideline on the current indications of probiotics or prebiotics in practical gastroenterology. Recommendations of the guideline are summarized in *Table 24.1*. Interestingly, the guideline reports the specific probiotic strains that have proven useful for each specific clinical indication. The use of probiotics for acute gastroenterological conditions is reviewed in depth in the other chapters of this book. Briefly, the major indications include prevention and treatment of acute diarrhea, treatment of lactose maldigestion, coadjuvant therapy for eradication of *Helicobacter pylori* infection, bacterial translocation, and necrotizing enterocolitis in pre-term infants. On the other hand, probiotic therapy for chronic gastroenterological disorders such as IBS, IBD, and colon cancer is still a matter of experimental and clinical research.

24.3.2 Acute Diarrhea

Probiotics are useful as treatment of acute infectious diarrhea in children. Different strains, including *Lactobacillus reuteri*, *L. rhamnosus strain* GG, *L. acidophilus*, and the yeast *Sacharomyces cerevisae* (boulardii) (*Table 24.1*), have been tested

in controlled clinical trials and were proven useful in reducing the severity and duration of diarrhea. Several meta-analyses of controlled clinical trials have been published, as well as a systematic review from the Cochrane Centre (Allen et al., 2004).

Table 24.1

Evidence-based indications of probiotics in gastroenterology (*Cont'd* p. 958)

Disorder	Product
Treatment of acute infectious diarrhea in children	*L. rhamnosus* GG
	L. reuteri ATTC 55730
	L. acidophilus + *B. infantis* (Infloran strains)
	S. cerevisiae (boulardii) lyo
Treatment of acute infectious diarrhea in adults	*Enterococcus faecium* LAB SF68
Prevention of antibiotic associated diarrhea in children	*S. cerevisiae* (boulardii) lyo
	L. rhamnosus GG
	Bacillus lactis Bb12 + *S. thermophilus*
Prevention of antibiotic associated diarrhea in adults	*Enterococcus faecium* LAB SF68
	S. cerevisiae (boulardii) lyo
	L. rhamnosus GG
	L. casei DN-114 001in fermented milk with *L. bulgaricus* + *S.thermophilus*
	Bacillus clausii (Enterogermina strains)
	L. acidophilus CL1285 + *L. casei* Lbc80r
Prevention of nosocomial diarrhea in children	*L. rhamnosus* GG
	B. lactis BB12 + *S. thermophilus*
	B. lactis BB12
	L. reuteri ATTC 55730
Prevention of *C. difficile* diarrhea in adults	*L. casei* DN-114 001 in fermented milk with *L. bulgaricus* + *S.thermophilus*
	L. acidophilus + *B. bifidum* (Cultech strains)
	S. cerevisiae (boulardii) lyo
Coadjuvant therapy for *H. pylori* eradication	*L. rhamnosus* GG
	Bacillus clausii (Enterogermina strains)
	AB yoghurt with unspecified lactobacilli and bifidobacteria
	S. cerevisiae (boulardii) lyo
	L. casei DN-114 001 in fermented milk with *L. bulgaricus* + *S.thermophilus*

Table 24.1

Disorder	Product
Reduces symptoms associated with lactose maldigestion	Regular yogurt with *L. bulgaricus* + *S.thermophilus*
Alleviates some symptoms of irritable bowel syndrome	*B. infantis* 35624
	L. rhamnosus GG
	VSL# 3 mixture
	L. rhamnosus GG, *L. rhamnosus* LC705,*B. breve* Bb99 and *Propionibacterium freudenreichii* subsp. *shermanii*
	B. animalis DN-173 010 in fermented milk with *L. bulgaricus* + *S.thermophilus*
Maintenance of remission of ulcerative colitis	*E. coli* Nissle 1917
Prevention and maintenance of remission in pouchitis	VSL# 3 mixture of 8 strains (1 *S. thermophilus*, 4 *Lactobacillus*, 3 *Bifidobacterium*)
Prevention of necrotizing enterocolitis in preterm infants	*B. infantis, S. thermophilus* and *B. bifidum*
	L. acidophilus + *B. infantis* (Infloran strains)
Prevention of post-operative infections	Synbiotic 2000: 4 bacteria strains and fibers including the prebiotic inulin

Data extracted from the Global Guideline "Probiotics and Prebiotics in Gastroenterology," published on-line by the World Gastroenterology Organization (http://www.worldgastroenterology.org)

A large number of clinical trials have tested the efficacy of probiotics in the prevention of acute diarrheal conditions, including antibiotic-associated diarrhea, nosocomial and community acquired infectious enteritis, and traveler's diarrhea (Sazawal et al., 2006). A variety of different types of probiotics show promise as effective therapy for the prevention of antibiotic associated diarrhea (*Table 24.1*). Several published meta-analysis of controlled trials and the Cochrane systematic review conclude that some probiotics can be used to prevent antibiotic-associated diarrhea in children and adults (Johnston et al., 2007).

Prophylactic use of probiotics has proven useful for the prevention of acute diarrhea in infants admitted into the hospital ward for a chronic disease condition. Probiotics may also be useful in the prevention of community acquired diarrhea, but there is lack of data from community based trials evaluating the effect on acute diarrhea unrelated to antibiotic usage (Sazawal et al., 2006).

Several studies have investigated the efficacy of probiotics in the prevention of traveler's diarrhea in adults, but methodological deficiencies, such as low compliance with the treatment and problems in the follow-up, limit the validity

of their conclusions. A recent meta-analysis suggests that probiotics may offer a safe and effective method to prevent traveler's diarrhea (McFarland, 2007).

The efficacy of probiotics in the prevention of acute diarrhea induced by radiation in cancer patients has been tested in placebo-controlled, double-blind studies. The probiotic mixture VSL#3 and the *L. casei* DN-114 001 strain significantly reduced the severity of radiation-induced diarrhea (Delia et al., 2007; Giralt et al., 2008).

24.3.3 Lactose Maldigestion

Lactose malabsorption is the result of lactase deficiency in brush border epithelial cells of the small intestine. Due to this deficiency, a fraction of the ingested lactose is not absorbed in the small intestine. Patients may develop gastrointestinal symptoms such as diarrhea, flatulence, abdominal bloating, and pain after ingestion of lactose containing food. Prevalence of lactase deficiency in adult populations is relatively high, and varies between 5 and 15% in Northern European and American countries and 50–100% in African, Asian, and South American countries. These subjects tend to eliminate milk and dairy products from their diet and their calcium intake may be compromised. The bacteria used as starter culture in yogurt (*Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*) can improve lactose digestion and eliminate symptoms in lactase deficient individuals. A number of controlled studies have demonstrated lactose digestion and absorption as well as reduction of gastrointestinal symptoms in lactase deficient individuals consuming yogurt with live cultures (Gill and Guarner, 2004).

24.3.4 Helicobacter Pylori Infection

Probiotics have been tested as a strategy for eradication of *Helicobacter pylori* infection of the gastric mucosa. Some strains of lactic acid bacteria are known to inhibit *in vitro* the growth of *Helicobacter pylori*. However, administration of one of these strains in a specially designed yogurt was not effective for the eradication of *H. pylori* infection (Wendakoon et al., 2002). On the other hand, several clinical studies have tested the efficacy of different probiotic strains in combination with the standard therapy of antibiotics. Adding probiotics can increase eradication rates after triple or quadruple anti-*H. pylori* antibiotic regimes

(Tong et al., 2007). Several lactobacilli and bifidobacteria, as well as *Bacillus clausii,* appear to reduce the side-effects of the antibiotic therapy and improve compliance. A recent meta-analysis of 14 randomized trials suggests that supplementation with probiotics can be effective in increasing eradication rates and may be considered helpful for patients with eradication failure (Tong et al., 2007).

24.3.5 Bacterial Translocation

Bacterial translocation of gut bacteria can produce systemic infections and sepsis. These complications have been shown to occur in some pathologic conditions associated with mucosal barrier dysfunction such as postoperative sepsis, severe acute pancreatitis, multisystem organ failure, necrotizing enterocolitis, etc. A randomized study involving 95 liver transplant patients compared the incidence of infections among three groups of patients submitted to different prophylaxis procedure: selective bowel decontamination with antibiotics, administration of live *L. plantarum* supplemented with fermentable fiber (as a synbiotic), and administration of heat-killed *L. plantarum* with the fiber supplement (Rayes et al., 2002). Post-operative infections were recorded in 15 out of 32 patients (48%) in the antibiotics group, 4 out of 31 (13%) in the live *L. plantarum* group, and 11 out of 32 (34%) in the heat-killed *L. plantarum* group; the difference between the antibiotics and live *L. plantarum* groups was significant. In a second study by the same group, patients were randomized to receive a synbiotic preparation (including four probiotic strains, and four fermentable fibers) or a placebo consisting only of the four fibers (Rayes et al., 2005). Post-operative infection occurred in only one patient in the treatment group (n = 33), in contrast to 17 out of 33 in the placebo group. The difference was highly significant. However, another clinical study performed with patients submitted to elective abdominal surgery found no effect of synbiotic treatment (4 bacteria strains plus oligofructose) on prevention of post-operative infections (Anderson et al., 2004). In this trial, synbiotic treatment after surgery was delayed until patients were able to tolerate oral nutrition. In contrast, the liver transplant studies introduced synbiotic therapy by naso-gastric tube immediately after surgery. Another clinical study in surgical patients has recently confirmed that pre- and post-operative enteral feeding with a synbiotic preparation is significantly more effective in preventing infectious complications that post-operative synbiotic treatment only (Sugawara et al., 2006). These data suggest that early

administration of live probiotics with prebiotics may become a useful and effective therapy to prevent post-operative infections.

24.3.6 Necrotizing Enterocolitis

Necrotizing enterocolitis is a severe clinical condition that may occur in low birth weight neonates due to immaturity and dysfunction of the gut mucosal barrier. Several controlled studies have demonstrated that the use of probiotic mixtures in low birth weight infants significantly reduces the incidence and severity of necrotizing enterocolitis and may also prevent mortality by this critical condition. Meta-analysis of the published trials suggests that probiotics reduce the risk of developing necrotizing enterocolitis by two thirds and risk of death by one half (Deshpande et al., 2007). These data are impressive since very few other strategies have proven effective in decreasing the incidence of necrotizing enterocolitis in preterm infants.

24.3.7 Irritable Bowel Syndrome

Fermentations taking place in the colon generate a variable volume of gas. However, some gut bacteria degrade metabolic substrates without producing gas, and even some other species may consume gas, particularly hydrogen. Symptoms of abdominal pain, bloating, and flatulence are commonly seen in patients with IBS. Hypothetically, administration of appropriate bacteria strains could reduce gas accumulation within the bowel in these patients and induce symptomatic improvement.

Table 24.2 shows data from double-blind controlled clinical trials testing probiotics in patients with IBS. In most studies, both probiotic and placebo treatment decreased the scores of abdominal pain up to some extent. This is a common observation in trials with IBS patients, which respond to placebo at variable rates. However, several studies have shown significant therapeutic gain of probiotics over placebo assessed by increased rate of responders to treatment or increased relief in symptom scores. A consistent finding of the published studies is the reduction of abdominal bloating and flatulence by probiotic treatment. Studies using *Bifidobacterium* strains appear to have higher rate of therapeutic success in adult IBS patients. In addition, a recent trial with 90 breastfed babies with infantile colic has shown that the probiotic *L. reuteri* may improve colicky

Table 24.2

Clinical studies testing efficacy of probiotic strains in the treatment of irritable bowel syndrome

Study	Test intervention	Duration (weeks)	Outcome			P
			Endpoint	Test	Control	
Nobaek et al. (2000)	*L. plantarum*	4	Pain response rate	36%	18%	NS
			Flatulence response rate	44%	18%	<0.05
Kim et al. (2003)	Probiotic mix: VSL#3	8	Overall response rate	33%	38%	NS
			Bloating score (3)	−13.7%	−1.7%	<0.05
Kajander et al. (2005)	Probiotic mix: LGG[a], *L. rhamnosus, Propionibacterium freudenreichii, and Bifidobacterium breve*	24	Symptom score (3)	−42%	−6%	<0.05
O'Mahony et al. (2005)	Two intervention arms: *Lactobacillus salivarius (LS)* and *Bifidobacterium infantis (BI)*	8	Symptom score (3)	LS: −13%	−16%	NS
				BI: −46%		<0.05
Niv et al. (2005)	*L. reuteri*	24	Symptom score (3)	improved	improved	NS
Bausserman and Michail (2005) (1)	LGG[a]	6	Overall response rate	44%	40%	NS
Kim et al. (2005)	Probiotic mix: VSL#3	8	Flatulence score (3)	−21%	−9%	<0.05
Whorwell et al. (2006) (2)	*B. infantis*	4	Pain score (3)	−89%	−58%	<0.05
Gawronska et al. (2007) (1)	LGG[a]	4	Overall response rate	33%	5%	<0.05
Guyonnet et al. (2007)	*Bifidobacterium animalis*	6	QoL response rate	65%	48%	<0.05

[a]*LGG*, *Lactobacillus rhamnosus* GG

(1) Children; (2) Women; (3) change versus score at entry; *NS*, not significant; *QoL*, health-related quality of life

symptoms within 1 week of treatment (Savino et al., 2007). Disordered bowel habits (diarrhea, constipation, or alternating habit) are common in subjects with IBS, but efficacy of probiotics for improvement of bowel habit in IBS has not been proven consistently. Probiotics need to be evaluated further, but they do appear to be useful for the control of the symptoms related with the altered handling or perception of intestinal gas.

24.3.8 Inflammatory Bowel Diseases

The IBD, Crohn's disease, ulcerative colitis, and pouchitis are chronic conditions of unknown etiology characterized by persistent mucosal inflammation at different levels of the gastrointestinal tract. Typically, these diseases exhibit undulating activity with bouts of uncontrolled, chronic mucosal inflammation, followed by remodeling processes that occur during periods of remission. The precise etiologies of these chronic inflammatory conditions remain to be elucidated and therefore, the available medical therapies can only control up to some extent the eruptions of disease activity, but fail completely to eradicate or permanently cure such diseases. However, the pathophysiological mechanisms that lead to the mucosal inflammatory lesions have been unveiled at least in part during the past few years. These mechanisms result from complex interactions of environmental, genetic, and immunoregulatory factors.

Infectious diseases are produced by specific microbial agents that possess the capacity of transmitting the disease to susceptible individuals. An infectious origin of Crohn's disease or ulcerative colitis is not supported by such criterion since transmission of these diseases has never been documented. However, there is a substantial body of evidence implicating the enteric bacteria in the pathogenesis of both Crohn's disease and ulcerative colitis (Sartor, 2008). Luminal bacteria appear to provide the stimulus for immuno-inflammatory responses leading to mucosal injury. In Crohn's disease, fecal stream diversion reduces inflammation and induces mucosal healing in the excluded intestinal segment, whereas infusion of intestinal contents quickly reactivates the disease (D'Haens et al., 1998). In ulcerative colitis, short-term treatment with an enteric-coated preparation of broad-spectrum antibiotics rapidly reduced metabolic activity of the flora and mucosal inflammation (Casellas et al., 1998). The presence of bacteria within the intestinal lumen is the critical condition that triggers mucosal inflammation in IBD.

Currently, two broad hypotheses have arisen regarding the fundamental nature of the pathogenesis of IBD (Strober et al., 2007). The first argues that primary dysregulation of the mucosal immune system leads to excessive immunologic responses to normal microbiota. The second suggests that the changes in the composition of gut microbiota and/or deranged epithelial barrier function elicit pathologic responses from the normal mucosal immune system (*Figure 24.3*). In either case, abnormal communication between gut microbial communities and the mucosal immune system is being incriminated as the core defect leading to IBD in genetically susceptible individuals.

The normal mucosal defense is based mainly on the production of IgA antibodies that are secreted to the gut lumen and neutralize microbes in the lumen, thus avoiding mucosal inflammation. In IBD, however, mucosal production of IgG antibodies against intestinal bacteria is highly increased, and mucosal defense relies on both IgG mediated responses within the tissue and hyperactivated lymphocytes in the lamina propria reacting against bacterial antigens (Macpherson et al., 1996). These events result in inflammation and tissue injury. The altered immune response is not specifically addressed or polarized towards a single group of potential pathogens, but involves a large and undefined number

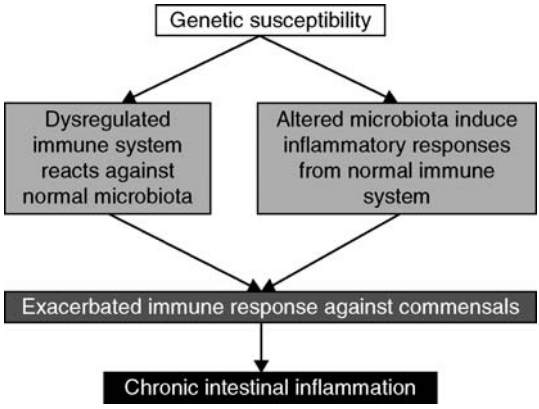

Figure 24.3

In patients with IBD, including Crohn's disease, ulcerative colitis and chronic pouchitis, mucosal lesions are caused by an aggressive immune response against gut bacteria. Most recent hypothesis suggests that either primary dysregulation of the mucosal immune system leads to excessive immunologic responses to normal microbiota, or changes in the composition of gut microbiota elicit pathologic responses from the normal mucosal immune system. Genetic susceptibility in combination with abnormal communication between gut microbial communities and the mucosal immune system is being incriminated as the core defect leading to chronic intestinal inflammation.

of commensal species belonging to the common enteric microbiota. As mentioned, a microbial imbalance in the gut ecosystem could explain the abnormal reactivity of the mucosal immune system against enteric bacteria.

24.3.9 The Gut Microbiota in IBD

Several studies have shown that the composition of the fecal microbiota differs between subjects with IBD and healthy controls (Guarner, 2005). Molecular studies show that a substantial proportion of fecal bacteria (30–40% of dominant species) in patients with active Crohn's disease or ulcerative colitis belong to phylogenetic groups that are unusual in healthy subjects. These remarkable changes could be secondary to disease activity but they are not observed in patients with infectious diarrhea.

On the other hand, studies have shown reduced diversity of bacterial species in both fecal and mucosa-associated communities in patients with IBD (Frank and Pace, 2008; Manichanh et al., 2006; Ott et al., 2004). Manichanh et al. (2006) employed a metagenomic approach for exhaustive investigation of bacterial diversity in Crohn's disease and found a striking reduction of Firmicutes in patients in remission as compared to healthy controls. Reduction in bacterial diversity has also been documented in ulcerative colitis patients. In addition, a recent study in individuals with quiescent ulcerative colitis followed during 1 year observed that the composition of the microbiota was highly variable over time (Martinez et al., 2008). Temporal instability in the microbiota may be a consequence of low biodiversity and suggests that the intestinal ecosystem in IBD may be more susceptible to environmental influence. These findings might explain why IBD patients have a high proportion of bacteria that are unusual in healthy subjects.

Other interesting studies have focused on the mucosa-associated microbiota. High concentrations of mucosal adherent bacteria have been observed in patients with clinically active disease, either ulcerative colitis or Crohn's disease. No significant differences were found between the two clinical entities, but the concentrations of mucosal bacteria increased progressively with the severity of mucosal inflammation. Bacteria identified by the molecular probes were commensal commonly present in feces. Most studies have shown a reduced presence of common anaerobes with low pathogenic potential, such as some *Bacteroide*s species, and an increased presence of aerobic enterobacteria able to invade. The fluorescent *in situ* hybridization (FISH) technique demonstrated bacterial

invasion of the mucosa in most colonic mucosa specimens from ulcerative colitis and Crohn's disease patients, but not in any of the mucosal specimens from controls (Kleessen et al., 2002). Invading bacteria in ulcerative colitis mucosa belonged to various genera, including *Proteobacteria, Enterobacteriaceae, Bacteroides/Prevotella* cluster, *Clostridium,* and sulfate-reducing bacteria. Crohn's disease mucosal samples harbored mainly *Proteobacteria, Enterobacteriaceae*, and *Bacteroides* species. Mucosal invasion by *Bifidobacterium* or *Lactobacillus* species was not detected. In another study, Macfarlane et al. (2004) investigated bacterial colonization in rectal biopsies from ulcerative colitis patients by culture-based methodologies and FISH. The authors observed that bifidobacteria were present in considerably low numbers in patients as compared to controls.

The demonstration of mucosal invasion by bacteria may explain why the immune system responds with high titers of IgG antibodies against bacterial antigens, as shown by several studies in IBD patients. Altered composition and/or structure of the gut microbiota would be the main environmental factor triggering immuno-inflammatory responses in individuals with genetic susceptibility for IBD.

24.3.10 Probiotics in IBD

Within the gastrointestinal tract, the inflammatory capacity of commensal bacteria is varied. Some resident bacteria are pro-inflammatory, whereas others attenuate inflammatory responses. In experimental settings, some commensal non-pathogenic bacteria activate mucosal immuno-inflammatory responses in mucosal specimens from Crohn's disease patients, whereas a probiotic *Lactobacillus* strain is able to down-regulate inflammation (Borruel, 2002). Thus, a favorable local microecology could restore the homeostasis of the mucosal immune system and lead to the resolution of the lesions.

Several probiotic strains have been tested in controlled clinical trials with patients with inflammatory bowel diseases, including ulcerative colitis, Crohn's disease, and pouchitis. In ulcerative colitis, three randomized controlled trials investigated the effectiveness of an orally administered enteric-coated preparation of viable *Escherichia coli* strain Nissle 1917 as compared with mesalazine, the standard treatment for maintenance of remission (› *Table 24.3*). These studies concluded that this strain has an equivalent effect to mesalazine in maintaining remission. Zocco et al. (2006) randomized 187 patients to three open-label arms (*Lactobacillus rhamnosus* strain GG, *L.* GG plus mesalazine, and mesalazine only)

Table 24.3

Clinical studies testing efficacy of probiotic strains in maintenance of remission in ulcerative colitis

Study	Intervention		Duration (months)	Relapse rate (%)		
	Test	Control		Test	Control	P
Kruis et al. (1997)	*E. coli* Nissle	Mesalazine	3	14	12	NS
Rembacken et al. (1999)	*E. coli* Nissle	Mesalazine	12	33	27	NS
Ishikawa et al. (2003)	*B. breve, B. bifidum, L. acidophillus*	None	12	27	90	<0.01
Kruis et al. (2004)	*E. coli* Nissle	Mesalazine	12	36	34	SE
Zocco et al. (2006)	LGG	Mesalazine	12	15	20	NS
	LGG + Mesalazine			17		NS

NS, not significant; *SE*, significant equivalence; *LGG*, *Lactobacillus rhamnosus* GG

and demonstrated that the two *Lactobacillus* arms experienced a prolonged relapse-free, similar to that achieved only with mesalazine treatment. Finally, a study with 21 ulcerative colitis patients in remission and on standard medication (mesalazine and others) tested a *Bifidobacterium* fermented milk in a non-blinded design. Those receiving supplements of bifidobacteria had fewer relapses during the 12 month follow-up period (Ishikawa et al., 2003). Probiotic therapies may have equivalent efficacy than the conventional drug therapies for maintenance of remission in ulcerative colitis. In contrast, the efficacy of probiotics to treat active ulcerative colitis has not been demonstrated, although two small pilot studies suggested that some *Bifidobacterium* strains added to standard drug therapy may be beneficial for the treatment of mild to moderately active disease (Furrie et al., 2005; Kato et al., 2004).

The efficacy of *Lactobacillus rhamnosus* GG to maintain the remission of Crohn's disease was tested in a randomized, double blind trial with patients undergoing curative surgery (*Table 24.4*). The probiotic showed no effect in the prevention of clinical and endoscopic recurrence as compared with the placebo (Prantera et al., 2002). Two other clinical trials confirmed the lack of efficacy of this strain to maintain remission of Crohn's disease. Likewise, postoperative recurrence of Crohn's disease could not be prevented by *L. johnsonii* or a synbiotic preparation (*Table 24.4*). Two small studies have evaluated probiotics in patients with active Crohn's disease but neither demonstrated convincing

Table 24.4

Clinical studies testing efficacy of probiotic strains in maintenance of remission in Crohn's disease

Study	Intervention		Duration (months)	Relapse rate (%)		
	Test	Control		Test	Control	P
Prantera et al. (2002)	LGG	Placebo	12	16	11	NS
Schultz et al. (2004)	LGG	Placebo	6	60	67	NS
Bousvaros et al. (2005)	LGG	Placebo	24	31	17	NS
Marteau et al. (2006)	*L. johnsonii*	Placebo	6	49	64	NS
Van Gossum et al. (2007)	*L. johnsonii*	Placebo	3	15	14	NS
Chermesh et al. (2007)	Synbiotic *2000*	Placebo	24	25	20	NS

NS, not significant; LGG, *Lactobacillus rhahmnosus*, GG

efficacy. Thus, there is no evidence to support the use of probiotics as therapy of Crohn's disease.

The VSL#3 mixture is a commercial probiotic product that has been proven highly effective for maintenance of remission of chronic relapsing pouchitis, after induction of remission with antibiotics (Gionchetti et al., 2000; Mimura et al., 2004). The VSL#3 mixture is characterized by a high bacterial concentration (300 billion/g of live microorganism) and the presence of a cocktail of eight different bacterial species. In the first study, a relapse occurred in only 3 out of 20 patients of the VSL#3 group and in all the 20 patients of the placebo group. Interestingly, all patients on remission in the probiotic arm had relapses within 4 months after stopping probiotic treatment at conclusion of the trial. In the second study, the probiotic mixture was administered in a once-a-day schedule, and similar efficacy was demonstrated (Mimura et al., 2004). Treatment with VSL#3 was also effective in the prevention of the onset of pouchitis after ileal pouch-anal anastomosis (Gionchetti et al., 2003). In contrast, *L. rhamnosus* GG was ineffective as a primary therapy for the induction of a clinical or endoscopic response in patients with chronic pouchitis (Kuisma et al., 2003).

The therapeutic manipulation of the luminal microecology with probiotics and prebiotics has attracted high expectation as a strategic area for the control and prevention of inflammatory bowel diseases. The evidence for the use of probiotics, and in particular the probiotic mixture VSL#3, in inflammatory bowel disease is the strongest in the case of pouchitis. In addition, the *E. coli* Nissle strain appears to be equivalent to mesalazine in maintaining remission of ulcerative colitis. However, studies of probiotics in Crohn's disease have been disappointing, and the Cochrane systematic review concluded that currently

there is no evidence to suggest that probiotics are beneficial for the maintenance of remission in Crohn's disease (Rolfe et al., 2006). Further research is needed to optimize the use of probiotics or prebiotics for these indications.

24.3.11 Food Allergy

Prevalence of allergic diseases in western societies is increasing at an alarming rate whereas it is much lower in the developing World. It has been suggested that this may be the result of inappropriate microbial stimulus during infancy due to improved hygienic conditions. Some epidemiological and experimental studies have indicated that stimulation of the immune system by certain microbes or microbial products may be effective in the prevention and management of allergic diseases. The effectiveness of probiotic therapy in the prevention of atopic eczema has been clearly demonstrated in randomized controlled trials (Kalliomaki et al., 2001). Likewise, well designed studies have provided evidence that specific strains of probiotics can be effective for treatment of a subset of patients with atopic eczema (Viljanen et al., 2005). A single study has reported efficacy of a formula supplemented with *Lactobacillus* GG in infants with cows' milk allergy (Majamaa and Isolauri, 1997). Infants receiving the probiotic showed significant improvement in both clinical symptoms and markers of intestinal inflammation compared with the placebo group. However, little is known about the efficacy of probiotics in preventing other types of food allergy.

24.3.12 Colon Cancer

Experimental studies clearly demonstrate a protective effect of probiotics, such as certain *Lactobacillus* and *Bifidobacterium* strains, or of the combination of prebiotics and probiotics, against colon cancer. They can prevent the establishment, growth, and metastasis of transplantable and chemically induced tumors. Several possible mechanisms of protection have been identified (Rafter et al., 2004).

Human intervention trials, trying to corroborate the evidence obtained in experimental studies, have intrinsic difficulties due to the natural history of the disease (difficulty in selection of subjects at high risk and requirement of long-term follow-up). Such studies require the validation of intermediate endpoints that can be used as valid biomarkers of colon cancer risk (Rafter et al., 2004). A Japanese study including 398 subjects with previous history of colonic polyp

followed for 4 years found that oral administration of *L. casei* reduced the degree of atypia in the recurrent polyps (Ishikawa et al., 2005). The SYNCAN study is another human intervention trial, which tested a synbiotic (oligofructose plus two probiotic strains) in patients at risk of developing colonic cancer and looked at intermediate endpoints that were used as biomarkers of cancer risk. This study found a reduction in epithelial cell proliferation in subjects on treatment with the synbiotic preparation, which was not observed in subjects taking placebo (Rafter et al., 2007). Likewise, genotoxicity assays of colonic biopsy samples indicated a decreased exposure to genotoxins in the synbiotic treated subjects. Epithelial cell hyperproliferation and high levels of gene damage in epithelial cells are recognized as markers of risk for colon cancer. Thus, the Rafter study suggests that the synbiotic preparation can decrease the expression of biomarkers of colorectal cancer. Further clinical studies are needed to translate the experimental findings to practical human applications.

24.4 Summary

- The human gut harbors large and diverse microbial communities which influence both the physiology and pathology of the host.
- Microbial ecology in the gut can be modulated by pharmacological and nutritional intervention with probiotics and prebiotics. A balanced microbial environment would likely promote symbiotic functions of gut microbes and enhance the health of host.
- In controlled human studies, probiotics have been used safely and successfully for improving metabolic functions of the microbiota (lactose digestion, reduction of gas related symptoms in IBS), protecting against infections (acute infectious diarrhea, bacterial translocation), and modulating inflammation (maintenance therapy in ulcerative colitis and pouchitis).
- Clinical research is currently focused at improving efficacy of probiotics in the prevention and control of chronic disorders of unknown etiology, such as IBD and colon cancer.
- The development of improved tools to investigate gut colonization will provide a more complete picture of the actual scenario in inflammatory diseases and colon cancer.
- Future interventional approaches should aim toward the accurate modulation of microbial communities with proven anti-inflammatory or anti-carcinogenic properties.

List of Abbreviations

FAO	Food and Agriculture Organization
FISH	fluorescence *in situ* hybridization
IBD	inflammatory bowel diseases
IBS	irritable bowel syndrome
IgG, IgA	immunoglobulin G, immunoglobulin A
NS	not significant
QoL	quality of life
WHO	World Health Organization

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25 Probiotics and Allergy

Seppo Salminen · Erika Isolauri

25.1 Introduction

The atopic diseases include atopic eczema, allergic rhinitis and asthma. The prevalence of atopic diseases has been on the rise for several decades, particularly in high-income industrialized nations (ISAAC, 1998; Sibbald et al., 1990). As potential explanations for the increased prevalence of atopic diseases the so-called hygiene hypothesis has been suggested. The hypothesis suggests that the continuously increased hygiene in the environment and the food supply results to reduced exposure to a variety of microbes and consequently to a less diverse intestinal microbiota from early life onwards and/or to changes in the gut microbiota from early life. Such changes reflect the exposure to microbes in industrially processed foods and the improved hygiene of the living environment of the mother and infant.

A cross-sectional study evaluated randomly selected people born before, during and after the World War II, thus exposed to markedly different diets and living conditions in their infancy (Isolauri et al., 2004). The latter is considered a critical period for the consolidation of the immune responder type. The prevalence of the atopic phenotype was shown to have steadily become more common over a long period of time. Moreover, increasingly prevalent sensitization to aeroallergens, but not to dietary allergens was found, suggesting that allergy may represent a constellation of diseases which respond differently to various environmental changes. Also, self-reported symptoms of atopic disease combined with relevant objective measurements, reactivity in skin prick tests, or concentrations of specific IgE and IgG4 antibodies in serum, have consistently suggested a true increase in the prevalence. Furthermore, there is evidence that the farming environment may have a protective effect on children when compared with the living city environment (Riedler et al., 2001).

Reversing the progressive increase in atopic diseases and specifically food associated reactions such as atopic eczema would represent an important breakthrough for health care and well-being in Western societies. However, the recent

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efforts to reduce their risk show unanimously that the understanding of the development of atopic diseases and of possible causes of the epidemic is far from being complete. It is likely that there are distinct etiological factors and pathogenetic mechanisms underlying the heterogeneous manifestations of atopic disease, such as asthma and atopic eczema which although are frequently defined as single conditions, they may include several phenotypes (Isolauri et al., 2002; Salminen and Isolauri, 2006).

Previous strategies of prevention through allergen avoidance during pregnancy, breast-feeding, and infancy have more recently been called into question. Alternative strategies are urgently required with respect to food allergy. These include among others the use of probiotics, and prebiotics, targeting the prevention and treatment of allergic reactions.

The research with probiotics has been intensive, but detailed insight is required to focus into the potential critical windows when probiotics should be administered, the mechanisms of how they impact their effect and the environmental elements which might carry anti-allergenic potential. Such knowledge will help to assess current therapies and to develop novel preventive therapies for the future. In order for satisfactory preventive measures to be put in practice, better genetic and molecular characterization of atopic eczema is required.

25.2 Gut Microbiota and the Hygiene Hypothesis

The hygiene hypothesis conceives the increase in the prevalence of atopic disease to be related to reduced exposure to microbes at an early age as a result of modern public health practices such as improved sanitation and living conditions, vaccinations and antimicrobial therapy, together with decreasing family size (Strachan, 1989; Yazdanbakhsh et al., 2002). The same progression is seen in human nutrition. A shift in food preservation from drying and natural fermentation to industrial processes including heat treatment and even radiation treatment has taken place during the last century. Thus, heat treatments such as pasteurization, UHT-treatment and sterilization of most foods and notably milk have significantly reduced the microbial exposure associated with food intake. Farm milk has almost completely disappeared and due to varying pasteurization or heat-treatment procedures only minor amount of bacteria are present in the milks currently available (Isolauri, 2001; Vicini et al., 2008).

People living in high-income hygienic environments lack the pressure of microbial stimulation from food and environment although there may be

differences for instance between city and farm environments and their impact (Riedler et al., 2001). According to the hygiene hypothesis, this stimulation is required to hinder the consolidation of the atopic Th2-skewed responder type which has been shown to be crucial for the maintenance of successful pregnancy and prevails at birth and during the first months of life.

Indeed, the earliest and most massive source of microbial exposure is associated with the acquisition of the gut microbiota, which still remains an unexplored organ of host defense. Indeed, the initial compositional development of the gut microbiota is considered a key determinant in the development of normal gut barrier functions and healthy host-microbe interactions (Grönlund et al., 2008; Gueimonde et al., 2007; Isolauri et al., 2002). The establishment of the early indigenous microbiota has a particularly strong impact on the immunophysiological regulation in the gut. In addition, it has been suggested that constant stimulation of the intestinal immune system by the diverse indigenous intestinal microbiota may outweigh the importance of occasional infections. This process leads to the repeated immunological provocation for the maturation of the host immune defense. It has been suggested that the immune priming may be essential particularly for the gut-associated immune system to perform well the two opposing functions: mounting a brisk response to pathogens and maintaining hyporesponsiveness to innocuous antigens.

25.3 The Establishment of the Gut Microbiota

The development of gut microbiota is a step-wise well controlled process. The current understanding has underlined the hypothesis that microbial colonization begins immediately following birth, and initially, facultative anaerobic strains dominate. However, there is evidence that the first exposure to microbes may take place earlier (Martin et al., 2003; Satokari et al., 2008). The major inoculum may be provided during the birth process by the contact with the mother's microbes which form the basis for microbiota development. Thereafter, lactic acid bacteria and coliforms become the predominant microorganisms, and bifidobacteria form the great majority of the gut microbiota of the healthy breast-fed child (Salminen et al., 1998; Favier et al., 2002). After weaning, the type of diet determines the relative distribution of species. Breastfeeding encourages the growth of bifidobacteria, while formula-fed infants have a more complex microbiota which includes bifidobacteria, bacteroides, clostridia and streptococci (Grönlund et al., 2008; Salminen and Gueimonde, 2005). Molecular methods indicate,

however, that the greatest difference in the microbiota of breast fed and formula fed infant lies in the bifidobacterial composition, while the lactic acid bacteria composition appears to be rather similar (Favier et al., 2002). *Bifidobacterium longum, Bifidobacterium infantis* and *Bifidobacterium breve* are frequently found in fecal samples of breast fed infants, whereas the most common lactobacilli in breast-fed and formula fed infant feces constitute *Lactobacillus acidophilus* group microorganisms, such as *L. acidophilus*, *L. gasseri* and *L. johnsonii*. After weaning an adult-type gut microbiota is rapidly reached (Salminen and Guiemonde, 2007). Healthy microbiota is defined as the normal microbiota appropriate of the age that both preserves and promotes wellbeing and absence of disease.

It is important to recognize certain scarcely studied areas of interest:

1. The transfer of microbiota from the mother to the infants
2. The impact of breast milk on the intestinal microbiota of the infant
3. The exchange of microbes between the mother and the breastfed infant by physical contact between them.

It has been recently demonstrated that there is significant transfer of lactic acid bacteria and bifidobacteria from the mother to the infant which impacts the microbiota composition and activity (Gueimonde et al., 2007; Grönlund et al., 2008). Thus, the microbiota of the mother is also of great interest for the health of the infant. Breast milk is never sterile but contains several bacteria and these bacteria are among the first colonizers of the gastrointestinal tract (Gueimonde et al., 2007; Grönlund et al., 2008). Among the common bacteria in breast-milk are bifidobacteria, which form the basis of infant gut microbiota. In addition, lactic acid bacteria and bacteria from the mother's skin have been reported in breast-milk. These bacteria significantly influence the early colonization process for the gut microbiota and later on facilitate the development of normal or aberrant microbiota with an impact on the child health.

25.4 Gut Microbiota Differences Predisposing to Atopic Diseases

Our report on the distinct patterns of neonatal gut microbiota in infants in whom atopic diseases was or was not developing has been the basis for the first prevention study with pre- and postnatal probiotic treatment (Kalliomäki et al., 2001a). This study still remains the one exploring gut microbiota composition

and aberrances in infants prior to any signs of atopic diseases were observed and comparing the deviations with later health outcomes. Microbiological analysis was conducted in infants ($n = 76$) at 3 weeks and 3 months after delivery using traditional culture methods, fluorescent *in situ* hybridization and gas chromatographic analysis of the bacterial cell wall fatty acids (Kalliomäki et al., 2001a, b). These children were then followed for 2 years, and then the diagnosis of atopic sensitization was confirmed using skin prick tests, and atopic eczema by clinical assessment and the general Hanifin criteria (Hanifin, 1984). After the 2 year clinical follow-up, the study was able to demonstrate, that differences in the neonatal gut microbiota precede the development of atopic diseases suggesting new modalities for early diagnosis of risk and possibilities for prevention. The predisposing differences were focused on early microbiota at 3 weeks of age and were demonstrated by significant differences in bacterial cellular fatty acid profiles. The numbers of clostridia and bifidobacteria in fecal samples indicated an excess of clostridia and decreased levels of bifidobacteria in those infants developing atopic disease later. Similar findings have also been reported to precede obesity development and this may indicate another source of early low-grade inflammatory condition (Kalliomäki et al., 2001a, b, 2008). This information is quite distinct from the large number of reports indicating that infants and children already expressing atopic diseases have differences in gut microbiota.

It was later demonstrated that differences in the composition of the *Bifidobacterium* microbiota are present at the species level and that the bifidobacteria usually found mainly in adults, such as *Bifidobacterium adolescentis*, may be important. It has also been reported that allergic mothers transfer more the *B. adolescentis* type bifidobacteria to their infant than healthy mothers, perhaps increasing the risk of atopy development later due to differences in microbiota succession and its impact on immune development. Such development differs from the suggested indicators presented for example by researchers who conducted their analysis on young infants already diagnosed with eczema and not associated with early microbiota and potential risk potentiation (Gore et al., 2007). Recently, another nested case control study reported no differences in the microbiota of infants in whom atopic eczema was or was not developing (Penders et al., 2006). However, the diagnosis of the children with eczema differed from the cohort of the Kalliomäki et al. study and the Hanifin criteria were not used, but instead total and specific IgE in capillary blood was used with parent reported rash during the first year and thus, no clinical assessment of the eczema was conducted. Also the microbiota analysis methods were significantly different and the results are not comparable to the first report of Kalliomäki and coworkers (2001).

Therefore, it is still important to further characterize the differences in the microbiota composition and activity as these appear to be important factors that may initiate the programming for later disease development.

25.5 The Gut Microbiota as Source of Health Promoting Bacteria

The interactions between microbes and the host have recently prompted active research subsequent to the demonstration that the generation of immunophysiological regulation is mediated by a coordinated interaction between the innate and adaptive immune systems. The innate immune system is able to distinguish microbial antigens through molecules such as Toll-like receptors and CD14, which recognize conserved pathogen-associated molecular patterns, including unmethylated CpG motifs characteristic of bacterial DNA and the bacterial endotoxin lipopolysaccharide. Initial signals to counter interleukin (IL)-4 and thereby IgE generation, and IL-5 –associated eosinophilic inflammation may stem from components of the innate immunity.

The dendritic cell constitutes an essential factor in CD4 + T-cell priming and differentiation. The Th1-promoting potential of the healthy gastrointestinal microbiota is possibly associated with two structural components of bacteria, the lipopolysaccharide portion of gram-negative bacteria (endotoxin) and a specified CpG motif in bacterial DNA (Hartmann et al., 1999). Non-pathogenic microbes elicit an immunosuppressive effect on intestinal epithelial cells by inhibition of the transcription factor IkappaB/NFkappaB pathway thus demonstrating a direct prokaryotic anti-inflammatory effect (Neish et al., 2000). Accumulating evidence further indicates that the establishment and maintenance of intestinal and systemic tolerance may be dependent on suppressive cytokines such as IL-10 and transforming growth factor (TGF)-β produced by regulatory T cells. These cells actively control or suppress the function of other cells, including both Th1 and Th2 cells; indeed, TGF-β has been implicated in protection against Th1-type autoimmune disease (Fukaura et al., 1996) as well as Th2-mediated atopic disease. TGF-β and IL-10 appear to function in a synergistic fashion.

The role of the intestinal microbiota in oral tolerance induction, the unresponsiveness to non-pathogenic antigens encountered at the mucosal surface, was

investigated in germfree mice (Sudo et al., 1997). Animals kept in germ-free conditions exhibit impaired development of the intestinal immune system resulting in defective oral tolerance formation and atopic type immune responsiveness. Reconstitution of the intestinal microbiota with bifidobacteria, however, restores the susceptibility to oral tolerance induction, but only if performed at the neonatal age. Furthermore, lactobacilli have also been observed to suppress atopic immune responsiveness in both animal models and allergic humans. These results suggest that in affecting the development of gut-associated lymphoid tissue at an early age the gut microbiota directs the regulation of systemic and local immune responsiveness. On the other hand, it has been reported that susceptibility to nasal and oral tolerance induction to the major birch pollen allergens are not dependent on the presence of the microbiota (Repa et al., 2008).

The gut microbiota provides maturational signals for the gut-associated lymphoid tissue, particularly for the IgA plasma cells, conferring the first line of host immunological defense. Clinical studies following the microbiota development in vaginally born infants and in infants born by caesarean section showed major differences in the culturable microbiota. Colonization was associated with the maturation of humoral immune mechanisms, particularly of circulating IgA- and IgM-secreting cells (Grönlund et al., 2000).

25.6 Probiotics for Counteracting Microbiota and Immune Response Deviations

The term probiotic has been defined as living microorganisms which upon ingestion in certain numbers exert health benefits beyond inherent general nutrition (WHO, 2002). Also bacterial cells per se can have probiotic properties and today the viability is a key issue as we understand that the methodology for the viability assay may have to be further developed and changed (Salminen and Benno, 1998; Ouwehand and Salminen, 1998). The term probiotic requires that the health benefits and the safety of probiotic organisms have to be demonstrated in a scientific manner – otherwise the term is misused (WHO, 2002). Earlier probiotics were considered to exert their impact on host health by modulating the intestinal microbiota composition or activity. However, it is now clear that an impact does not always require changes in intestinal microbiota or so called 'colonization' or temporary colonization of the human gastrointestinal tract as the probiotic organism can exert its effects locally or during transient passage

through the gastrointestinal system (Isolauri et al., 2002). In fact, recent reports indicate only temporary colonization as no permanent settlement in the gut has been demonstrated (Gueimonde et al., 2006).

Probiotics by definition belong to the beneficial bacteria in the healthy gut microbiota. An important question arises, what is the healthy gut microbiota? There is currently more information on microbiota deviations and on the role of the microbiota in different diseases including allergic diseases (Juntunen et al., 2003; Kalliomäki et al., 2001a, b; Wen et al., 2008). It appears that the compositional development of gut microbiota differs between infants developing atopic manifestations and those who do not. Healthy infants harbor a natural predominance of bifidobacteria with specific species and strains. At 3 weeks of age, the bacterial cellular fatty acid profiles in fecal samples differed between those infants who later manifested atopic sensitization and those who did not. Also, atopic subjects have more *Clostridium* species and fewer *Bifidobacterium* species in stools compared with atopic subjects. Furthermore, a recent study utilizing traditional plate culture methods showed that children who developed allergy were less often colonized with bifidobacteria during their first year of life as compared to those who did not develop allergic disease (Björkstén et al., 2001; Kalliomäki et al., 2001a, b). Furthermore, allergic infants appear to be colonized mainly with *B. adolescentis* whereas healthy infants, again, mainly with *B. longum* and *B. bifidum*. The *Bifidobacterium* species isolated from allergic infants were shown to induce proinflammatory cytokine production as compared to those from healthy infants (reviewed by: Isolauri and Salminen, 2008), suggesting a need for more precise characterization, at genus, species and strain levels, of the healthy gut microbiota as a source of probiotics. Recent studies in animal models have indicated a protective function of intestinal lactic acid bacteria against other immune mediated diseases such as type 1 diabetes providing further evidence that probiotics should be isolated from members of the healthy human gut microbiota (Wen et al., 2008). These findings emphasize the characterization of gut microbiota as a source of potential future probiotics.

The classification of a strain as probiotic requires that the beneficial physiological effects of the particular strain have been scientifically proven. The criteria for a probiotic include that the strain be of human origin, be safe in human use and stable to acid and bile, and that it adheres to the intestinal mucosa. The most frequently used genera fulfilling these criteria are lactobacilli and bifidobacteria and as a result the probiotics currently assessed belong to these genera.

25.7 Probiotics in the Management and Risk Reduction of Eczema

Our working hypothesis is that specific probiotics form a useful in risk reduction tool in reducing the risk of atopic eczema with food and gut involvement, i.e., in cases with documented food allergy. This hypothesis provides a working target for reducing the risk of atopic disease: impaired barrier functions of the skin epithelium and gut mucosa, sustained inflammation associated with aberrant. Probiotics may include good candidates to reverse the deviations. The old doctrine has been to avoid allergens, as the reason for allergy was thought to be primary exposure to allergens. The new strategy includes the use of modified or degraded antigens such as the use of partially or extensively hydrolyzed formulas for infants. This approach is highlighted for instance in the 6-year follow-up study of von Berg and coworkers (2008). It is now thought that the exposure to microbes and other antigens is needed to develop tolerance. Probiotics can act as factors that regulate inflammation and enhance gut barrier function. They may also prevent deviations in intestinal microbiota.

There are data on record concerning the beneficial effects of probiotics in the treatment of atopic eczema (see *Table 1* for detailed list). In double-blind placebo-controlled trials, infants with atopic eczema were given probiotic-supplemented extensively hydrolyzed formula containing either *L. rhamnosus* GG or *Bifidobacterium lactis* BB-12; this resulted in significant improvement of the skin condition along with a decrease in markers of intestinal and systemic allergic inflammation as compared to infants receiving unsupplemented formula (Majamaa et al., 1997; Isolauri et al., 2000). These results, recently repeated in older children with the same condition (Rosenfeld et al., 2003), indicated that probiotics may have potential also in controlling inflammatory responses beyond the gut. In a larger trial with three arms (placebo, *L. rhamnosus* GG, or a mixture of *L. rhamnosus* GG, *L. rhamnosus* LC 705, *B. breve* and *Propionibacterium freudenreichii*) 230 infants were given probiotics and the results suggested that *L. rhamnosus* GG alone was effective in infants with IgE associated eczema while the probiotic combination had no therapeutic effect (Viljanen et al., 2005). On the other hand, in a smaller study by Brouwer and coworkers (2006) probiotics were not effective, however; the study was conducted in a population with intrinsic eczema and no food allergy, thus supporting the hypothesis of gut involvement as a prerequisite for a successful probiotic effect. Similarly, no effects were observed in another study using *L. rhamnosus* (Folster-Holst et al., 2006).

Table 25.1

Efficacy of probiotics in studies on the treatment of atopic eczema in infants (*Cont'd* p. 987)

Probiotic strain and dose	Number of subjects/type of intervention	Outcome/reference
Lactobacillus rhamnosus GG (ATCC 53013) 5×10^8/cfu/day	31 children with eczema/ Extensively hydrolyzed formula with and without probiotic	Improvement in SCORAD in probiotic group Majamaa et al. (1997)
Lactobacillus rhamnosus GG (ATCC 53013) 10^8/cfu/day Bifidobacterium *lactis* Bb12 10^9/cfu/day	27 breastfed infants with eczema/Extensively hydrolyzed formula with and without probiotic	Significant improvement in SCORAD Isolauri et al. (2000)
Lactobacillus rhamnosus GG (ATCC 53013) 10^{10}/cfu/day (either viable or heat-killed preparation)	27 infants with eczema	SCORAD, but discontinued due to adverse effects caused by non-viable probiotic
Bifidobacterium breve 5–15 $\times$ 10^9/cfu/day	17 infants/Extensively hydrolyzed formula with prebiotic and with and without probiotic	Decreased eczema severity reported with probiotic Hattori et al. (2003)
Lactobacillus rhamnosus 19070 and *Lactobacillus reuteri* DSM 12246	58 children with eczema/ cross over study for 6 weeks/ placebo skim-milk powder with dextrose	Improvement of eczema with SCORAD, use of medicines and global assessment/Rosenfeldt et al. (2003)
Lactobacillus rhamnosus GG (ATCC 53013), *or probiotic combination (Lactobacillus rhamnosus* GG (ATCC 53013), *Lactobacillus rhamnosus LC 705, Bifidobacterium breve, Propionibacterium freudenreichi* ssp *shermanii)*	252 infants with eczema, Extensively hydrolyzed formula with prebiotic and with and without probiotic or probiotic mix	Viljanen et al. (2005) SCORAD improvement in *Lactobacillus rhamnosus* probiotic alone in IgE associated eczema, no impact with probiotic mix
Lactobacillus fermentum VRI-003PCC 2×10^9 cfu/day	56 children with eczema, probiotic or control (maltodextrin)	Improvement in global assessment and SCORAD
Lactobacillus rhamnosus and *Bifidobacterium lactis* at 2×10^{10} cfu/day	60 children with eczema,	Improvement in children with Age associated patters
Lactobacillus rhamnosus Lcr35, 3,6 $\times$ 10^9 with prebiotic in the placebo and active	48 children with eczema, *L rhamnosus* and prebiotic,	Passeron et al. 2006 Beneficial effects reported

Table 25.1

Probiotic strain and dose	Number of subjects/type of intervention	Outcome/reference
Lactobacillus rhamnosus GG (ATCC 53013) versus at 2×10^{10} cfu/day, and *microclystalline cellulose*	53 infants (1–55 months) with eczema in a double-blind placebo controlled study	Folster-Holst et al. (2006) No improvement
Lactobacillus rhamnosus GG (ATCC 53013) versus *microclystalline cellulose* at 1×10^{10} cfu/day	106 infants 3–12 months old with eczema, double blind placebo-controlled study	Gruber et al. (2007) No significant effect reported

In a similar study, a combination of *L. rhamnosus* and *B. lactis* improved atopic eczema in food sensitized children (Sistek et al., 2006).

The reports demonstrate that the effects of probiotics are strain-specific, and also depend on the immunological condition of the host. Furthermore, probiotic combinations should be planned using careful *in vitro* preclinical studies on their properties and strain interactions, prior to proceeding to clinical trials in humans. Nevertheless, the results suggest that specific probiotics may have a protective effect against the development of chronic debilitating atopic diseases, according to the hypothesis of atopic eczema as a gatekeeper in the development of IgE-mediated atopic manifestations (Hanifin, 2002). However, each strain has specific properties and these can even vary depending on the production methods of the strain, the processing conditions of the product and the method of application, i.e., in a food matrix or in a freeze-dried powder form, or sometimes even in the non-viable or dormant form (*Table 25.1*).

25.8 Primary Prevention of Atopic Eczema

The interest in using probiotic treatment for the risk reduction of atopic diseases has focused especially on atopic eczema. Probiotics administered pre- and postnatally for 6 months to children at high risk of atopic diseases, succeeded in reducing the prevalence of atopic eczema to half as compared with that in infants receiving placebo (Kalliomäki et al., 2001a, b, 2003). However, no reduction was observed in antigen-specific IgE generation, while the combination of *B. lactis* and *L. rhamnosus* GG influenced sensitization in a high risk population (Huurre et al., 2008). In a subgroup of the Kalliomäki study population, probiotic

supplementation increased the concentration of TGF-ß in breast milk (Rautava et al., 2002), identifying one potential mechanism useful in the primary prevention of atopic eczema.

The prevention studies available today provide a basis for comparing the effects of different probiotic bacteria, although the strains are often not well defined and do not fulfill the probiotic criteria provided by the WHO expert group (WHO, 2002). *Table 25.2* summarizes some of the recent probiotic prevention studies and the differences which could have an impact on the result.

Table 25.2

Probiotics and primary prevention studies of atopic eczema: differences influencing the outcome

Probiotic studied	Result and factors potentially influencing the result	Reference
Lactobacillus rhamnosus GG	Positive effect, both pre and postnatal probiotic administration	Kalliomäki et al. (2001, 2004, 2007)
Lactobacillus rhamnosus GG	No effect, confusing factors include exceptionally long breastfeeding period (over 9 months in probiotic and placebo group) and source of probiotic	Kopp et al. (2008)
Lactobacillus acidophilus LAVRI-A1	*Lactobacillus acidophilus* LAVRI (later identified as *Lactobacillus acidophilus* LAFTI 10, without preclinical studies and demonstration of probiotic properties	Taylor et al. (2007)
Lactobacillus reuteri	Less IgE-associated eczema, different probiotic, lower dose	Abrahamsson et al. (2007)
Lactobacillus rhamnosus GG, *Lactobacillus rhamnosus* LC705, *Bifidobacterium lactis, Propionibacterium freudenreichii* ssp *shermanii*, with a galacto-oligosaccharide	Tended to reduce IgE-associated (atopic) diseases, different strain combination, short prenatal treatment time	Kukkonen et al. (2007)
Lactobacillus rhamnosus HN001 and *Bifidobacterium lactis* HN019	*Lactobacillus rhamnosus* clinically effective but *Bifidobacterium lactis* not effective; results support earlier reports on the efficacy of another *Lactobacillus rhamnosus* strain	Wickens et al. (2008)
Bifidobacterium lactis BB12 and *Lactobacillus rhamnosus* GG combined	Risk of sensitisation could be reduced by the use of probiotics during pregnancy and lactation; longer treatment time, combination of two probiotics	Huurre et al. (2008)

Notwithstanding these recent studies, the specific immunomodulatory properties of probiotic strains and their safety should be precisely characterized (*Table 25.2*). The probiotic performance of strains differs; different bacteria or modifications/components thereof have different adherence sites, immunological effects and varied effects on the healthy versus inflamed mucosal milieu. In addition, the food matrix may modify these effects. The human diet contains several potential immunomodulatory compounds including dietary fatty acids and vitamins. Due to the interaction between nutrients, the effects of diet beyond single supplements need to be determined for the development of specific clinical applications in defined target populations.

The investigations of the gut microbiota deviations form the basis for atopic disease prevention studies using probiotics. They suggest that early microbiota is important and that the transfer of microbiota from mother to infant has a significant effect on later risk. Thus, the scintists planned the first prevention study on early microbiota differences and risk reduction by administering the chosen probiotic to counteract the deviations by prenatal administration to the mother, thus influencing the transfer of microbiota to the infant, and by postnatal administration to the infant in order to reduce the risk of deviation during the first 6 months of the early feeding period. The results demonstrated significant reduction in atopic eczema in infants in the probiotic intervention group at 2 years, 4 years and the effect was still seen at 7 years (Kallimäki et al., 2001, 2003, 2007).

25.9 Probiotics and Factors Influencing Allergy Prevention Studies

As atopic diseases form a common burden especially to infants and children the probiotic approach should be further studied in this population. Based on the probiotic concept both the mechanisms and potential targets should be characterized taking into consideration the variability of both the host, of the specific probiotics used and of the environmental factors (*Figure 25.1*). These factors offer often clarification to the confusing results reported in different studies and emphasize the importance of not only selecting the right probiotics and probiotic combinations for each target population but also of assessing carefully the other factors that potentially influence the outcome. Potential targets for future research are summarized in *Table 25.3*.

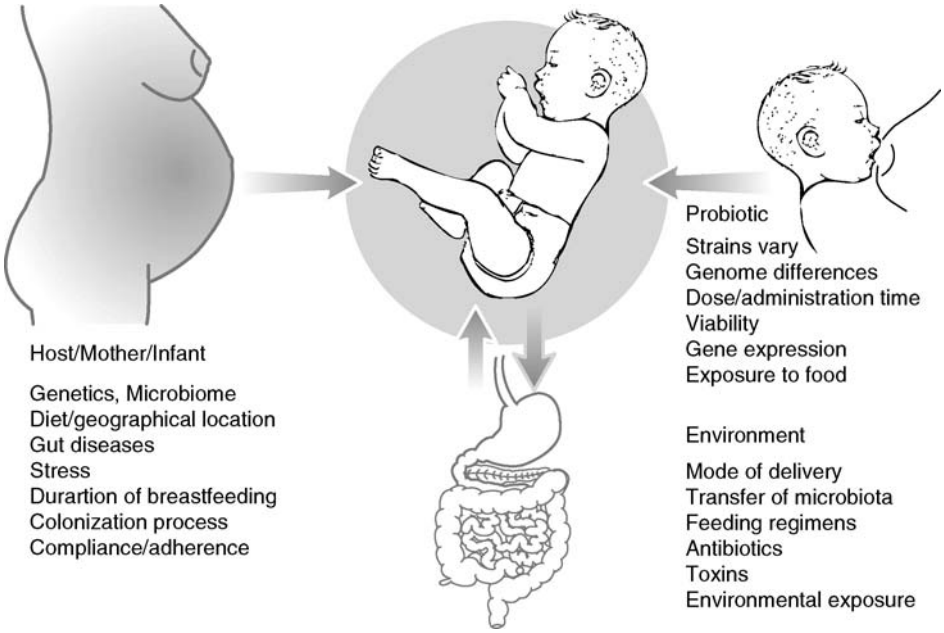

Figure 25.1
Factors affecting probiotic efficacy in atopic eczema risk reduction and symptom alleviation.

25.10 Conclusion

The intestine's mucosal surface provides a defense barrier against antigens encountered by the enteric route. In this system a balance is generated and maintained between the host and the microbiota. In intestinal inflammation, the integrity of the barrier is disrupted, a greater amount of antigens traverses the mucosal barrier and the routes of transport are altered, possibly evoking aberrant immune responses and release of proinflammatory cytokines with further impairment of the barrier function. Restoration of the properties of unbalanced indigenous microbiota forms the rationale of probiotic therapy. However, an important function of probiotics is related to their immunomodulatory effects. These include immune-enhancing as well as anti-inflammatory activities beyond "colonization" or "temporary colonization", as probiotics may exert their effects locally or during transient passage through the gastrointestinal system. In the future, the properties of specific dietary compounds, such as probiotics in an optimal food matrix should be exploited in order to develop specific prophylactic and therapeutic interventions. However, no single supplement can be expected to resolve the epidemic of atopic diseases. The challenge

Table 25.3

Potential probiotic effects and mechanisms to be assessed in future probiotic research in allergic disease risk reduction and symptom alleviation

Effects	Action mechanisms	Potential risks
Nutritional management of atopic disease	Degradation/structural modification of enteral antigens, control of gut barrier function (normalizing and balancing barrier and permeability), aberrant gut microbiota, local and systemic inflammatory response, probiotic properties, impact of probiotic manufacture and technology on their action mechanisms	Adverse effects on innate immunity/infection (risk related to host and strain characteristics, risks related to aggravation of microbiota aberrancies by specific prebiotics)
Reduction in risk of atopic disease	Enhanced host defense- maturation and stimulation. Generation of anti-inflammatory cytokines, selection of specific probiotics with preclinical assessment and ability to influence microbiota and immune system, characterization of probiotic properies, impact of probiotic manufacture and technology on their efficacy	Directing the microbiota toward other adverse outcomes/Directing the immune responder type to other adverse outcomes (risk related to host and strain and prebiotic characteristics)

in terms of reducing the risk of atopic eczema is to further identify the mechanisms of the disease in order to identify specific targets for defined probiotics and dietary factors, and their optimal combinations.

There are several studies demonstrating the efficacy of specific probiotic preparations on both treatment and risk reduction of atopic eczema with food or intestinal involvement in infants. Studies on probiotics in other allergic states are not well characterized and these should be continued. There is some confusion about the importance of specific strains and strain characteristics in allergic disease and this requires rigorous updating. It is important to know the strain(s) properties and to conduct preclinical studies before the application of probiotics to clinical interventions.

The present data demonstrate that when the right probiotic strains are selected and used in defined settings with symptoms of food and intestinal origin, there are excellent possibilities for both primary and secondary prevention of atopic eczema and also prevention of sensitization. Other areas of probiotics and allergy need further studies prior to making concrete conclusions.

25.11 Summary

- New approaches in the fight against allergic diseases are called for, and these are especially needed for infants to both treat and prevent deviated immune responder pattern during and beyond infancy. Atopic eczema is the first and earliest of such conditions, and more importantly, may act as a portal for the development of immunoglobulin E-mediated atopic manifestations.
- There is now ample evidence that intestinal microbiota deviations or aberrancies are associated with both the development of allergic diseases, and the microbiota differences between allergic and non-allergic subjects are significant.
- Probiotics are live microbial food supplements which are beneficial for human health (WHO, 2002). Ways to modulate the health are often mediated via the intestinal microbiota by altering the composition or the activity of the microbiota.
- There is now abundant evidence that specific probiotic strains selected from the members of healthy gut microbiota may exhibit powerful anti-pathogenic and anti-inflammatory capabilities, and that several targets for the probiotic approach have emerged in atopic eczema. These include the degradation/structural modification of enteral antigens, the normalization of the properties of aberrant indigenous microbiota and of gut barrier functions, the regulation of the secretion of inflammatory mediators, and the promotion of the development of the immune system. It is now clear that specific probiotic strains can be used as adjuncts in the treatment of atopic eczema. However, improved understanding of the mechanisms and the strain specific effects of probiotics and probiotic combinations is needed for the characterization of specific future strains with anti-allergic potential.

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26 Potential Protective Effects of Probiotics and Prebiotics Against Colorectal Cancer

Philip Allsopp · Ian Rowland

26.1 Introduction

Colorectal cancer (CRC) is the fourth most frequent cause of cancer related mortality in the world. Approximately 944,000 new cases were diagnosed globally in 2000 and this accounts for 9.2% of all new cancer cases (IARC, 2000). In Western societies namely Europe, North America and Australasia, it is the second most prevalent cancer after lung/breast (Boyle and Langman, 2000). About 363,000 new cases were reported in Europe in 2000 and it affects 6% of men and women by age 75, in almost equal proportion.

26.2 Diet and Lifestyle Factors and CRC Risk

World-wide incident rates for CRC show an approximate 20-fold variation, with the developed world suffering the highest rates and India one of the lowest (IARC, 2000). These fluctuations are generally attributed to both genetic and environmental factors, especially diet. Studies in migrants moving from areas of low incidence to high incidence (e.g., Japan to USA) give additional support to the role of environmental factors in the aetiology of colorectal cancer, with reported incident rates of migrants and their descendants reaching those of the host country, sometimes within one generation (WCRF, 2007). The highest rates of CRC are seen within Hawaiian Japanese men with an incidence of 53.5/100,000 (IARC, 1997).

Evidence suggests that diet plays an important role in the aetiology of colorectal cancer, however, identifying conclusively which constituents

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(e.g., vegetables, meat, fiber, fat, micronutrients) exert an effect on risk has been more problematic due to inconsistent data. The 2007 World Cancer Research Fund report (WCRF, 2007) concluded that the epidemiological evidence was convincing or probable for associations between overweight/obesity (waist circumference), processed meat, alcohol and increased risk of colorectal cancer. Fiber, garlic, milk and calcium are associated with decreased risk.

26.2.1 Colon Carcinogenesis

Approximately 15% of all CRC is due to genetic predisposition, with a further 60% the result of sporadic tumors that appear to develop from adenomatous polyps. Adenomas are well demarcated clumps of epithelial dysplasia, classified into three histological types, tubulovillous, tubular and villous, which increase in prevalence with age, being present in 24–40% of people over 50 years old (Ponz de Leon and Roncucci, 2000). It should be noted that only about 5% of polyps develop into malignancies.

Adenomas and carcinomas develop through a stepwise accumulation of somatic mutations indicating the importance of genetic damage in the process. While the precise sequence of genetic events is not completely understood, it involves inactivation of various tumor suppressing genes (e.g., APC, p53), activation mutations in proto-oncogenes (e.g., k-ras, c-myc), and loss of function in DNA repair genes (e.g., hMLH1, hMSH2). This archetypal multistep model has been termed the "adenoma-carcinoma sequence" (Fearon and Vogelstein, 1990).

It has been postulated that the development of an adenomatous polyp is a consequence of genetic damage to and subsequent transformation of a colonocyte within the colonic crypt presumably from a luminal agent. The altered mucosal cells spread laterally and downward to form new crypts, that connect to and eventually replace existing crypts (Shih et al., 2001).

The origin and identity of the luminal agents involved in CRC have not been fully elucidated although it is clear that fecal extracts contain substances with genotoxic, and tumor promoting activity which may be derived from unabsorbed dietary residues, endogenous secretions and gut bacterial

metabolites of both (Hughes et al., 2008; Hughes and Rowland, 2003) as discussed below.

26.2.2 Role of the Gut Flora in Cancer

The relationship between colorectal cancer, diet, and gut microbiota is complex and intimate. The substances entering the colon from the ileum and the resident microbiota represent key determinants of colon physiology. These, together with the innate biology of the colon (e.g., epithelium, motility), are pertinent to colorectal carcinogenesis. The concentration of bacteria resident in the colon increases distally with an estimated 1,000 different species resident within a healthy adult colon (Holzapfel et al., 1998). Once the microbiota is established, little qualitative variation in the composition occurs over time, although there is extensive evidence that the metabolic activity of the microbiota can be modulated by diet, especially non-digestible carbohydrates (fiber, oligosaccharides) (Mallett and Rowland, 1988). With the capacity for the microbiota to modulate colonic conditions established, it becomes obvious why analyzing their dynamic interaction with the colonic environment and mucosa is of such importance in terms of CRC and why there is currently intense interest in dietary modulation of microbiota with food ingredients, such as pro and prebiotics.

Evidence from a wide range of sources supports the view that colonic microbiota is involved in the aetiology of cancer (reviewed by Rowland, 2009). The main pieces of evidence are:

1. Human feces have been shown to be mutagenic and exert tumor promoting activity *in vitro* and genotoxic substances of bacterial origin have been isolated (Gill et al., 2007; Venturi et al., 1997).
2. Intestinal bacteria can produce, from dietary components, substances with genotoxic, carcinogenic and tumor-promoting activity (Rowland, 2009).
3. Gut bacteria can activate procarcinogens to DNA reactive agents.
4. Germ-free rats treated with the carcinogen 1,2-dimethylhydrazine have a lower incidence of colon tumors than similarly treated rats having a normal microbiota (Reddy et al., 1975).
5. Germ-free rats fed human diets exhibit lower levels of DNA adducts in tissues than conventional rats (Rumney et al., 1993).

26.3 Probiotics and Prebiotics and CRC

It follows from the above, that modification of the gut microbiota may interfere with the process of carcinogenesis and this opens up the possibility for dietary modification of colon cancer risk. Probiotics and prebiotics, which modify the microbiota by increasing numbers of lactobacilli and/or bifidobacteria in the colon, have been a particular focus of attention in this regard. Evidence that probiotics and prebiotics can influence carcinogenesis is derived from a variety of sources:

1. Effects on bacterial enzyme activities.
2. Antigenotoxic effects *in vitro* and *in vivo*.
3. Effects on pre-cancerous lesions in laboratory animals.
4. Effects on tumor incidence in laboratory animals.
5. Epidemiological and experimental studies in humans.

26.3.1 Effects of Probiotics and Prebiotics on Bacterial Enzyme Activities

The ability of the colonic microbiota to generate a wide variety of mutagens, carcinogens and tumor promoters from dietary and endogenously-produced precursors is well documented (Rowland, 1995; Rowland and Gangolli, 1999; Rowland 2009). For example, the enzyme ß-glucuronidase is involved in the release in the colon from their conjugated form of a number of dietary carcinogens, including polycyclic aromatic hydrocarbons. Similarly, bacterial ß-glycosidase hydrolyzes the plant glycoside cycasin to a carcinogen in the gut. It should be noted however that glycoside hydrolysis by intestinal microbiota can result in the generation of potential anti-carcinogenic and anti-mutagenic substances in the form of flavonoids such as quercetin (Rowland, 1995). A major role for the intestinal microbiota has been identified in the metabolism of the primary bile acids cholic and chenodeoxycholic acids to deoxycholic and lithocholic acids respectively, which are thought to possess tumor-promoting activity (Hughes et al., 2008). Other potential tumor-promoters, namely ammonia, phenols and cresols, are also generated by deamination of amino acids such as tyrosine by intestinal bacteria (Hughes et al., 2008).

The reaction of nitrite with secondary amines and amides can lead to the formation of *N*-nitroso compounds, many of which possess mutagenic and

carcinogenic activity. There is evidence from germ-free rat studies that nitrosation can occur under neutral pH conditions by an enzymic process catalyzed by intestinal bacteria (Massey et al., 1988). Another bacterially-catalysed reaction yielding a reactive substance capable of causing DNA damage and mutation, is the conversion of the cooked food carcinogen 2-amino-3-methyl-3H-imidazo (4,5-*f*)quinoline (IQ) to its 7-hydroxy derivative. The latter, unlike its parent compound is a direct-acting mutagen (Carman et al., 1988).

In general, species of *Bifidobacterium* and *Lactobacillus*, have low activities of enzymes involved in carcinogen formation and metabolism by comparison to other major anaerobes in the gut such as bacteroides, eubacteria and clostridia (Saito et al., 1992). This suggests that increasing the proportion of LAB in the gut could modify, beneficially, the levels of xenobiotic metabolizing enzymes. Studies have been carried out in laboratory animals and humans in order to acquire a greater understanding of the way in which administration of specific probiotics and prebiotics affect gut microbiota metabolism.

26.3.1.1 Studies in Laboratory Animals

The effects of probiotics and prebiotics on gut bacterial enzymes have been studied in conventional flora animals and also germ free rats associated with a human fecal microbiota, so called "Human Flora Associated" (HFA) rats. These studies are summarized in *Table 26.1* and some examples are discussed in more detail below.

In a conventional rat study, supplementation of a high meat diet (72% beef) with *L. acidophilus* (10^9–10^{10} organisms/day) significantly decreased by 40–50% the activity of fecal β-glucuronidase and nitroreductase (Goldin and Gorbach, 1976). Interestingly the modulating effect of the *lactobacillus* strain was dependent on the type of basal diet fed – no significant effects were seen when the rats were fed a grain based diet. In an analogous study in HFA rats, Cole et al. (1989) demonstrated a significant reduction in β-glucuronidase and β-glucosidase activities when *L. acidophilus* was fed for 3 days, with the effect persisting for 7 days after dosing ceased.

These changes in enzyme activities seen after consumption of LAB would in theory be expected to result in changes in rates of metabolism of their substrates *in vivo*, although only if the enzymes catalyzed the rate limiting step in their metabolism. Goldin and Gorbach (1984a) have confirmed this by showing that a reduction in activity of the bacterial enzymes nitroreductase, azoreductase

Table 26.1

Effects of probiotics and prebiotics on bacterial enzyme activity and metabolic end products in laboratory animals (*Cont'd* p. 1003)

Species	Endpoint	Pro/prebiotic	Result	Author
F344 rat	Fecal β-glucuronidase	*L. acidophilus* (10^9–10^{10} cells/day)	Decreased the activity of β-glucuronidase by 40–50%	Goldin and Gorbach (1976)
Lister hooded rat (HFA)	β-glucuronidase and β-glucosidase activity	*L. acidophilus* or *B. adolescentis* (10^9 cells/day for 3days)	A significant decrease in enzyme activity for *L. acidophilus* only	Cole et al. (1989)
F344 rat	Fecal levels of enzyme reaction products after administration of test substances	*L. acidophilus*	Animals given *L. acidophilus* had significantly lower free amines in feces and 50% less of conjugates	Goldin and Gorbach (1984)
Rat	Fecal SCFA levels	Neosugar (10–20% in diet)	Significantly increased SCFA concentration in faeces	Tokunga et al. (1986)
Germ free Lister hooded rat	Various cecal enzymes	TOS (5%w/w in diet for 4 weeks) or TOS + *B. breve*	β-glucuronidase and nitrate reductase activities, pH and the conversion of IQ to 7-OHIQ significantly reduced in cecal contents of the TOS-fed rats. Bacterial β-glucosidase activity was increased in TOS fed rats	Rowland and Tanaka (1993)
Male Sprague-Dawley rats	Fecal enzymes and ammonia	*B. longum* (freeze dried) and inulin (5%)	Significant decrease in β-glucuronidase and ammonia. Probiotic plus prebiotic was more effective	Rowland et al. (1998)
Minipigs	Fecal enzymes	*3 Lactobacillus species*	Significantly reduced in β-glucuronidase and azoreductase	Haberer et al. (2003)
Male winstar rat	Fecal β-glucuronidase	*P. jensenii* 702	No effect	Huang et al. (2003)
Male F344 rats	Colonic β-glucuronidase	*Clostridium butyricum* – CBM588	No affect	Nakanishi et al. (2003)

Table 26.1

Species	Endpoint	Pro/prebiotic	Result	Author
Male SD rats	Fecal enzymes	*B. longum*	Reduced β-glucosidase, β-glucuronidase, tryptophanase and urease	Choi et al. (2005)
BALB/c mice	Fecal enzymes	*L. delbrueckii* subsp. *bulgaricus* and *S. thermophilus*	Reduction of β-glucuronidase and nitroreductase in tumor bearing mice following DMH challenge	De Moreno de le blanc et al. (2005)
Male Jcl:ICR mice	β-glucuronidase (cecal and colonic contents)	*Butyrivibrio fibrisolvens* MDT-1 or *C. butyricum*	*B. fibrisolvens* significantly reduced β-glucuronidase activity	Ohkawara et al. (2005)
Male Sprague-Dawley rats	Fecal enzymes	*L. casei*	Significantly reduced β-glucuronidase ($P < 0.01$)	Villarini et al. (2008)

and β-glucuronidase in rats given oral lactobacilli, was matched by a decrease (of about 50% in comparison to controls) in the excretion in feces of the reaction products of the enzymes.

Studies on the influence of NDOs on gut bacterial enzyme activities in laboratory animals have concentrated on fructo-oligosaccharides and galacto-oligosaccharides. In conventional flora rats fed a purified diet containing tyrosine and tryptophan, the incorporation of a fructo-oligosaccharide ("Neosugar") into the diet at 0.4–10% reduced the fecal concentration of the potential tumor promoter *p*-cresol (Hidaka et al., 1986). The effect was related to dose of the NDO. Higher dietary concentrations of Neosugar (up to 20%) were found to increase short chain fatty acids (SCFA) and total daily excretion of neutral and acid sterols (Tokunga et al., 1986).

The effect of ingestion of galactooligosaccharide (GOS; 5% w/w in diet for 4 weeks) with or without *B. breve*, was studied in HFA rats (Rowland and Tanaka, 1993). In the GOS-fed animals, an increase in bifidobacteria and lactobacilli numbers in the caecum was seen, associated with significant decreases in β-glucuronidase and nitrate reductase activities, pH, and in the conversion of IQ to its directly genotoxic derivative 7-OHIQ. Bacterial β-glucosidase activity was increased presumably as a consequence of elevated numbers of LAB which have a high activity of this enzyme. In a study by Rowland et al. (1998) in rats given *B. longum*, inulin or both, an increased effect of the synbiotic combination on enzyme activities and fecal bacterial metabolites was reported. For example,

feeding of *B. longum* alone resulted in a 30% decrease in activity of β-glucuronidase whereas a 55% decrease was seen in rats given diet supplemented with the probiotic/prebiotic combination.

Choi et al. (2005) demonstrated a potent protective effect of *B. longum* SPM1205 and Duolac™ (*L. casei, L. rhamnosus, L. lactis, L. plantarum* and *B. longum*) supplementation in Male SD rats. Probiotic treatment caused significant reductions in β-glucosidase, β-glucuronidase, tryptophanase and urease, although Duolac™ was consistently more potent than *B. longum* SPM1205.

In addition to studies on lactic acid producing bacteria, there is evidence that less conventional probiotics can modulate enzyme activity in laboratory animals. Particular attention has been paid to butyrate producing organisms, in the light of the beneficial trophic effects of this short chain fatty acid on the colonic epithelium For example, Nakanishi et al. (2003) investigated the influence of *Clostridium butyricum* (CB) – CBM588 alone, and with high amylase maize starch (HAS) on AOM induced ACF and colonic β-glucuronidase activity in F344 rats. CB had no effect on ACF formation or β-glucuronidase activity although combined treatment with CB and HAS resulted in a significant reduction in ACF ($P < 0.05$) and β-glucuronidase ($P < 0.05$) activity. The authors suggest that the prebiotic activity of HAS alongside the probiotic activity of CB resulted in a significant symbiotic effect.

In a study by Ohkawara et al. (2005) supplementation of butyrate producing bacteria *Butyrivibrio fibrisolvens* MDT-1 (10^9 cfu/dose, 3 times/week for 4 weeks) significantly reduced the β-glucuronidase activity ($P < 0.05$) in both cecal and colonic contents.

No significant reduction was noted in rats supplemented with the cell homogenate of *Butyrivibrio fibrisolvens* indicating the need for intact bacterial cells. In contrast rats administered *Propionibacterium jensenii* 702 (10^{10} cfu/day) had significantly elevated β-glucuronidase activity after 36 days ($P < 0.05$), however this undesirable effect was not evident after 81 days. Such contrasting results raise safety concerns and justify the need for further research into the safety of short and long term supplementation of *Propionibacterium* as a probiotic (Huang et al., 2003).

26.3.1.2 Studies in Human Subjects

A number of studies have been carried out on the effects of pro-, pre and synbiotics on human subjects which have included measurement of bacterial enzyme activities (*Table 26.2*). Goldin and Gorbach (1984b) studied volunteers

Table 26.2

Effects of probiotics and prebiotics on bacterial enzyme activities and metabolic end products in man (*Cont'd* p. 1006)

Subjects	Endpoint	Probiotic/prebiotic	Result	Author
21 Healthy subjects	Fecal enzyme activities	Milk supplemented with *L. acidophilus* (1×10^9 viable bacteria per day)	Fecal β-glucuronidase activity was reduced from 1.7–2.1 units to 1.1 units in all subjects	Goldin and Gorbach (1984a)
14 Colon cancer patients	Fecal β-glucuronidase activity	*L. acidophilus* (given as a fermented product, between 1.5×10^{11} and 6×10^{11} cfu/day)	A 14% decrease in mean β-glucuronidase activity after 2 weeks	Lidbeck et al. (1991)
20 Healthy male subjects (40–65) years old)	Fecal β-glucuronidase and β-glucosidase activity	*L. casei* (strain Shirota) 3×10^{11} cfu/day	Significant decrease in β-glucuronidase and β-glucosidase activity ($P < 0.05$)	Spanhaak et al. (1998)
Nine healthy adults	Fecal β-galactosidase β-glucosidase and β-glucuronidase	Olifus™ (a commercial fermented milk containing *L. acidophilus* (3×10^9 bacteria/day) strain A1, *B. bifidum* B1 (3×10^{10} bacteria/day), *Strep. lactis* and *Strep. cremoris* (3×10^{10} bacteria/day)	No change in fecal β-galactosidase and β-glucuronidase. Significant increase β-glucosidase activity	Marteau et al. (1990)
Three male and nine female healthy subjects	Fecal β-glucosidase and β-glucuronidase activity.	Digest™ containing viable *L. acidophilus* (strain DDS1) (3 eight oz cups of milk containing 2×10^6 cfu/ml per day)	A decrease in β-glucuronidase and β-glucosidase activity	Ayebo et al. (1980)
21 Young women aged 21–35 years with severe premenstrual syndrome	19 fecal enzyme activities	*L. acidophilus* and *B. bifidum* (1×10^9 of each type of bacteria/capsule – three capsules per day	Reduction in β-glucosidase activity. A decrease in β-glucuronidase activity	Bertazzoni Minelli et al. (1996)

Table 26.2

Subjects	Endpoint	Probiotic/prebiotic	Result	Author
Human volunteers	Viable bacterial count	Soy bean oligosaccharides (SOE) 10 g/day	No significant difference in levels of *p*-cresol, indole or phenol between various dietary periods	Hayakawa et al. (1990)
Open parallel human study (n = 28)	Fecal azoreductase activity	*L. rhamnosus* and *Propionibacterium freudenreichii*	Significant reduction in azoreductase activity ($P < 0.05$)	Ouwehand et al. (2002)
DBPCT healthy volunteers (n = 19)	Fecal enzyme activity	Isomalt (Palatinit™)	Significant decrease in β-glucosidase ($P < 0.05$)	Gostner et al. (2006)
Human IBS volunteers DBPCT (n = 54) 6 months	Fecal ß-glucosidase and ß-glucuronidase activity	*L. rhamnosus* GG, *L. rhamnosus* Lc705, *Propionibacterium freudenreichii* subsp. *shermanii* JS and *B. breve* Bb99	A reduction in ß-glucuronidase was evident in 67% of subjects in the probiotic group vs. 38% in the placebo group although was not significant ($P = 0.06$). No effect with ß-glucosidase	Kajander et al. (2007)
DBPC prospective study Child liver transplant patients	Fecal ß-glucosidase, ß-glucuronidase and urease activity	*L. casei* strain DN-114001	Decrease in urease and significant decreases in β-glucuronidase and β-glucosidase ($P < 0.05$)	Pawłowska et al. (2007)
Randomized crossover healthy volunteers	Fecal ß-glucuronidase, ß-glucosidase activity	Lactulose, Raftilose (synergy 1) *L. casei* Shirota, *B. breve*	Lactulose and Synergy 1 decreased ß-glucuronidase *L. casei* Shirota *B. breve* caused nonsignificant decreases	De Preter et al. (2008)

consuming milk supplemented with 10^9 viable lactobacilli per day. Prior to lactobacilli feeding, fecal β-glucuronidase activity ranged between 1.7 and 2.1 units. This declined in all 21 subjects after consumption of lactobacilli to a mean value of 1.1 units. The activity returned to baseline values 10 days after

consumption of LAB ceased. Lidbeck et al. (1991) supplemented the diets of 14 colon cancer patients with *L. acidophilus* as a fermented milk product (approximately 3×10^{11} lactobacilli per day) for a period of 6 weeks and fecal microbiota, fecal bile acids and β-glucuronidase activity were measured. Coincident with changes in microbiota (an increase of lactobacilli in feces and a decrease in the numbers of *E. coli*) was a 14% decrease in β-glucuronidase activity. A decrease in total bile acids and deoxycholic acid of 15 and 18%, respectively, was also observed. Similar results were obtained by Spanhaak et al. (1998) who reported a significant decrease in the activity of fecal β-glucuronidase and β-glucosidase activity in a group of twenty healthy male subjects given *L. casei* (approximately 10^{11} cfu/day for a 4 week test period).

Marteau et al. (1990) studied nine healthy volunteers before (period 1), during (period 2), and after (period 3) ingesting 100 g/day of a fermented milk product ("Olifus") containing *L. acidophilus* (10^7 cfu/g), *B. bifidum* (10^8 cfu/g) and *Strep. lactis* (10^8 cfu/g) and *Strep. cremoris* (10^8 cfu/g) for 3 weeks. Fecal azoreductase and β-glucuronidase activities did not change throughout the three periods. Nitroreductase activities dropped significantly in period 2 and remained at a low level during period 3. There was no change in β-galactosidase activity but β-glucosidase activity significantly increased in period 2 and returned to baseline levels in period 3. *In vitro* the dairy product showed a high β-glucosidase activity that was related to the presence of *B. bifidum*. The decrease in nitroreductase activity still persisted 3 weeks after cessation of ingestion of the fermented dairy product suggesting more prolonged modifications of the colonic flora.

Ayebo et al. (1990) assessed the effect of consumption of non-fermented milk containing *L. acidophilus* (2×10^6 cfu/ml) on fecal β-glucuronidase and β-glucosidase in a cross-over study in elderly human subjects. Low fat milk was given as a control and diets were consumed for a period of 4 weeks. Fecal counts of lactobacilli rose during the period of probiotic consumption by approximately one order of magnitude. β-glucuronidase activity decreased slightly after 4 weeks of *Lactobacillus* feeding and there were inconsistent effects on β-glucosidase activity.

A double-blind placebo-controlled trial (DBPCT) (n = 86) carried out by Kajander et al. (2007) investigated the influence of a multispecies probiotic preparation (*L. rhamnosus GG, L. rhamnosus Lc705, Propionibacterium freudenreichii* subsp. *shermanii JS and B. breve Bb99*) on the intestinal microbiota. A non-significant decrease ($P < 0.06$) in ß-glucuronidase activity was noted with

reductions evident in 67% of subjects in the probiotic group compared to 38% in the placebo.

Effects in human studies of prebiotics and synbiotics on toxic bacterial metabolites in feces are few and generally have yielded inconsistent or negative results. Tanaka et al. (1983) reported no effect of GOS (3 or 10 g/day) on fecal ammonia, but did show that simultaneous ingestion of GOS and *B. breve* reduced ammonia concentration in 4 out of 5 subjects. Similarly a study in human volunteers given soy bean oligosaccharides; (10 g/day) with or without simultaneous consumption of *B. breve* demonstrated no significant effects on fecal pH or amino acid breakdown products (*p*-cresol, phenol and indole), despite changes in fecal bifidobacteria numbers (Hayakawa et al., 1990).

A DBPC human intervention (n = 19) carried out by Gostner et al. (2006) supplemented Isomalt (30 g/d) and noted a significant reduction in β-glucosidase activity ($P < 0.05$) but β-glucuronidase, sulfatase, nitroreductase and urease remained unchanged.

26.3.2 Anti-Genotoxicity of Probiotics and Prebiotics *In Vitro*

More direct evidence for protective properties of probiotics and prebiotics against cancer has been obtained by assessing the ability of cultures to prevent DNA damage and mutations (which is considered to be an early event in the process of carcinogenesis) in cell cultures or in animals (› *Table 26.3*).

The effect of LAB on the induction of mutations by a wide variety of model carcinogens *in vitro* has been studied using the Ames Salmonella assay. The carcinogens used include N-nitrosocompounds N-methyl-N-nitro-N-nitrosoguanidine (MNNG) and N-methylnitrosourea (MNU) heterocyclic amines (e.g., IQ and related compounds) and aflatoxin B1. Overall the results indicate that the various LAB can inhibit genotoxicity of dietary carcinogens *in vitro*. The degree of inhibition was strongly species dependent. For example Pool-Zobel et al. (1993) demonstrated that *L. casei* and *L. lactis* inhibited the mutagenic activity of nitrosated beef by over 85%, whereas *L. confusus* and *L. sake* had no effect. Similar protective effects using the Ames test have been seen for six different species of bifidobacteria (*B. adolescentis, B. bifidum, B. breve, B. infantis, B. lactis, B. longum*) against benzo[a]pyrene induced mutagencity (Lo et al., 2004), for several species of *Lactobacillus* against 4-nitroquinoline-1-oxide and N-methyl-nitro-N-nitrosoguanidine (Caldini, 2008; Caldini et al., 2005; Cenci

Table 26.3

Antigenotoxicity of probiotics and prebiotics in vitro and in vivo (*Cont'd* p. 1010)

Target	Endpoint	Mutagen	Probiotic/prebiotic	Result	Author
In vitro studies					
Salmonella typhimurium TA 98	*In vitro* mutagenicity (Ames)	Trp-P-1, Trp-P-2 and Glu-P-1	Lactic acid bacteria isolated from a traditional Chinese cheese	Lyophilized cells of all strains inhibited Trp-P-1 and Trp-P-2 mutagenicity. Some strains inhibited Glu-P-1	Zhang et al. (1990)
Salmonella typhimurium TA 1538	*In vitro* mutagenicity (Ames)	Nitrosated beef extract	Ten isolated *Lactobacillus* strains	*L. casei* and *L. lactis* inhibited mutation by >85% and *L. sake* and *L. confusus* had no effect	Pool-Zobel et al. (1993)
Salmonella typhimurium	*In vitro* mutagenicity (Ames)	Glu-P-1, Glu-P-2, IQ, MeIQ, MeIQx, Trp-P-1, Trp-P-2	22 strains of intestinal bacteria	The majority of strains inhibited mutagenicity	Morotomi et al. (1986)
Salmonella typhimurium TA100 and TA97	*In vitro* mutagenicity (Ames)	Nitrovin and 2-aminofluorene	Nine strains of LAB	Significant anti-genotoxic activity exerted by six of the nine strains tested	Ebringer et al. (1995)
Salmonella typhimurium TA100	*In vitro* mutagenicity (Ames)	Benzo[a]pyrene B[a]P	*B.adolescentis, B.bifidum, B. breve, B. infantis, B. lactis, B. longum*	All 6 Bifidobacterial species significantly reduced mutagenicity of B[a]P ($P < 0.05$)	Lo et al. (2004)
Escherichia coli PQ37	*SOS Chromotest*	Furazolidone (10 μM)	*Lactobacillus casei* Shirota, *L. acidophilus* T_{20}, *Strep. salivarius, B. lactis Bb-12.*	All strains showed high levels of antigenotoxicity ranging from 64% *L. casei* Shirota to 92% shown by *B. lactis* Bb-12	Raipulis et al. (2005)
Escherichia coli PQ37	*SOS Chromotest*	4-NQO MNNG	65 strains of Lactobacilli	*L. acidophilus* A9 significantly reduced MNNG genotoxicity	Caldini et al. (2005)

Table 26.3 (*Cont'd* p. 1012)

Target	Endpoint	Mutagen	Probiotic/prebiotic	Result	Author
Escherichia coli PQ37	SOS Chromotest	4-NQO Furacolidone	*Saccharomyces boulardii (cerevisiae)*	Significant antigenotoxic activity towards 4-NQO and Furacolidone	Toma et al. (2005)
Escherichia coli PQ37	SOS Chromotest	4-NQO MNNG	25 strains of *Lactobacillus*	12 produced high inhibition, 9 moderate and 4 were inactive against 4-NQO. 1 Highly active 13 moderate and 11 were inactive against MNNG	Corsetti et al. (2008)
Escherichia coli PQ37	SOS Chromotest	4-NQO MNNG	21 strain of *Bacilluse* species	All species had high inhibitory activity against 4-NQO. Activity against MNNG was moderate or high	Cenci et al. (2008)
Escherichia coli PQ37	SOS Chromotest	4-NQO	67 probiotic strains	46% of strains exerted high antigenotoxicity	Cenci et al. (2002)
Escherichia coli PQ37	SOS Chromotest	4-NQO, MNNG	*L. rhamnosus, L. casei, L. plantarum, L. brevis, Lactobacillus* spp.	All species significantly reduced 4-NQO genotoxicity *L. brevis*, *L. plantarum* and *L. casei* significantly reduced MNNG genotoxicity	Caldini et al. (2008)
HT29 human colon cells	Genotoxicity (Comet assay)	Fecal water following incubation with probiotic	*B. lactis* Bb12, *L. plantarum, B. spp.* 420, *L. bulgaricus, Enterococcus faecium, Strep. thermophilus*	All species except *Strep. thermophilus* significantly reduced DNA damage	Burns and Rowland (2004)
HT29 human colon cells	Genotoxicity (Comet assay)	Fecal water incubated with fermentation supernatant	*L. plantarum* and *B. lactis* Bb12 plus prebiotics	Significantly reduced genotoxicity ($P < 0.05$) in all synbiotic combinations except *B. lactis* Bb12 and inulin	Burns and Rowland (2004)

HT29 human colon cells in vitro	Genotoxicity (Comet assay)	Plumbagin, H_2O_2	55 different LAB species	45% reduced Plumbagin induced damage 20% reduced H_2O_2 induced damage 13% of LAB were genotoxic	Koller et al. (2008)
In vivo studies					
Female, 4 week old BALB/c mice	*In vitro* binding and in vivo mutagenicity in liver	B(a)P, AFB1, IQ, MeIQ, MeIQx, PhIP and Trp-P-2	*L. acidophilus* and *B. longum*	Bacterial strains tested were able to bind carcinogens *in vitro*. No effect on *in vivo* mutagenicity or absorption	Bolognani et al. (1997)
Male Sprague-Dawley rats	*In vivo* DNA damage in colon (Comet assay)	MNNG and DMH	*L. acidophilus, L. gasseri, L. confusus, B. longum, B. breve, Strep. thermophilus, L. acidophilus*	Most lactic acid bacteria tested strongly inhibited genotoxicity in the colon. *Strep. thermophilus* had no effect. Heat treatment abolished probiotic effect	Pool-Zobel et al. (1996)
F344 rats (human flora associated)	*In vivo* DNA damage in colon (Comet assay)	DMH	Lactulose (3% in diet)	Lactulose significantly decreased extent of DNA damage ($P < 0.05$)	Rowland et al. (1996)
Female, 4 week old BALB/c mice	*In vitro* binding and *in vivo* mutagenicity in liver	B(a)P, AFB1, IQ, MeIQ, MeIQx, PhIP and Trp-P-2	*L. acidophilus* and *B. longum*	Bacterial strains tested were able to bind carcinogens *in vitro*. No effect on *in vivo* mutagenicity or absorption	Bolognani et al. (1997)
Male CD-1 mice	Micronucleated normochromatic erythrocytes (MNNE)	Aflatoxin B1	*Saccharomyces cerevisiae* (1×108 live cells/g)	Reduced MNNE induced by AFB1 in mice fed *Saccharomyces*	Madrigal-Santillan et al. (2006)

Table 26.3

Target	Endpoint	Mutagen	Probiotic/prebiotic	Result	Author
Male F344 rats	*In vivo* DNA damage in peripheral leukocytes	DMH (s.c.)	*Bacillus polyfermenticus* (3.1 × 108 cfu/d)	BP significantly reduced ($P < 0.05$) tail length (54%) and tail moment (55%)	Park et al. (2007)
Male F344 rats	Genotoxicity of fw on HT29 cells using comet assay	AOM (s.c.)	Synergy 1, *L. rhamnosus* and *B. lactis* or both	Synergy 1 diets reduced exposure to fw genotoxicity	Klinder et al. (2004)
Male Sprague-Dawley rats	Comet assay with rat colonocytes	DMH (gavage)	*L. casei*	L.casei significantly decreased basal DNA damage and also following DMH treatment	Villarini et al. (2008)
Male F344 rats	Comet assay with leukocytes	DMH (s.c.)	*Bacillus polyfermenticus*	DNA damage induced by DMH significantly reduced (54%)	Park et al. (2007)
Colon obtained from Sprague Dowley rat	Comet assay with isolated rat colonocytes	MNNG	*L. acidophilus* 317/402	Significant reduction in DNA damage with whole culture ($P < 0.05$) and isolated pallet ($P < 0.001$)	Nersesyan (2001)

et al., 2002, 2008; Corsetti et al., 2008) and for *L. casei* against IQ and other cooked food mutagens (Tavan et al., 2002).

Burns and Rowland (2004) incubated six different probiotic strains with genotoxic fecal water (FW) separately, then sedimented the bacteria and exposed HT29 cells to the resulting fecal water supernatant. All strains except *Strep. thermophilus* showed a significant reduction of FW genotoxicity, and *B. lactis* Bb12 was also shown to reduce genotoxicity in a dose dependant manner. Incubations of *L. plantarum* with fructan prebiotics including inulin, raftiline and raftilose (patented forms of inulin and oligofructose) and another commercial fructo-oligosaccharide (Actilight) also showed the significant reductions in FW genotoxicity on HT29 cells, whereas fermentation with galactooligosaccharide (Vivinal) and maltodetrin (Fibersol) were less effective.

A study by Koller et al. (2008) investigated the genoprotective activity of 55 species of lactic acid bacteria (LAB) when exposed to HT29 cells. They showed that although some of the LAB strains reduced plumbagin and H_2O_2 induced genotoxicity, 13% of LAB strains actually induced DNA damage in the colon cell line. They also showed that activity was dependant on LAB viability and such ambivalent activity between LAB species highlights the need for species specific research.

Nersesyan (2001) demonstrated significant antigenotoxic activity of cultured *L. acidophilus* 317/402 (24 h culture) when incubated with isolated colon rat tissue. The whole growth culture of lactobacilli ($P < 0.01$) and pelleted lactobacilli ($P < 0.001$) both significantly reduced DNA damage in rat coloncytes following MNNG challenge.

It seems likely that the above results are a consequence of binding of the mutagens by the LAB (Bolognani et al., 1997; Zhang et al., 1991). Whether such a mechanism operates *in vivo* is questionable, since binding appears to be highly pH dependent and easily reversed and does not appear to affect uptake of carcinogens from the gut, neither does it have any apparent effect on *in vivo* mutagenicity in the liver (Bolognani et al., 1997); nevertheless there is growing evidence that probiotics and prebiotics can reduce genotoxicity in the colon.

26.3.3 Anti-Genotoxicity of Probiotics and Prebiotics *In Vivo*

Using the technique of single cell microgel electrophoresis (Comet assay), Pool-Zobel et al. (1996) investigated the ability of a range of species of LAB to inhibit DNA damage in the colon mucosa of rats treated with the carcinogens MNNG or

1,2-dimethylhydrazine (DMH). All the strains of lactobacilli and bifidobacteria tested – *L. acidophilus* (isolated from a yoghurt), *L. gasseri, L. confusus*, *B. breve* and *B. longum*, prevented MNNG-induced DNA damage when given at a dose of 10^{10} cells/kg body weight, 8 h before the carcinogen. In most cases the DNA damage was reduced to a level similar to that in untreated rats. *Streptococcus thermophilus* was not as effective as the other LAB strains.

The protective effect was dose dependent: doses of *L. acidophilus* representing 50 and 10% of the original dose were less effective in reducing MNNG-induced DNA damage. Importantly, heat-treatment of *L. acidophilus* abolished its antigenotoxic potential indicating the importance of viable cells. Similar results were obtained when the LAB strains were tested in rats given DMH as the DNA damaging agent. Again, all the lactobacilli and bifidobacteria strongly inhibited DNA damage in the colon mucosa, whereas *Strep. thermophilus* was much less effective. There was evidence of strain differences in antigenotoxic effects: Of three strains of *Strep. thermophilus*, two were ineffective and one exhibited protection against DNA damage.

The Comet assay has also been used to evaluate the effect of a prebiotic, lactulose, on DNA damage in the colonic mucosa. Rats that were fed a diet containing 3% lactulose and given DMH, exhibited less DNA damage in colon cells than similarly treated animals fed a sucrose diet. In the latter animals, the percentage of cells with severe DNA damage comprised 33% of the total compared with only 12.6% in the lactulose-fed rats (Rowland et al., 1996).

Horie et al. (2003) investigated the influence of a probiotic mixture consisting of *Streptococcus fecalis, Clostridium butyricum* and *Bacillus mesentericus* on the carcinogenicity of 2-amino-9H-pyrido[2,3-b]indole (2-amino-alpha-carboline;) in human-flora-associated mice. Supplementation led to a significant reduction of DNA adducts (as measured by 32P-high-performance liquid chromatography) in the colonic tissue of probiotic fed rats.

Klinder et al. (2004) also showed that prebiotic and synbiotic supplementation (8 months) caused a reduction in the genotoxicity of fecal and cecal samples obtained from azoxymethane. Park et al. (2007) also showed significant antigenotoxic activity in DMH challenged rats when supplemented with *Bacillus polyfermenticus* (3×10^8 cfu/d). The increased DNA damage in leukocytes and reduced plasma antioxidant status exerted by DMH treatment was ameliorated following *Bacillus polyfermenticus* supplementation.

Vallarini et al. (2008) investigated the antigenotoxic effect of *L. casei* supplementation in rats before and after DMH treatment. They isolated rat colonocytes and hepatocytes and using the Comet assay showed significant antigenotoxic

effects in colonocytes before and after DMH, although no protective activity was noted in hepatocytes.

Oberreuther-Moschener et al. (2004) demonstrated that the fecal water samples from human volunteers (n = 9) consuming probiotic yoghurt (*L. acidophilus* 145 and *B. longum* 913) elicited significantly more oxidized DNA pyrimidine bases, however the overall fecal water genotoxicity was significantly lower compared to the standard yogurt ($P < 0.05$). Moreover, the fecal water of volunteers supplemented with the probiotic yoghurt also had significant antigenotoxic effects ($P < 0.05$) against H_2O_2 induced DNA damage on HT29-clone19A cells.

Rafter et al. (2007) investigated the influence of 12 weeks synbiotic supplementation (*L. rhamnosus* GG (LGG) + *B. lactis* Bb12 + a commercial fructan preparation-Synergy 1) on selected cancer biomarkers in polypectomized and cancer patients. Synbiotic supplementation resulted in significant reductions in DNA damage in the colonic mucosa of polyp patients.

The above results provide evidence that both LAB and prebiotics may have protective effects against the early stages of colon cancer.

26.3.4 Effect of Probiotics and Prebiotics on Pre-Cancerous Lesions in Laboratory Animals

Aberrant crypts (AC) are putative pre-neoplastic lesions seen in the colon of carcinogen treated rodents. In many cases a focus of two or more crypts is seen and is termed an aberrant crypt focus (ACF). Aberrant crypts are induced in colonic mucosa of rats and mice by treatment with various colon carcinogens such as azoxymethane (AOM), DMH and IQ. The findings of significantly more ACF with four or more crypts in rats with tumors compared with those without tumors suggests that large ACF may be a predictor of eventual tumor incidence (Pretlow et al., 1992). Studies (*Table 26.4*) have employed various treatment regimes with differences in the sequence of exposure to carcinogen and probiotic/prebiotic to allow conclusions to be drawn about the stage of carcinogenesis affected. In the majority of studies the protocol involved feeding the probiotic for about 1 week, followed by dosing with carcinogen and then continued probiotic administration until animals were killed prior to ACF assessment. Thus the probiotic treatment covered both initiation and early promotion stages. In the "promotion" protocol, the rats were dosed with carcinogen prior to probiotic treatment.

Table 26.4

Effect of probiotics/prebiotics and synbiotics on colonic aberrant crypt foci in laboratory animals (*Cont'd* p. 1018)

Species	Carcinogen	Probiotic/prebiotic	Result	Author
Male F344 rats	AOM (s.c.)	Lyophilized *B. longum* (1.5% and 3% dietary)	Significant inhibition of total ACF ($P < 0.01$). Significant reduction in total AC per colon ($P < 0.001$)	Kulkarni and Reddy (1994)
Male aF344 rats	AOM (s.c.)	*B. longum* (1×10^8 cells/g of feed, rats fed *ad libitum*) lactulose (2.5%) or both	Significant reduction in ACF in rats consuming BI, L, BI + L (*P, 0.05*). Rats fed BI + L had significantly fewer ACF than rats consuming BI or L alone	Challa et al. (1997)
Male F344 rats	DMH (i.p.)	*Bifidobacterium* spp. (6×10^9 cells/animal/day) in cell suspension, or fermented milk. Skim milk powder	Significant ($P < 0.05$) inhibition of AC: 61% (*Bifidobacterium*) 51% (skim milk) 49% (fermented milk)	Abdelali et al. (1995)
Male Sprague-Dawley rats	AOM (s.c.)	*B. longum* (4×10^8 viable cells/g of diet) or inulin (5%w/w dietary)	Total ACF decreased by 74% in rats treated with probiotic + prebiotic (synbiotic effect). Numbers of the large ACF (>4 AC per focus) were significantly decreased ($P < 0.05$) by 59% in rats fed probiotic + prebiotic	Rowland et al. (1998)
Male wistar rats	DMH (gavage)	Skim milk, skim milk + bifidobacteria (10^9/day) skim milk + fructooligosaccharide and skim milk + bifidobacteria + fructooligosaccharide; *L. acidophilus* (10^8)	Inconsistent results	Gallaher et al. (1996)
Weanling male F344 rat	AOM (s.c.)	Inulin (10%) in diet	No significant effect on ACF but reduced the number of AC/cm^2	Rao et al. (1998)
F344 rat	AOM (s.c.)	*L. acidophilus* NCFMTM (lyophilized) in diet	Significant suppression of colonic ACF	Rao et al. (1999)

Male F344 rat	AOM (s.c.)	Oligofructose (10%) or inulin (10%) in diet	Significant inhibition of ACF/colon – more pronounced for inulin ($P < 0.0006$) than for oligofructose ($P < 0.02$). Crypt multiplicity also inhibited in animals fed inulin ($P < 0.02$) or oligofructose ($P < 0.04$)	Reddy et al. (1997)
Rats	AOM (s.c.)	*L. casei* Shirota	Reduced number of large ACF	Yamazaki et al. (2000)
Male Sprague Dawley rats	DMH, AOM	*B. longum, L. casei, L. acidophilus*	Significant inhibition of ACF only when rats fed a high fat diet	Bolognani et al. (2001)
Male Fischer rats	AOM	*L.acidophilus* NCFMTM	Significant suppression of ACF	Chinathalapally et al. (1999)
Mice ()	DMH (s.c.)	Inulin (10%)	Inulin significantly reduced total ACF ($P < 0.05$), Oligofructose decreased total ACF ($P = 0.08$)	Buddington et al. (2002)
		Oligofructose (10%)		
Fischer 344 rats	DMH	Inulin – Raftiline™ (5 and 10%)	Raftiline (5 and 15%) and Raftilose (15%) reduced the total number of ACF	Poulsen et al. (2002)
		Oligofructose – Raftilose ™ (5 and 10%)		
Male F344 rats	AOM (s.c.)	Long chain Inulin (Raftilin™) 10% diet	Significant reduction in ACF in both the proximal and distal colon ($P < 0.001$)	Verghese et al. (2002a)
Male F344 rats	AOM (s.c.)	Long chain Inulin (Raftilin™) 0, 2.5, 5.0,10% in diet	Decrease the incidence of ACF ($R^2 = 0.9915$) and total crypts ($R^2 = 0.9828$) in a dose dependant manner ($P < 0.01$)	Verghese et al. (2002b)
Male F344 rats	AOM (s.c.)	*Clostridium butyricum* (CBM588), High Amylose maize starch (HAS)	CB had no effect on ACF, HAS showed a decrease trend	Nakanishi et al. (2003)
			Mixture of both significantly decreased ACF 35% ($P < 0.05$)	

Table 26.4

Species	Carcinogen	Probiotic/prebiotic	Result	Author
Male Sprague-Dawley rats	DMH (gavage)	FOS 60 g/kg (fructoligo-95P; Meiji) XOS 60 g/kg (xylooligo-95P; Suntory)	FOS and XOS decreased mean number of multicrypt clusters of aberrant crypts by 56 and 81%, respectfully ($P < 0.05$)	Hsu et al. (2004)
Jcl:ICR mice	DMH	*Butyrivibrio fibrisolvens*	Significant reduction of ACF and aberrant crypts	Ohkawara et al. (2005)
Male F344 rats	AOM (s.c.)	Inulin (Raftiline™) 150 g/kg Oligofructose (Raftilose™) 150 g/kg	Oligofructose and Inulin significantly increased the number of large ACF's but fewer tumors ($P < 0.05$)	Jacobson et al. (2006)
Male F344 rats	DMH (s.c.)	*Bacillus polyfermenticus* (BP) (3.1 × 108 cfu/d)	Significant inhibition of ACF ($P < 0.05$). Rats fed high fat low fiber diet + BP had 50% less ACF than rats fed high fat low fiber diet	Park et al. (2007)
Male F344 rats	DMH (s.c.)	*Bacillus polyfermenticus SCD* (3 × 106 cfu/d)	Significant inhibition ($P < 0.05$) of ACF (40%)	Lee et al. (2007)
Male Sprague-Dawley rats	DMH (s.c.)	FOS	Significant inhibition of ACF in a dose dependant manner ($P < 0.001$)	Sung and Choi (2008)

26.3.4.1 Effect of Probiotic Treatment Alone

A variety of studies have been carried out using AOM or DMH to determine the effects of specific probiotics on ACF formation. Kulkarni and Reddy (1994) reported inhibition in ACF formation of about 50% when male F344 rats were fed *B. longum* in the diet (1.5 and 3% of a lyophilized culture containing 2×10^{10} cfu/g) for 5 weeks and injected subcutaneously with AOM once weekly for 2 weeks. Since dietary treatments were started 5 weeks prior to administration of the carcinogen dose results do not allow deductions to be made about the stage of carcinogenesis affected. There were no differences between the animals fed the 1.5 and 3% *B. longum* diets.

A similar study was carried out by Challa et al. (1997) who observed a 23% reduction in total colonic ACF and a 28% reduction in total AC in rats given a diet containing 0.5% *B. longum* (1×10^8 viable cells/g of feed). Animals were fed the experimental diet before treatment with AOM and throughout the experiment.

Abdelali et al. (1995) compared the effects of *Bifidobacterium* spp. administered in diet and also fed as a fermented milk product. The amounts of organisms consumed were similar (6×10^9 cells/day). DMH was given 4 weeks after the LAB and the latter treatments continued for a further 4 weeks before ACF assessment. The dietary bifidobacteria appeared to be slightly more effective in reducing ACF than the bifidobacteria – fermented milk (61 and 49% reduction respectively). Interestingly however, skim milk alone reduced ACF numbers by 51%.

Rowland et al. (1998) in a study of *B. longum* (6×10^9 cfu/day) in AOM-treated Sprague Dawley rats, demonstrated a significant reduction of 26% in total ACF by comparison to control animals. The changes were seen in only small ACF (1–3 AC per focus). Since the probiotic treatment began 1 week after the carcinogen exposure, the results indicate an effect on the early promotional phase of carcinogenesis.

Not all ACF studies with probiotics have yielded positive effects. Gallaher et al. (1996) who used a "promotion" protocol with *B. longum* and *L. acidophilus*, obtained inconsistent results, which they attributed to differences in ages of rats when DMH was administered.

Supplementation with the probiotic strain *L. acidophilus* NCFMTM significantly suppressed AOM-induced ACF in a dose dependant manner (Rao et al., 1999). Yamazaki et al. (2000) supplemented rats with *L. casei*, which resulted in a significant reduction in the number of large ACF.

Marotta et al. (2003) showed that supplementation of a probiotic mixture significantly reduced ratio of ACF/colon and of aberrant crypt per colon and per each single focus ($P < 0.05$) in AOM challenged rats.

Supplementation of butyrate producing bacteria *Butyrivibrio fibrisolvens* MDT-1 (10^9 cfu/dose, 4 weeks) once a week or 3 times a week reduced ACF formation by 25 and 60%, respectfully, in DMH challenged rats. The number of aberrant crypts was also significantly reduced by 40 (once per week) and 70% (three times per week) (Ohkawara et al., 2005).

Bacillus polyfermenticus supplementation (3.1×10^8 cfu/d) has been shown to decrease the incidence (50%) of DMH induced ACF in F344 rats fed with a high fat-low fiber diet (Park et al., 2007). Moreover, in a similar study by Lee et al. (2007) supplementation with *Bacillus polyfermenticus SCD* (3×10^6 cfu/d) led to a 40% reduction of ACF.

The basal diet appears to be important in modulating the protective effects of probiotics on ACF. Bolognani et al. (2001) reported that when rats were fed a basal diet high in starch and low in fat, treatment with various strains of LAB or inulin resulted in no consistent reduction in ACF numbers (induced by methyl-N-nitrosourea, 1,2-dimethylhydrazine, or AOM). However, when the diet was changed to a high fat low carbohydrate diet (i.e., more similar to a Western diet) treatment with *L. acidophilus* or inulin significantly decreased AOM-induced colonic ACF.

26.3.4.2 Prebiotic Treatment and Colonic ACF

Prebiotics alone appear to give inconsistent results on carcinogen induced ACF induction which may be partly a consequence of differences in carcinogen and treatment regimes used. For example Rao et al. (1998) reported that inulin (10% in diet) had no significant effect on total ACF in colon, or their multiplicity, in F344 rats, although curiously a significant decrease in ACF/cm^2 of colon was reported. A study by Gallaher et al. (1996) on *Bifidobacterium* spp. and FOS (2% in diet) gave inconsistent results with only 1 out of 3 experiments showing a decrease in DMH-induced ACF. In contrast, Verghese et al. (2002b), reported a a dose dependant decrease in the incidence of ACF and total crypts ($P < 0.01$) after inulin supplementation (0, 2.5, 5 and 10 g/100 g diets) in AOM challenged rats.

The effects of prebiotics on ACF may be dependent on the chain length of the oligosaccharides, since a number of studies report more potent inhibition by longer chain inulin than by FOS.

Reddy et al. (1997) compared short- (FOS) and long-chain (inulin) oligosaccharides incorporated at a level of 10% in diet on AOM-induced ACF in rats. The oligosaccharides were fed before carcinogen treatment and throughout the

experiment and significant decreases of approximately 25 and 35%, respectively in total ACF were reported. The decreases seen were almost entirely in the smaller ACF (<3 AC per focus) and inhibition by inulin appeared to be more pronounced than that of FOS.

Buddington et al. (2002) investigated the influence of inulin or FOS (10% diet w/w) supplementation on ACF occurrence in DMH challenged mice. Inulin, but not FOS fed mice had significantly lower ACF numbers than controls. In a similar study by Poulsen et al. (2002) in DMH-treated rats long-chained inulin fructan (5 or 15%) significantly inhibited the numbers of total ACF after 5 and 10 week supplementation, whereas short-chained fructan (15%) inhibited the number of total ACF after 5 and 10 week but significantly increased the numbers of medium and large ACF after 10 week.

Some studies have found that fructans have differential effects on ACF and tumors. For example Jacobson et al. (2006), reported that FOS or long chain inulin (15% in diet) increased the number of ACF but significantly reduced the tumor incidence. A study by Caderni et al. (2003) showed similar results when rats were fed the synbiotic containing Synergy 1 alongside *L. rhamnosus* GG, *L. delbrueckii* subsp. *rhamnosus* and *B. lactis* Bb12. Supplementation caused increased ACF multiplicity after 16 weeks, however significantly reduced tumor incidence following 32 weeks in AOM challenged rats.

There are limited studies on prebiotics other than FOS and inulin in this area. Challa et al. (1997) demonstrated a small reduction (22%) in total ACF in AOM treated F344 rats when the synthetic, non-digestible disaccharide lactulose was incorporated in the diet at 2%. Hsu et al. (2004) compared the influence, of FOS (60 g/kg) and XOS supplementation on DMH induced aberrant crypts in rats. FOS and XOS decreased the mean number of multicrypt clusters of aberrant crypts by 56 and 81%, respectfully ($P < 0.05$).

Buecher et al. (2003) investigated the influence of the NSAID celecoxib and FOS administered alone and together in AOM challenged rats. No significant reduction in ACF was observed with either FOS or celecoxib alone. However, the combination of FOS and celecoxib resulted in a 61% reduction in the number of ACF ($P < 0.01$).

26.3.4.3 Synbiotic Treatments and Colonic ACF

Both Challa et al. (1997) and Rowland et al. (1998) studied the effect of combined treatment of probiotic and prebiotic on ACF numbers. The combination of

B. longum and lactulose resulted in a 48% inhibition of colonic ACF, which was significantly greater than that achieved by either *B. longum* or lactulose alone (Challa et al., 1997). Similarly, Rowland et al. reported a decrease in total ACF of 74% in rats given *B. longum* + inulin (by comparison to 29 and 21% reduction achieved by *B. longum* or inulin alone). Importantly, the combined administration of probiotic and prebiotic reduced large ACF by 5% whereas the individual treatments had no effect (Rowland et al., 1998). Nakanishi et al. (2003) showed that supplementation with *Clostridium butyricum* (CB) in AOM challenged rats had no significant affect on ACF occurrence. However, CB supplemented alongside high amylose maize starch (a poorly digestible carbohydrate with prebiotic activity) decreased the number of ACF significantly ($P < 0.05$) indicating a degree of synbiotic activity.

26.3.5 Effect of Probiotics and Prebiotics on Colon Tumor Incidence in Laboratory Animals

Goldin and Gorbach (1980) investigated the effect of *L. acidophilus* on colon tumor incidence in rats treated with DMH (*Table 26.5*). A reduction in colon cancer incidence (40% vs. 77% in controls) was evident in animals receiving *L. acidophilus* after 20 weeks but no difference was discernible at 36 weeks suggesting that the lactobacilli had increased the latency period, or induction time, for tumors.

Administration of dietary *B. longum* (0.5% lyophilized *B. longum* in diet, 1×10^{10} live bacterial cells/day) significantly inhibited the formation of IQ-induced colon and liver tumors and multiplicity (tumors/animal) of tumors in colon, liver and small intestine in male rats (Reddy and Rivenson, 1993). The percentage decrease in tumor incidence was 80% in liver and 100% in colon. In female rats, dietary supplementation with *Bifidobacterium* cultures also decreased the IQ induced mammary carcinogenesis to 50% and liver carcinogenesis to 27% of that on the control diet, but the differences were not significant. There were however significant changes in tumor multiplicity in the mammary gland.

Goldin et al. (1996) investigated the effect of *L. rhamnosus* GG in DMH treated rats given either before, during or after DMH exposure (initiation + promotion protocol) or after (promotion protocol) the carcinogen treatment. Using the former protocol, a significant decrease was seen in the incidence of colon tumors (71% vs. 100% in control rats), and the number of tumors per tumor bearing animal (1.7 vs. 3.7 in controls). However, when *L. rhamnosus* GG was

Table 26.5

Effect of probiotics and prebiotics on tumor incidence in laboratory animals (*Cont'd* p. 1024)

Species	Endpoint	Carcinogen	Probiotic/ prebiotic	Result	Author
F344 rats	Incidence of colon tumors	DMH	*L. acidophilus*	Colon tumor incidence lower in probiotic fed animals (40% vs. 77% in controls)	Goldin and Gorbach (1980)
F344 rats	Colon, liver, mammary tumors (incidence and multiplicity)	IQ in diet	*B. longum* (1×10^{10} live bacterial cells in diet)	Suppression of colon ($P < 0.05$), liver ($P < 0.05$) and mammary (NS) tumor incidence	Reddy and Rivenson (1993)
C57BL/CJ-*Min*/ + mice	Colon tumor incidence	N/A	Short chain FOS (5.8%)	Significant reduction in colon tumors ($P < 0.01$)	Pierre et al. (1997)
C57BL/6J *Min* mice	Small intestine and colon tumors	N/A	Inulin (2.5% in diet)	Non significant increase in adenomas in small intestine	Mutanen et al. (2000)
C57BL/6J *Min* mice	Small intestine and colon tumors	N/A	Inulin (10% in diet)	Significant increase in number and size of adenomas in distal small intestine. Non significant decrease in size of colon tumors	Pajari et al. (2003)
Male F344 rats	Incidence and multiplicity of colon tumors	DMH (s.c.)	*L. rhamnosus* GG ($2–4 \times 10^{10}$ organisms/ day	Lower incidence of tumors ($P < 0.012$) and tumor multiplicity ($P < 0.001$) when rats given *L. rhamnosus* GG throughout experiment. No effect when *L. rhamnosus* GG given after DMH	Goldin et al. (1996)
Male Sprague Dawley rats	Incidence, size, multiplicity of colon tumors	DMH	*L. acidophilus, B. animalis, L. rhamnosus, Strep. thermophilus*	*L. acidophilus* reduced incidence, size and number of malignant tumors	McIntosh et al. (1999)
Male F344 rats	ACF, tumors	AOM	LGG, + B. lactis Bb12 + Inulin/ FOS (synergy 1)	Increase in ACF, decrease in colon tumors	Caderni et al. (2003)

Table 26.5 (*Cont'd* p. 1025)

Species	Endpoint	Carcinogen	Probiotic/ prebiotic	Result	Author
Mice	Tumor incidence	DMH	*L. tolerans*, *L. acidophilus*, *L. fermentum*, and *L. rhamnosus* supernatants	Decreased colon tumor and microadenoma incidence	Fukui et al. (2001)
Rat	Tumor incidence	AOM	*L. casei* Shirota	Decreased number of rats with colon tumors and number of tumors per rat	Yamazaki et al. (2000)
Male F344 rats	Tumor incidence	AOM (s.c.)	*L. rhamnosus* GG, *B. lactis* Bb 12 (5×108 cfu/g per strain+/− Synergy 1 (100 g/kg, w/w)	Probiotic treatment reduced tumor incidence (not significant) Synergy 1 treatment alone and with probiotic reduced adenoma ($P < 0.01$) and tumor ($P < 0.01$) incidence compared to control and probiotic alone	Femia et al. (2002)
Male Sprague-Dawley rats	Tumor incidence	AOM (s.c.)	Probiotic mixture	Probiotic supplementation resulted in 45% reduction the incidence of rats with tumor and a reduction in number of tumors.	Marotta et al. (2003)
Male Wistar rats	Tumor incidence	DMH (s.c.)	*Lactococcus lactis nz9000*, 5×10^9 cells/ day	No significant effects	Li and Li (2003)
Male F344 rats	Tumor incidence	AOM (s.c.)	*L. rhamnosus* GG, *L. delbrueckii rhamnosus, B. lactis* Bb12, (5×108 cfu/g per strain – Synergy 1(100 g/kg, w/w)	Synbiotic treatment reduced incidence and number of tumors ($P < 0.01$)	Caderni et al. (2003)

Table 26.5

Species	Endpoint	Carcinogen	Probiotic/ prebiotic	Result	Author
Male F344 rats	Tumor incidence	AOM (s.c.)	Inulin (Raftiline™) 150 g/kg Oligofructose (Raftilose™) 150 g/kg	Rats fed Oligofructose significantly reduced the tumor incidence ($P < 0.05$). Inulin and Oligofructose reduced tumor frequency per animal ($P < 0.05$)	Jacobson et al. (2006)
Balb/c mice	Transplanted mammary tumor growth	–	FOS or Inulin 15% in diet	Reduced tumor size	Taper and Roberfroid 1999
CH3 mice	Transplanted mammary tumor metastasis	–	FOS or Inulin 15% in diet	Reduced metastatic spread	Taper and Roberfroid (2000)
CH3 mice	Transplated liver tumor growth	–	FOS or Inulin 15% in diet	Reduced tumor size	Taper and Roberfroid 1999
NMRI mice	Transplated ascites tumor growth	–	FOS or Inulin 15% in diet	Increased life span	Taper and Roberfroid (2002)

DMH: 1,2 dimethylhydrazine; *AOM*: Azoxymethane; *sc*: subcutaneous administration; IQ 2-amino-3-methylimidazo/4,5-f/quinoline

administered after DMH, no decrease in tumor incidence was seen indicating that the effect of the LAB was on the initiation stage rather than on the promotion stage of tumorigenesis. In this study, the rats were fed basal diets either high or low in fat content. Although the decrease in colon tumor incidence induced by the probiotic was similar on the two diets, the effects on tumor multiplicity were more pronounced in the animals fed a high fat diet.

McIntosh et al. (1999) examined a number of freeze-dried probiotic strains [*L. acidophilus* (Delvo Pro LA-1), *L. rhamnosus* GG, *B. animalis* (CSCC1941), and *Strep. thermophilus* (DD145)] for their influence on DMH-induced intestinal tumors in rats fed a high fat semi-purified diet. *L. acidophilus* was the most effective strain reducing colon tumor incidence by 25% with tumor burden being reduced from 10 in control to 3 in the *L. acidophilus* treated rats.($P < 0.05$). Large

intestinal tumor mass index was also lower for the *L. acidophilus* treatment than for control (1.70 and 0.10, respectively, $P < 0.05$), and importantly no adenocarcinomas were present in the colons suggesting that *L. acidophilus* may be capable of suppressing the conversion of benign to malignant tumors.

Not all studies with lactic acid bacteria (LAB) have proved positive – Li and Li (2003) failed to see any protective effect on tumor incidence of *Lactococcus lactis* NZ9000 (5×10^9 cells/day) supplementation.

There are fewer reports on prebiotic and synbiotics than on probiotics in terms of tumor incidence but overall the studies indicate protective effects. Jacobson et al. (2006) compared the incidence of tumors in AOM challenged rats following consumption of FOS or inulin (15% diet w/w). Significantly less rats developed colon tumors in the FOS group ($P < 0.05$) compared to the control diet. The total number of tumors developed per rat was significantly reduced following both FOS ($P < 0.01$) and inulin ($P < 0.05$) supplementation. However supplementation had no effect on the malignancy of the tumors.

Femia et al. (2002) investigated the protective effects of prebiotic (Synergy 1), probiotic (*B. lactis* Bb12 and *L. rhamnosus* GG, (5×10^8 cfu/g diet) or synbiotic combination of the two, against AOM-induced colon tumors in rats. Prebiotic fed groups (prebiotic and synbiotic groups) resulted in lower adenoma ($P < 0.001$) and adenocarcinoma ($P < 0.05$) incidence than in the rats not given prebiotic (probiotic and control). Prebiotic and synbiotic groups had in fact nine cancers over 84 tumors (11%) ($P = 0.04$), while controls and probiotic groups had 19 cancers over 83 tumors (23%).

Caderni et al. (2003) showed that synbiotic supplementation (Synergy 1 plus *L. rhamnosus* GG, *L. delbrueckii* subsp. *rhamnosus*, and *B. lactis* Bb12) significantly reduced the number of rats with AOM induced colon tumors ($P < 0.01$), as well as the number of tumors per rat ($P < 0.01$).

A mouse model has recently been developed (*Min* mice) in which the animals are heterozygous for a non-sense mutation of the *Apc* gene, the murine homologue of APC. These mice, which develop spontaneous adenomas throughout the small intestine and colon within a few weeks, have been used for testing of chemopreventive agents targeted against cancerous lesions. Results from studies on inulin and FOS in this model have been conflicting, with both inhibitory and stimulatory effects on tumors reported. In one study *Min* mice were fed various diets containing wheat bran, resistant starch or FOS (5.8% in diet) for 6 weeks. Tumor numbers remained unchanged from the control (fiber free diet) in the mice fed either wheat bran or resistant starch, but a significant reduction in colon tumors was observed in rats receiving the diet supplemented with FOS.

Furthermore, 4 out of the 10 FOS fed animals were totally free of colon tumors (Pierre et al., 1997). These results contrast markedly with those of Mutanen and co-workers using the same model. In the first of her studies, *Min* mice fed a purified high fat (40% energy) diet with 2.5% inulin had higher numbers of adenomas in the small intestine than the control animals fed the high fat diet alone (49.3 ± 16.3 vs. 34.6 ± 7.1) although the results were not significant (Mutanen et al., 2000). The tumor incidence in the large intestine was also higher (again non significantly) in the inulin fed mice (100% vs. 71%). A subsequent study (Pajari et al., 2003) using a higher inulin dose (10%) confirmed these results with increases being seen in the number of adenomas in the small intestine and colon (as before non-significant) and significant increases in tumors in the distal small intestine after 9 weeks of treatment. Interestingly, although the adenoma size in the small intestine was significantly increased in the inulin-fed mice, in the colon the size was reduced from 3.72 to 2.54 mm (non significant). Increases were also seen in the accumulation of cytosolic ß-catenin in adenoma tissue in inulin fed mice, which is associated with hyperproliferation.

26.3.5.1 Effects of Prebiotics on Other Tumor Sites

Taper and Roberfroid (1999) investigated the effects of inulin, FOS or pectin (15% in the diet) on the growth of intramuscularly transplanted mouse tumors, belonging to two tumor lines – TLT (a mammary tumor) and EMT6 (a liver tumor). The growth of both tumor lines was significantly inhibited by supplementing the diet with nondigestible carbohydrates. In subsequent studies, the same authors demonstrated that FOS or inulin (15% in diet) reduced the incidence of mammary tumors induced in Sprague-Dawley rats by methylnitrosourea; and decreased the incidence of lung metastases of a malignant tumor implanted intramuscularily in mice (Taper and Roberfroid, 2005).

26.3.6 Probiotics, Prebiotics and Cancer Human Epidemiological Studies

There are no specific epidemiological studies on pro- and prebiotics and CRC and relatively few on fermented milk products (*Table 26.6*). A case-control study in the Netherlands showed that certain fermented dairy products may confer a protective effect against breast cancer. The results indicated that consumption

Table 26.6

Effect of probiotics and prebiotics on cancer in humans – epidemiological studies

Subjects	Type of cancer	Probiotic/ prebiotic	Result	Author
289 Population controls and 133 breast cancer cases	Breast cancer	Fermented milk products (yoghurt, buttermilk and kefir)	Fermented milk consumption (>225 g/day) reduced odds ratio to 0.5	van't Veer et al. (1989)
Control group 182 men and 245 women. Cancer cases 109 men and 62 women	Small and large colon adenoma, and colon cancer	Yoghurt consumption)	Inverse relationship yoghurt consumption (0.5–1/ day) with risk of large adenomas in men and women	Boutron et al. (1996)
152 Proximal colon cancer patients, 201 distal colon cancer patients and 618 general population controls	Colon cancer	Fermented milk consumption	Inverse association of colon cancer with the consumption of fermented milk	Young and Wolf (1988)
746 Colon cancer patients and 746 controls	Colon cancer	Yoghurt	Protective effect against colon cancer	Peters et al. (1992)
331 Men and 350 women with adenomatous polyps of colon/ rectum and controls (9,159 men and 8,585 women)	Colorectal adenomas	Fermented dairy products	Inverse association (nonsignificant) between yoghurt consumption and adenomas in men and women	Kampmann et al. (1994a)
232 Colon cancer patients and 259 population controls	Adenocarcinoma of colon	Fermented dairy products	Positive, significant association (OR 1.52) in men; negative, nonsignificant association in women	Kampmann et al. (1994b)

of > 225 g per day of fermented dairy products (yoghurt, buttermilk, curds and kefir) reduced the odds ratio to 0.50 (van't Veer et al., 1989).

Results from a case-control study by Boutron et al. (1996) showed a significant ($P = 0.03$) inverse relationship between risk of large colonic adenomas

in both men and women and consumption of moderate amounts (0.5–1 pot per day) of yoghurt. The odds ratios were 0.6 and 0.5, respectively for the two levels of yoghurt consumption. There was no relationship between CRC risk and yoghurt consumption. Other population-based case control studies have provided evidence of inverse associations of CRC risk and consumption of fermented dairy products (Young and Wolf, 1988) and yoghurt (Peters et al., 1992). Kampman et al. (1994a) reported a non-significant inverse relationship between yoghurt consumption and colonic adenomas. This finding, however, was not confirmed in a further case control study in the Netherlands of CRC risk and fermented dairy products, which revealed a small significant positive association in men (OR 1.52) and a small, non-significant inverse association in women (OR 0.77) (Kampman et al., 1994b).

The fact that epidemiological studies have not provided any conclusive evidence for a role for pro- or prebiotics in the prevention of CRC is perhaps not surprising given the long period of development required for cancer (10–25 years) and the relatively recent introduction of pro and prebiotics on the market.

26.3.7 Effects of Probiotics and Prebiotics in Human Intervention Studies

For human intervention trials (*Table 26.7*), cancer is an impractical endpoint in terms of numbers of subjects, cost, study duration and ethical considerations. An alternative strategy employed in recent studies is to use early or intermediate biomarkers of cancer such as DNA damage and cell proliferation in colonic mucosa and genotoxic activity of fecal extracts ("fecal water") (Gill and Rowland, 2002).

Hayatsu and Hayatsu (1993) examined the effect of 3 week oral administration of *L casei* in six healthy non-smokers on the urinary mutagenicity of dietary fried ground beef using the Ames assay (*Salmonella typhimurium* TA 98, with S9 mix). The six people were divided into two groups, one group for administration of the *L. casei* 3×10^{10} cells/day and another group for supplementation with *L. casei* 1.5×10^{11} cells/day for 3 weeks. Urine was collected before meat meals and 0–12 and 12–24 h urines were collected after the meat meal. A suppressive effect of *L. casei* administration was observed (6–67% of the control group mutagenicity) when control urinary mutagenicity was compared to test sample results.

Table 26.7

Effect of probiotics and prebiotics on cancer/cancer biomarkers in human intervention studies (*Cont'd* p. 1031)

Subjects	Endpoint	Probiotic/ prebiotic	Result	Author
Healthy male and female subjects (n = 18)	Fecal water (FW) cytoxicity in HT29 cells and genotoxicity (Comet assay)	Dairy products vs. low dairy product diet	Decreased cytotoxicity of fecal water during high dairy product intake effect. No effect on genotoxicity	Glinghammar et al. (1997)
Healthy subjects (n = 6)	Urinary mutagenicity after fried beef consumption	*L. casei*	Decrease in urinary mutagenicity ($P < 0.001$)	Hayatsu and Hayatsu (1993)
Healthy subjects (n = 11)	Urinary and fecal mutagenicity after fried beef consumption	*L. acidophilus*	Probiotic consumption decreased urinary mutagen excretion by 50–70% and fecal mutagen excretion by 30%	Lidbeck et al. (1992)
Patients with colonic adenomas (n = 20)	Cell proliferation in rectal mucosa biopsies	*L. acidophilus* and *B. bifidum*	LAB adm inistration reduced rectal proliferation only in patients with high basal proliferation rates	Biasco et al. (1991)
Polypectomized (n = 43) and colon cancer (n = 37) patients	Various biomarkers in rectal biopsies. Various FW end points	Synergy1 (12 g/d) and *B. lactis* Bb12 and *L. delbreuckii* subsp. *rhamnosus* GG	Reduced colorectal proliferation (biopsy). Reduced capacity of FW to; induce necrosis in colonic cells, exert genotoxicity	Rafter et al. (2007)
			FW enhanced epithelial barrier function	
Adenoma patients (n = 42) Adenoma free patients (n = 28)	Bile acid and Butyrate concentrations	subcutaneous FOS	Elevated butyrate in adenoma patients, Reduced lithocholic acid ($P < 0.02$), increased cholic acid ($P = 0.02$), ursodeoxycholic acid and total bile acids ($P < 0.05$) in ademona free patients	Boutron-Ruault et al. (2005)

Table 26.7

Subjects	Endpoint	Probiotic/ prebiotic	Result	Author
Healthy human female volunteers (n = 9)	Genotoxicity of FW in HT29 cells	Probiotic yoghurt containing *L. acidophilus* 145 and *B. longum* 913 1 × 109/g (300 g/d)	The genotoxicity of FW on HT29 cells was significantly reduced afterprobiotic supplementation ($P < 0.05$)	Oberreuther-Moschener et al. (2004)
Patients with resected adenomas/early cancers	Adenoma recurrence	*L. casei* Shirota ~3 × 10^{10}/d for 4 years	Non significant reduction in adenoma numbers. Significant reduction in adenomas with moderate or severe atypia after 4 year	Ishikawa et al. (2005)

FW Fecal water; Synergy 1 FOS/Inulin mixture

Lidbeck et al. (1992) carried out a study involving 11 subjects fed, as part of their diet, fried hamburgers which are a source of pyrolysate mutagens detectable in urine. *Lactococcus* milk was given as a control (10^{10}–10^{11}/day) 2 days prior to 6 days after dietary supplementation with fried hamburgers. The probiotic *Lactobacillus acidophilus* was given to the second group at a dose of 1–5 × 10^{11} cells per day starting again 2 days before the hamburger addition and lasting for a further 6 days afterwards. Consumption of LAB decreased urinary excretion of mutagens by 50–70% and excretion of fecal mutagens was decreased by 30%.

Increased cell proliferation in the mucosal crypts is considered to be a marker of elevated cancer risk. In a study of the effect of LAB on cell proliferation in the rectal mucosa, Biasco et al. (1991) administered six capsules containing 10^9 *L. acidophilus* and 10^9 *B. bifidum* daily for a period of 3 months to 20 patients with colonic adenomas. Four rectal biopsies were taken at baseline and after treatment and cell proliferation in the upper part of the rectal mucosa crypts assessed by tritiated thymidine incorporation. Overall no significant differences were detected in crypt cell proliferation before and after treatment. Eight patients having elevated cell proliferation rates, however, showed a significant decrease in proliferation after LAB (0.21±0.03 vs. 0.10±0.03, $P < 0.03$).

Aso et al. (1995) investigated the administration of *L. casei* (Bioloactis powder, 3 g/day) on the recurrence of superficial transitional cell carcinoma

of the bladder after trans-urethral resection in 125 patients. In patients with either primary multiple tumors or single recurrent tumors, the recurrence free rate increased from 54.9% in placebo group to 79.2% in the *L. casei* group. There was no significant effect however in patients with recurrent multiple tumors, who had very poor prognosis. The authors suggest that a stimulation of the immune system by the lactobacilli may be an important factor in its effect on the patients.

An open pilot study carried out by Boutron-Ruault et al. (2005) investigated the influence of short-chain fructo-oligosaccharides (sc-FOS, 2 × 5 g/d) on parameters that are indicative of colon cancer in adenoma and adenoma free patients. Interestingly, butyrate concentrations were lower in the adenoma patients (n = 42) compared to the adenoma free patients (n = 28), although sc-FOS supplementation significantly elevated the butyrate concentrations ($P < 0.05$) in ademona patients. Supplementation in adenoma free patients decreased the fecal lithocholic acid ($P < 0.02$) whilst cholic acid ($P = 0.02$), ursodeoxycholic acid ($P = 0.05$) and total bile acids ($P = 0.03$) were all significantly increased. The lack of significant effects in adenoma patients would indicate that sc-FOS supplementation may have a role in the prevention of cancer in healthy humans.

Oberreuther-Moschner et al. (2004) reported a study in which nine healthy volunteers were given for a period of 6 weeks either a standard yoghurt or a probiotic yoghurt (300 g/d) which included the strains *L. acidophilus* 145 and *B. longum* 913. Feces were collected from the volunteers and fecal water was prepared and incubated with human colon HT29 cells. DNA strand breaks and oxidized DNA bases were determined by the Comet assay. The probiotic yoghurt significantly reduced fecal water genotoxicity compared with standard yoghurt. However, probiotic intervention also increased oxidative DNA damage. This was attributed either to pro-oxidative activity of the LAB strains or stimulation of endogenous defense systems. However, the balance of effects favored protection, since fecal water from the probiotic group reduced overall genetic damage.

In a larger scale, randomized, double blind, placebo-controlled trial, patients with resected polyps (n = 37) or colon cancer (n = 43) were given, for 12 week, a synbiotic food supplement composed of the prebiotic Synergy 1 (oligofructose-enriched inulin) and the probiotics *L. rhamnosus* GG and *B. lactis* Bb12 (Rafter et al., 2007) The effect of synbiotic consumption on a battery of intermediate biomarkers for colon cancer was examined. The intervention significantly reduced colorectal proliferation as assessed by *in vitro* [^{3}H]thymidine incorporation

and autoradiography in colorectal biopsy samples. Given the correlation between colorectal proliferative activity and colon cancer risk, these results suggest that synbiotics might be beneficial for patients with an increased risk of colon cancer. In addition, in the polyp patients, the synbiotic intervention was associated with a significant improvement in barrier function as assessed by trans-epithelial resistance (TER) of Caco-2 cell monolayers after exposure to fecal water samples. This anti-promotion effect may reflect changes to the balance of short-chain fatty acids and secondary bile acids (deoxycholic acid and lithocholic acid) in the samples because these gut microbial metabolites have been shown to influence TER, beneficially and adversely respectively, in this system. Genotoxicity assays of colonic biopsies and fecal water indicated a decreased exposure to genotoxins in the polyp patients at the end of the intervention period.

Thus several CRC biomarkers were altered favorably by the intervention and the results show consistency with animal studies conducted in parallel (Femia et al., 2003). Also of interest was the observation that the polyp patients and cancer patients appeared to respond differently to the synbiotic, as evidenced by the different biomarkers being affected to a different extent. This may have been due to the fact that the intestinal microbiota was more refractory to changes induced by the synbiotic in the cancer patients than in the polyp patients.

At present, recurrence of polyps after resection is the strongest surrogate marker for colon cancer in dietary intervention studies, but is not frequently used because of the time and expense of conducting such studies. However, recently the effect of *L. casei* Shirota on polyp recurrence has been reported (Ishikawa et al., 2005). The subjects were patients who had two or more colorectal tumors (adenomas or early cancers) removed at least 3 months before recruitment to the study. They were randomized (approx 100 per group) to either control or *L. casei* treatment ($\sim 3 \times 10^{10}$ cfu/d). Tumors developing at the end of the second and fourth years of intervention were assessed by colonoscopy. The incidence of tumor recurrence in the controls was approximately 60% after 2 years. Multivariate adjusted odds ratios for occurrence of at least one tumor in the *L. casei group* after 2 and 4 years were 0.76 (95% CI 0.50–1.15) and 0.85 (0.56–1.27), respectively. The occurrence rate of tumors with moderate or severe atypia (considered to be more likely to become malignant) was significantly reduced at 4 years in the group given the probiotic (OR 0.65 [CI 0.43–0.98]). There was no difference in the size of tumors that developed.

26.4 Mechanisms of Anticarcinogenicity and Antigenotoxicity

26.4.1 Binding of Carcinogens

There are a large number of reports describing the adsorption or binding *in vitro* by LAB and other intestinal bacteria, of a variety of food-borne carcinogens including the heterocyclic amines formed during cooking of meat, the fungal toxin Aflatoxin B1, benzo(a)pyrene and the food contaminant AF2 (Bolognani et al., 1997; Morotomi and Mutai, 1986; Orrhage et al., 1994; Zhang and Ohta, 1991; Zhang et al., 1990). In several of these studies, a concomitant decrease in mutagenicity was reported. The extent of the binding was dependent on the mutagen and bacterial strain used, in general greatest binding was seen with the heterocyclic amines and least with Aflatoxin B1 and AF2. The adsorption appeared to be a physical phenomenon, mostly due to a cation exchange mechanism.

However, although binding represents a plausible mechanism for the inhibition of genotoxicity and mutagenicity by LAB *in vitro*, it does not appear to have any influence *in vivo*. Bolognani et al. (1997) demonstrated that simultaneous administration to mice of LAB with various carcinogens had no effect on absorption of the compounds from the gastrointestinal tract, nor did it affect the *in vivo* mutagenicity of the carcinogens in the liver. It should be noted that these results conflict with those of Zhang and Ohta (1993), who found that absorption from the rat small intestine of Trp-P-1 was significantly reduced by co-administration of freeze-dried LAB. However, the latter study was confounded by the use of rats that had been starved for 4 days, which would induce severe nutritional and physiological stresses on the animals.

26.4.2 Effects on Bacterial Enzymes, Metabolite Production

The studies listed in *Table 26.1* and *26.2* demonstrate that the increase in concentration of LAB as a consequence of consumption of LAB and/or prebiotics leads to decreases in certain bacterial enzymes purported to be involved in synthesis or activation of carcinogens, genotoxins and tumor promoters. This would appear to be due to the low specific activity of these enzymes in LAB (Saito et al., 1992). Such changes in enzyme activity or metabolite concentration have been suggested to be responsible for the decreased level of preneoplastic lesions or

tumors seen in carcinogen-treated rats given pro- and prebiotics (Reddy and Rivenson, 1993; Rowland et al., 1998). Although a causal link has not been demonstrated, this remains a plausible hypothesis.

26.4.3 Production of Anti Cancer Metabolites

The primary metabolites of LAB are the SCFAs acetoacetate, acetate and lactate (Macfarlane and Gibson, 1995). Acetate is the major SCFA found in human feces. In the host, it may be absorbed and utilized by peripheral tissues. Further, bacteria isolated from the human intestine are capable of utilizing acetate for the production of butyrate in the colon. Lactate and acetoacetate may form substrates for other members of the flora and may be degraded into other SCFA. Luminal SCFA, in particular butyrate, are potential anti-carcinogenic agents within the gut. As mentioned previously, there is considerable focus currently on synbiotics, the theory being that, the metabolites of the fermentation of the prebiotic by the probiotic will have anti cancer activity. The use of synbiotics has been shown to have additional beneficial effects on, for example ACF incidence, over the use of pro- or prebiotic separately (Challa et al., 1997; Rowland et al., 1998). It could be inferred that, the amplifying effect of using synbiotics or the effects observed with applying prebiotics on their own are due to the modulation in the SCFA composition of the luminal contents. Perrin et al. (2001) studied the effect of different forms of dietary fiber, a starch free wheat bran, a type 3 resistant starch and FOS on the prevention of ACF in rats. Their hypothesis was that, only fibers capable of releasing butyrate *in vitro* would be capable of preventing colon cancer. The resistant starch diet and the fructo-oligosaccharide diet both produced large quantities of butyrate and inhibited ACF formation, in contrast to the wheat bran diet that neither generated large amounts of butyrate nor protected against ACF formation. Mechanisms behind the anti-carcinogenic effects of SCFA have been most clearly demonstrated for butyrate. Butyrate is the preferred energy source of colonocytes and has been implicated in the control of the machinery regulating apoptosis and cellular differentiation (Basson et al., 2000; Hague et al., 1993). At a molecular level, butyrate affects gene expression via the phosphorylation and acetylation of histone proteins (Archer and Hodin, 1999). Butyrate is not produced by the lactic acid bacteria (LAB), however, certain probiotics may modify the ratio of SCFA in the colon. This remains one of their more likely mechanisms of anti-carcinogenic action within the colon.

26.4.4 Stimulation of Protective Enzymes

Many of the food-borne carcinogens such as heterocyclic amines and polycyclic aromatic hydrocarbons are known to be conjugated to glutathione, which appears to result in inactivation. The enzyme involved, glutathione transferase (GSH), is found in the liver and in other tissues including the gut. Challa et al. (1997) in a study of the effect of *B. longum* and lactulose on AOM-induced ACF in the colon, showed that the activity of GSH in the colonic mucosa was inversely related to the ACF numbers. Such a mechanism of protection would be effective against a wide range of dietary carcinogens.

26.4.5 Increase in Immune Response

In a study of tumor growth in DMH treated mice, yoghurt was found to suppress the inflammatory immune response with an increase in IgA secreting cells and in CD4 + T lymphocytes. In those animals, a marked reduction in tumors was seen (Perdigon et al., 1998). An immune mechanism was also proposed to explain the increase in time before tumor recurrence in bladder cancer patients given *L. casei* (Aso et al., 1995), although no supporting evidence for the mechanism was presented. However, Yokokura (1994) demonstrated the ability of oral administrations of *L. casei* Shirota to inhibit methylcholanthrecene-induced sarcomas in murine models. Further, isolating and culturing the animal splenocytes *in vitro*, allowed Yokokura to observe increased levels of the inflammatory immune response associated cytokine IL-2, in the group given the oral application of *L. casei* strain Shirota, as compared with the control group.

Such studies are consistent with the work of Schiffrin et al. (1996) and Marteau et al. (1997) who have provided evidence of modulation of the immune system in human subjects consuming probiotics. The changes seen were increased phagocytic activity of monocytes and granulocytes and increases in levels of antibody secreting cells although the significance of these changes in relation to tumor development has not been established.

The potential mechanisms of probiotic-induced immune suppression of carcinogenesis are complex. An inflammatory immune response produces cytokine activated monocytes and macrophages, which release cytotoxic molecules capable of lysing tumor cells *in vitro*. There is evidence from *in vitro* models that the inflammatory cytokines IL-1 and TNF-α exert cytotoxic and cytostatic effects on neoplastic cells. Furthermore, Natural killer (NK) cells are effective against

tumor cells and low NK cell activity has been associated with cancer risk (Takeuchi et al., 2001). Studies report increased NK cell activity and inflammatory type responses with the administration of some probiotic strains (Matsuzaki and Chin, 2000).

Dietary intervention studies in human volunteers have revealed increased NK cell activity in response to consumption of probiotics. Gill et al. (2001) used B. lactis HN019 to raise NK cell numbers in the peripheral blood of elderly volunteers; further, they demonstrated an *ex vivo* increase in the tumoricidal activity of the NK cells from the treatment group comparable to the control group. Nagao et al. (2000) also observed increases in NK cell activity, in human volunteers fed fermented milk *L. casei* Shirota preparation daily for 3 weeks. Studies in animal models provide evidence of a link between modulation of NK activity by probiotic consumption and tumor development. Takagi et al. (2001) reported that dietary *L. casei* strain Shirota inhibited methylcholanthracene-induced tumor development in mice. Elevated levels of NK cell activity in the treatment group mice were observed, as was the delayed onset of tumor development in comparison to the control group. Further, NK cell deficient mice did not show delayed tumor development in response to the treatment.

Anti-inflammatory effects have been reported for some probiotic strains. For example, Borruel et al. (2002) observed decreases in TNF production in human ileal specimens obtained from patients with Crohn's disease and treated *ex vivo* with *L. casei* DN114001 and *L. bulgaricus* LB10.

The establishment of oral tolerance within the host, in the long term, may mean that modulation of host immunity is a temporary event, confined to early probiotic intake. In general, however, in the rat the inhibition of carcinogenesis correlates with changes to immune activity in response to probiotic consumption (Roller et al., 2004). Further work is needed to assess the long-term effects of probiotics on host immunity in relation to anti-carcinogenesis.

26.4.6 Apoptotic Effects

The control of gene expression, cell growth, proliferation and cell death in multi-cellular organisms is dependent upon the complex array of signals received and transmitted by individual cells. Apoptosis or programmed cell death is one of the primary mechanisms by which multi-cellular organisms control normal development and prevent aberrant cell growth. Upregulation of apoptosis has received some attention recently as a potential mechanism of action of pro and prebiotics.

Hughes and Rowland (2001) fed 3 groups of rats one of three diets: basal, basal with oligofructose (5%w/w) or basal with long chain inulin (5%w/w), for 3 weeks. All animals were then dosed with 1,2-dimethylhydrazine and killed 24 h later. The mean number of apoptotic cells per crypt was significantly higher in the colon of rats fed oligofructose ($P = 0.049$) and long chain inulin ($P = 0.017$) as compared to those fed the basal diet alone. This suggests that prebiotics exert protective effects at an early stage in the onset of cancer, as the supplements were effective soon after the carcinogen insult. Comparison of the apoptotic indices between the two oligosaccharide diets showed no significant difference even though the mean apoptotic index was higher in animals fed long chain inulin.

Le Leu et al. (2005) investigated the apoptotic response 6 h after administration of the colon carcinogen AOM in rats fed lyophilized cultures of *L. acidophilus* and/or *B. lactis* in a semi-purified diet containing either low-resistant starch (RS) or moderate-RS (10% Hi-maize for 4 weeks). Rats fed the moderate-RS diet in combination with *B. lactis* had a significantly greater apoptotic response in the colon than those fed that diet without *B. lactis*. The synbiotic combination of RS and *B. lactis* significantly facilitated the apoptotic response to a genotoxic carcinogen in the distal colon of rats.

26.4.7 Effects on Tight Junctions

Other studies have looked at cellular and physiological events associated with tumor promotion in the colon. For example, one feature of colonic tumor promotion is a decrease in epithelial barrier integrity. Probiotics have been shown to improve this barrier function *in vivo*. Garcia Lafuente et al. (2001) used *L. brevis* and showed a decrease in radiolabeled mannitol uptake in the rat, and Kennedy et al. (2000) report a similar effect in IL-10 knockout mice fed *L. plantarum* v299. Commane et al. (2005) showed using an *in vitro* model of tight junction integrity (transepithelial resistance) that metabolic products (probably SCFA) derived from pro- and prebiotics fermentations were capable of improving tight junction integrity, suggesting that synbiotics may have anti tumor promoting activity.

26.5 Summary

- The evidence for anti cancer activity of pro and prebiotics from animal studies is quite compelling particularly for probiotics, with many reports indicating reductions in tumor incidence and size and in some cases malignancy.

- There is emerging evidence that consumption of synbiotics (combination of probiotics and prebiotics) is more effective than pro- or prebiotics alone.
- Studies in cell cultures and animals indicate that pro- and prebiotics can have effects at various stages in tumor development from reducing DNA damage in colon tissue, suppressing tumor promotion and inhibiting the change from benign to malignant tumors.
- Epidemiological studies have not provided any conclusive evidence for a role for pro or prebiotics in the prevention of colorectal cancer. This perhaps it is not surprising given the long period of development required for cancer (10–25 years) and the relatively recent introduction of pro and prebiotics on the market.
- There is a growing number of human intervention studies using biomarkers for colon cancer risk that suggest that probiotics, prebiotics and synbiotics may reduce risk. Clearly the current priority is to extend these into further large-scale, carefully controlled intervention studies in human subjects using biomarkers of cancer risk.
- Proposed mechanisms of action of probiotics and prebiotics in cancer include binding of carcinogens, alteration in gut microbiota formation and metabolism of carcinogens, stimulation of immune suppression of carcinogenesis, improvement in epithelial barrier function, anti-inflammatory effects, induction of apoptosis, and induction of protective enzymes.

List of Abbreviations

ACF	aberrant crypt foci
AOM	azoxymethane
CRC	Colorectal cancer
DMH	1,2-dimethylhydrazine
FOS	fructo-oligosaccharide
Glu-P-1	2-amino-6-methyldipyrido[1,2-a:3′,2′-d]imidazole
Glu-P-2	2-aminodipyrido[1,2-a:3′,2′-d]imidazole
HFA	human flora associated
IQ	2-amino-3-methyl-3H-imidazo(4,5-f)quinoline
LAB	lactic acid producing bacteria
MeIQ	2-amino3,4-dimethylimidazo[4,5-f]quinoline
MeIQx	2-amino-3,8-dimethylimidazo[4,5-f]quinoxaline
MNNG	N-methyl-N'-nitro-N-nitrosoguanidine

NDO	non-digestible oligosaccharide
PhIP	2-amino-1-methyl-6-phenylimidazo-[4,5b]pyridine
TOS	trans-galactosylated oligosaccharide
Trp-P-1	3-amino-1,4-dimethyl-5H-pyrido-[4,3-b]indole
Trp-P-2	3-amino-1-methyl-5H-pyrido[4,3b]indole

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27 Urogenital Applications of Probiotic Bacteria

Gregor Reid

27.1 Introduction

The urogenital tract extends from the perineal skin close to the anus, to the vulva, vagina, cervix, uterus, urethra, bladder and kidneys. The uterus, bladder and kidneys are regarded as being sterile, although it will not be surprising if molecular techniques discover that this is not necessarily the case. The importance of the urogenital tract in the health of women cannot be understated. Given its proximity to potential pathogens emerging from the rectum, exposure to sexually transmitted organisms, hormonal fluctuations that affect cells, use of tampons, contraceptives and douches, and the birthing process, it is remarkable that this area is not constantly infected. Nevertheless, it has been estimated that almost every female will have a vaginal or bladder infection at some point in her life.

27.2 Main Problems Associated with Microbes

Three types of urogenital infections afflict over a billion women each year. The most prevalent, bacterial vaginosis (BV) can present with odor and vaginal discharge along with vulvovaginal irritation. Classically, the diagnosis of BV was made by finding the presence of at least 20% of "Clue" cells (engulfed by Gram negative organisms) in the squamous cell population on microscopic examination of a saline suspension of vaginal discharge, associated with two of the following (Schwebke et al., 1996): (1) anterior fornix vaginal pH equal or greater than 4.7; (2) release of a fishy odor on addition of 10% KOH to the vaginal discharge (positive "whiff test"), or (3) presence of an increased thin homogenous white vaginal discharge. However, in all too many cases, symptoms and signs of BV are not obvious (Klebanoff et al., 2004).

As many microbes cannot be cultured, and as attempting to do so would be very expensive, other tests are performed. A simple Gram stain of a vaginal smear

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can identify "clue cells" engulfed by Gram negative organisms. It can also be used to establish a "Nugent" score in which the presence of mainly Gram positive rods (indicative of lactobacilli) is scored "normal" and the presence of clue cells, Gram negative rods and absence of lactobacilli is scored as "BV" (Dan et al., 2003; Nugent et al., 1991). This method is practical even for quick clinic use, but two problems arise from it. Firstly, it is likely to identify high numbers (25–29%) of so-called healthy women as having BV (Allsworth and Peipert, 2007; Reid et al., 2003a). This presents a dilemma for the physician as to whether or not to administer treatment. In most cases, the antibiotics of choice, metronidazole and clindamycin, are not prescribed without symptoms and signs being present, and/or the patient being at risk of a further complication. If no antimicrobial is administered, this leaves a sexually active woman at greater risk of acquiring a sexually transmitted infection (Cherpes et al., 2003; Sewankambo et al., 1997). The second problem is that a highly prevalent BV pathogen, *Atopobium vaginae* is a Gram positive rod, and therefore the Gram stain does not differentiate it from lactobacilli, unless the technician has been trained in their morphological differences and these are visible on the smear (Buton et al., 2004).

New methods have been introduced to detect BV, but they have not been widely used by physicians, patients or researchers. The BVBlue test is a chromogenic system that detects elevated sialidase produced by BV organisms in vaginal fluid (Myziuk et al., 2003). The FemExam detects elevated pH and proline iminopeptidase activity (West et al., 2003), but its cost is prohibitive and its effectiveness questionable (Reid et al., 2004).

Urinary tract infection (UTI) occurs with an average of 0.5 episodes/woman/year and a recurrence rate of between 27 and 48% (Hooton et al., 1996). When symptomatic, it presents with dysuria, frequency of micturition, urgency, occasional haematuria, suprapubic pain and significant loss of quality of life (Ellis and Verma, 2002). The uropathogens emerge from the rectum, ascend to the perineum and vagina, then enter the urethra and infect the bladder. Many of the most common uropathogen, *E. coli*, carry virulence factors (*papC* pilus associated with pyelonephritis; *hlyA* hemolysin; *cnf*1 cytotoxic necrotizing factor; PAI pathogenicity-associated island marker; *ibeA* invasion brain endothelium; and K1 antigen), which aid their pathogenesis (Obata-Yasuoka et al., 2003). The use of long term, low dose antibiotics while reasonably effective, still leads to 1–2 breakthrough infections per year, increases in antibiotic resistance and a range of side effects (Reid et al., 2003c). UTI recurrences are common, and in some cases may be due to the pathogens invading the vesical epithelium and

evading the host's responses and antibiotics (Justice et al., 2008). In such cases, and in patients with neurogenic bladder disease or spinal cord injury, bacteria form biofilms (Reid et al., 1992, 2003d), which appear to resist the host's response of IL-6, IL-8, polymorphonuclear neutrophils (PMNs) and gamma delta T-lymphocytes, as well as B-lymphocytes and antibodies including local sIgA (Uehling et al., 1999). The persistence of uropathogens in the bladder can increase the risk of cancer at that site (Davis et al., 1991) and elsewhere due to production of carcinogens by the organisms (Guarner and Malagelada, 2003; Nagase et al., 1983). Thus, the lack of symptoms and signs of infection, and the inability to eradicate biofilms, places the patient at risk of other problems.

Yeast vaginitis, caused mostly by *Candida albicans*, afflicts 1 in 5 black American women and 1 in 10 caucasians during any given 2 month period (Foxman et al., 2000), with 1 in 12 reporting four or more episodes per year. Patients generally present with a white vaginal discharge were characterized by non-homogenous caseous appearance, accompanied with vaginal and introital itching and irritation, and evidence of vaginal inflammation. The mere finding of yeast does not mean the patient is infected, and in many healthy women these organisms can be isolated along with non-pathogenic lactobacilli (Demirezen, 2002). Yeast colonization appears to involve at least three stages: adhesion, blastopore germination and epithelium invasion (Ferrer, 2000). *Candida albicans*' cell wall-bound Als adhesins mediate yeast-to-host tissue adherence and yeast aggregation (Otoo et al., 2008). They can be inhibited by sugars known to be part of the N-linked oligosaccharide chains of collagen IV, namely N-acetylglucosamine, L-fucose, methylmannoside and particularly N-acetyllactosamine, but not glucose, galactose, lactose or heparan sulfate (Alonso et al., 2001). The use of short courses of oral antibiotics seems to increase prevalence of asymptomatic vaginal *Candida* colonization and incidence of symptomatic yeast vaginitis (Xu et al., 2008). Locally acquired mucosal immunity appears to be the most important defense against yeast on the vaginal mucosa (Fidel and Sobel, 1996). However, a recent study has shown that the concentration of helper T cell type 1 (Th-1) cytokines (IL-2 and INF-γ) was higher in women with ongoing, recurrent, or cured vulvovaginal candidiasis than in controls ($P < 0.05$) (Fan et al., 2008). The Th-2 cytokine IL-4 was higher in women with severe infection than in those with mild to moderate VVC ($P < 0.05$), while the concentration of IL-13 was higher in cured women than in controls ($P < 0.05$). The authors suggested that these allergic vaginal responses might be the basis for changing the way the disease is treated in the future.

27.3 What Is the Microbial Composition of the Vagina, Cervix and Urethra?

In order to obtain a better understanding of the composition of the urogenital microbiota, new molecular techniques have been utilized. The combined use of chromotyping, ribotyping and plasmid typing was found to be more accurate in identifying urogenital lactobacilli than biochemical characteristics (Zhong et al., 1998), which was the method of choice up to the 1990s (Gilliland et al., 1975). Twelve different plasmid profiles were found amongst 100 vaginal isolates (Reid et al., 1996), but this method was not sufficiently specific to differentiate species. Chromosome typing (restriction endonuclease fingerprinting of chromosomal DNA) (chromotyping) is more specific and reproducible than plasmid content analysis, however, it is not easy to differentiate electrophoretic patterns with up to 100 bands. Ribotyping combines Southern hybridization of chromosomal DNA fingerprints with the use of *Escherichia coli* rRNA probes, thereby discriminating between species and individual strains of lactobacilli. The rRNA genes are separated and identified, making it possible to delineate species based on differences in the restriction fragment length polymorphisms of the rRNA genes. Specific regions of the rRNA genes have remained well conserved because of their functional importance, thus allowing the detection of a broad range of bacteria with 16S and 23S rRNA of *E. coli* as probes.

A Random Primed DNA Labeling Kit has been used to prepare whole-chromosomal probes for 20 *Lactobacillus* species, and a resultant analysis of vaginal samples from 215 healthy women showed *L. crispatus* to be the most commonly isolated species (Antonio et al., 1999). However, this method first cultures the bacteria, then identifies them. In a more recent study, *L. iners*, an organisms that does not grow on traditionally used lactobacilli media (Rogosa's and MRS), was found to be the predominant colonizer of the vagina, as determined by PCR-denaturing gradient gel electrophoresis (DGGE) and sequencing of the V2–V3 region of the 16S rRNA gene (Burton et al., 2003; Burton and Reid, 2002). Using DGGE, the microbiota of the vagina have been studied in various parts of the world, with some commonalities such as a high prevalence of *Lactobacillus iners* (Anukam et al., 2006a; Devillard et al., 2004; Vásquez et al., 2002).

Few studies have examined the microbiota of the cervix or urethra, but one used a recent restriction fragment length polymorphism analysis of the amplicons cloned from the mixtures of PCR products showing that the healthy male urethra was colonized by 71 clones, including *Pseudomonas, Streptococcus, Burkholderia,*

Ralstonia, Sphingomonas and various unidentified organisms (Riemersma et al., 2003). Earlier culture based studies of the distal urethra showed *Corynebacterium* to be most common in girls, lactobacilli to predominate after puberty and *Bacteroides melaninogeneicus* to be dominant post-menopause (Marrie et al., 1980). The mean number of species per physiological age group was 6.5 (premenarcheal), 7.7 (reproductive) and 10.3 (postmenopausal) with surprisingly aerobic gram-negative rods not isolated from any of the premenarcheal or reproductive-age subjects. A repeat of this study using molecular typing and a large sample size, would likely show a more diverse microbiota.

An approximately 600-bp region of the chaperonin 60 (Cpn60) gene, amplified by PCR with a single pair of degenerate primers, has been shown to have utility as a universal target for bacterial identification (Goh et al., 2000). This method amplifies, clones and sequences *cpn*60 gene segments from complex DNA templates extracted from complex microbial communities and helps identify the organisms from which they were derived (Hill et al., 2002). Combined with high-throughput sequencing, this genomics approach enables the characterization of the structures of even complex microbial communities. To date, a database of *cpn*60 gene sequences (*cpn*DB) from reference organisms covering a wide phylogenetic range has been generated with 3,000 distinct sequence entries. Analysis has been completed of 30,000 cloned *cpn*60 gene sequences from libraries representing the microbiota from various sources including the human vagina (Hill et al., 2005), but some findings have yet to be published. These and other molecular based methods will soon provide an accurate microbial map of the urogenital tract of males and females, thereby providing more information to design and undertake studies on how the organisms colonize in relation to health.

The microbiota of healthy pre-menopausal woman is dominated by *Lactobacillus* species, the most common of which are *Lactobacillus iners*, *Lactobacillus crispatus*, *Lactobacillus gasseri*, *Lactobacillus jenesenii,* followed by *Lactobacillus acidophilus, Lactobacillus fermentum, Lactobacillis plantarum, Lactobacillis brevis, Lactobacillis casei, Lactobacillus vaginalis, Lactobacillus delbrueckii, Lactobacillus salivarius, Lactobacillus reuteri,* and *Lactobacillus rhamnosus* (Anukam et al., 2005; Burton et al., 2003; Fredricks et al., 2005; Heinemann and Reid, 2005).

After menopause, it was once thought that the conditions were not conducive to survival of lactobacilli, but this is not true and some lactobacilli can be found in the vagina. However, the numbers are significantly increased when the woman is using hormone replacement therapy (HRT). Our studies and others have shown that HRT drugs such as premarin cause a multi-species microbiota, many with pathogenic potential such as *Bacteroides, Prevotella,* and *Gardnerella*,

responsible for BV, and *E. coli and Enterococcus,* associated with UTI, to be displaced for a simpler microbiota dominated by one or two species of *Lactobacillus* (Burton and Reid, 2003; Devillard et al., 2004; Heinemann and Reid, 2005). The restoration of lactobacilli has been associated with significantly reduced incidence of UTI (Raz and Stamm, 1993).

A recent genomic array analysis of post-menopausal women on HRTs, showed that BV was associated with a down-regulation of host antimicrobial defenses, 7-fold for colony stimulating factor, 10 fold for IL-1α, eight fold for IL-1β and 4 fold for IL-6, while use of HRT premarin appeared to up-regulate some defenses (Dahn et al., 2008).

While a vaginal tract dominated by lactobacilli appears to protect the host against some vaginal infections, it does not fully prevent colonization by other species. Pathogens such as *G. vaginalis*, can co-exist with *Lactobacillus* (Burton et al., 2003). Studies are needed to determine how, when and why the microbiota changes between an apparent healthy state and one which is associated with disease or increased risk of disease. Hormone levels are one influencing factor, along with douching and sexual practices, but the microbial response to these is not well understood (Bruce et al., 1973; Reid, 2001). Innate immunity likely also plays an important role in the switch to BV from a healthy state, through microbial-induced inhibition of Toll-like receptor expression and/or activity blocking pro-inflammatory immunity, lack of 70-kDa heat-shock protein production, and a deficit in vaginal mannose-binding lectin concentrations decreasing the capacity for microbial killing (Witkin et al., 2007).

The high prevalence of hydrogen peroxide (H_2O_2)-producing lactobacilli, and their ability to inhibit the growth of urogenital pathogens is believed to be associated with protection against infection (Dimitonova et al., 2007; Kaewsrichan et al., 2006; Kim et al., 2006b; Mijac et al., 2006; Ness et al., 2005). However, Alvarez-Olmos et al. (2004) and Rosenstein et al. (1997) found H_2O_2-producing lactobacilli in 85 and 91.7%, respectively, of women with BV, and this might indicate that H_2O_2 is not protective. In the case of yeast, acid and or other compounds are needed to displace or inhibit their presence (Kaewsrichan et al., 2006). The low pH of the vagina is believed to contribute more than H_2O_2 production to the inhibition of *G. vaginalis* (McLean and McGroarty (1996) with a 30% reduction in bacteriostatic effects seen when catalase was introduced to denature H_2O_2. Klebanoff et al. (1991) found that the toxicity of H_2O_2-producing lactobacilli was inhibited by the presence of catalase. Low concentrations of H_2O_2-producing lactobacilli must be combined

with myeloperoxidase and chloride in vaginal mucus, to be toxic toward *G. vaginalis*. One study suggested that agitation and pH 6.5 were needed for H_2O_2 production, which makes it less likely that significant quantities are produced in the relatively static, acidic mucosa of the vagina (Tomas et al., 2003). Peroxidase converts H_2O_2 into hypochlorous acid, thereby creating a microbicidal vaginal milieu by maintaining a balanced, non-toxic, steady state level of the H_2O_2 microbicides and HOCI. This reaction may drive superoxide anion-producing transformed cells into apoptosis (Bauer, 2001). The production of H_2O_2 can act to self-inhibit lactobacilli growth, and may be one factor in stopping the vaginal lactobacilli concentration from going over a level of around 10^8 viable organisms per ml fluid. In short, H_2O_2 is one of several defenses in the vagina.

27.4 Probiotics to Prevent and Treat Urogenital Infections

Probiotics are defined as "Live microorganisms which when administered in adequate amounts confer a health benefit on the host" (FAO/WHO, 2001). This is an important definition which unfortunately has not yet been embraced by all researchers and companies. Thus, the literature has papers on "dead probiotics" (which don't exist), probiotics based only upon *in vitro* data (human studies are required) or reference to studies with different formulations (which cannot be simply transferred to a different product form and content), while many companies persist on selling multi-strain "probiotics" that have no clinical rationale or testing. This has led to reviews and Cochrane papers or other analyses that fail to understand the concept and therefore pool together studies on non-probiotics with those of well documented products. This issue is pertinent for the urogenital tract as well as the gut, with a number of products on the market making false claims or misleading consumers into thinking their products have been tested and shown to confer benefits to urogenital health. Sadly, this detracts from the well-documented products and makes it difficult for consumers and health care professionals to know which products are reliable.

There remains a mistaken belief that by making a product with five or six representatives of *Lactobacillus* or other species found in the vagina, it must result in benefits. Assuming the organisms are alive in sufficient numbers at end of product shelf life, which is often not the case, one strain of Lactobacillus might produce a bacteriocin that inhibits others in the product, or one strain might

counteract the way the host's immune system responds (Fink et al., 2007). This can lead to failure of a product to provide benefits, such as with a Lactobacillus coated tampon designed to prevent BV and restore the normal microbiota (Eriksson et al., 2005).

A PubMed search of "probiotics AND vagina," as of August, 2008, revealed 92 publications of which only 14 were clinical trials. Of these, 6 in the list plus other studies showed that *L. rhamnosus* GR-1 and *L. reuteri* RC-14 could displace BV organisms and restore the normal flora and improve treatment of BV and yeast vaginitis (Anukam et al., 2006b, c; Cadieux et al., 2002; Martinez et al., 2009; Morelli et al., 2004; Reid et al., 1994, 2001a), one showed that *L. gasseri* (Lba EB01-DSM 14869) and *L. rhamnosus* (Lbp PB01-DSM 14870) can help prevent BV recurrences after antibiotic treatment (Larsson et al., 2008), one phase 1 study showed safe application of *L. crispatus* CTV05 (Czaja et al., 2007), and one study showed a potential for *L. crispatus* GAI 98322 to prevent recurrence of UTI (Uehara et al., 2006). This emphasizes that relatively few probiotics have been shown to improve urogenital health, even though a growing number of products claim to be effective.

In addition to being the most clinically documented strains for urogenital health in women, *Lactobacillus* GR-1 and RC-14 are two of the most extensively studied of all probiotics. This includes data on surface structures, hydrophobicity, capsules, growth in different conditions, by-products, interactions with the host, use with antimicrobials, and safety. They have been administered directly into the vagina and shown to displace BV pathogens (Burton et al., 2003) and cure the disease (Anukam et al., 2006b). They were the first to be shown to reach the vagina after oral administration (Reid et al., 2001a), a result independently confirmed (Morelli et al., 2004). Although oral administration does not deliver as high a count of the strains compared to direct vaginal instillation, there are several advantages to the oral route. (1) This is the natural way that lactobacilli reach the vagina. (2) The administration appears to help reduce the transfer of yeast and urogenital pathogenic bacteria from the rectum to the vagina (Reid et al., 2003a). (3) The capsules are easier to take each day than suppositories and they help to retain a lactobacilli-dominated flora (37% of patients with BV had a normal vaginal microbiota within one month compared to 13% with placebo; $p = 0.02$), restoring the conditions for indigenous strains to prosper. The failure of *L. rhamnosus* GG to be as effective in the vagina (Colodner et al., 2003) and of *L. acidophilus* NCDO 1748 to help cure BV (Fredricsson et al., 1989) further emphasizes that each product must be tested before concluding that it can provide tangible benefits for specific sites.

Some studies have supplemented antibiotics with probiotics to reduce side effects of the former. However, four studies with *L. rhamnosus* GR-1 and *L. reuteri* RC-14 have shown that the benefits go beyond reducing drug side effects. In one study of 24 patients prescribed 10 days of antibiotics, the conjoint use of the probiotic resulted in fewer follow-up cases of BV (Reid et al., 2003b). In two studies, the use of different antimicrobial agents plus one month of *L. rhamnosus* GR-1 and *L. reuteri* RC-14 doubled the cure rate of BV (Anukam et al., 2006a; Martinez et al. 2009b). The mechanisms are yet unclear, but the two probiotic strains can actually grow in metronidazole (Anukam and Reid, 2008), and their ability to reach the vagina might help displace BV pathogens more effectively. A fourth study showed that the lactobacilli therapy could improve the cure rate of patients with yeast vaginitis from 64 to 90% ($p = 0.003$) (Martinez et al., 2009). Follow-up *in vitro* studies using vaginal epithelial cells has shown that the two lactobacilli, and to some extent their culture supernatants, can reduce *Candida albicans* colonization and displace the yeast without inducing an adverse inflammatory reaction (Martinez et al. 2009c). The displacement might involve the biosurfactant activity discovered by our group (Velraeds et al., 1996) and shown to be somewhat effective against yeast (Velraeds et al., 1998). The ability of lactobacilli to reduce *Candida* biofilms is another possible mechanism, as could be the ability to bind (coaggregate) some pathogens. Mastromarino et al. (2002) reported that *Lactobacillus gasseri* 335 and *Lactobacillus salivarius* FV2 coaggregate with *G. vaginalis* and reduced their adherence.

Gardnerella, like *Candida,* are often found in the vagina of healthy women, yet patients with BV have 13-fold higher vaginal inflammatory IL-1beta levels and elevated anti-Gardnerella hemolysin (Gyh) IgA responses (Cauci et al., 2002a). BV subjects also have elevated sialidase and prolidase in vaginal fluids (Cauci et al., 2002b). The link with increased risk of HIV infection in women with BV, may be due to cathepsin, an estrogen-regulated protease D in vaginal secretions, as this molecule increases infectivity for CD4 + cells (El Messaoudi et al., 1999).

The ability of lactobacilli to modulate the vaginal immune response has not been well studied, to date. On the one hand, direct vaginal instillation of *L. rhamnosus* GR-1 has been shown to up-regulate some antimicrobial defenses (Kirjavainen et al., 2008), while on the other hand, a commercial candida skin test antigen did not invoke local immune stimulation, including changes in Th and proinflammatory cytokines, IgE, histamine, and prostaglandin, despite a natural modulation of vaginal cytokines over the course of the menstrual cycle (Fidel et al., 2003). A mannoprotein extract (MP) and secreted aspartyl proteinases (Sap) from *Candida albicans* has been shown to induce anti-MP and anti-Sap

vaginal antibodies and confer a high degree of protection against vaginitis (De Bernardis et al., 2002), thereby suggesting an intolerant capacity. The immune response to pathogens, indigenous organisms, probiotics and various strains that enter on a temporary basis, is likely complex and a fine balance between a Th-1 and Th-2 reaction exists (Steele and Fidel, 2002). Studies are needed to better understand these responses, especially to uncover why some women suffer repeated infections and others do not.

The issue is far from simple, when one considers that uropathogenic *E. coli* elevate inflammatory IL-6 in the bladder (Svanborg et al., 1994) and Gram negative BV organisms do likewise in the vagina (Donder et al., 2002), yet an avirulent *E. coli* 83972 holds promise to reduce symptomatic UTI in spinal cord injured patients (Darouiche et al., 2001), and *E. coli* Nissle which also induces an IL-6 response in the gut, has been shown to reduce inflammatory responses in the gut (Schultz, 2008).

Two other applications of probiotics of relevance to the urogenital tract are for bladder cancer and renal calculi. Lactobacilli can inhibit cancer cell proliferation and in some cases be cytotoxic (Seow et al., 2002). Although this does not explain why orally administered *L. casei* Shirota can reduce recurrences of bladder (Ohashi et al., 2002), it indicates that urogenital effects can be achieved via oral administration of a probiotic. Similarly, patients with *Oxalobacter formigenes*, a Gram-negative, anaerobic bacterium, in their gut have significantly reduced incidence of kidney stones (Kaufman et al., 2008), and a pilot study has shown that administration of probiotic *O. formigenes* can significantly reduce urinary oxalates (Hoppe et al., 2006).

27.5 Summary

- Probiotics are live microbial strains proven in a given formulation and viable count to provide measurable benefits to humans.
- The urogenital tract in women is inhabited by a range of indigenous and transient microbes. Pathogens primarily colonize from the subject's own rectal source, and then ascend to cause vaginal, cervical and urinary infections.
- The dominant presence of lactobacilli is associated with a healthy environment, except if yeast are also present in high numbers.
- Bacterial vaginosis is extremely common, even when odor and discharge are not. This condition increases the risk of sexually transmitted infections and preterm labor.

- The concept of probiotics for urogenital health, is to restore a lactobacilli flora (microbiota), displace pathogens and potential pathogens, and balance the immunological response between being antimicrobial and not inflammatory.
- Strains can be selected for their probiotic potential using *in vitro* experiments, but they do not become probiotic until tested in humans.
- *Lactobacillus rhamnosus* GR-1 and *Lactobacillus reuteri* RC-14 are by far the most documented probiotics for urogenital health with extensive data on properties and clinical functionality.
- Over the next 5–10 years, as a better understanding of the vaginal microbiota and its fluctuations are accrued, new approaches to probiotic therapy will emerge to the benefit of many millions of women around the world.

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Conflict of Interest

Dr. Reid no longer owns *Lactobacillus* GR-1 and RC-14.

List of Abbreviations

BV	Bacterial vaginosis
Cpn60	chaperonin 60 gene
DGGE	denaturing gradient gel electrophoresis
HRT	Hormone replacement therapy
H_2O_2	hydrogen peroxide
IL	Interleukin
INF-γ	interferon gamma
PMNs	polymorphonuclear neutrophils
Th-1	T cell type 1
Th-2	T cell type 2
UTI	Urinary tract infections
VVC	vulvovaginal candidiasis

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28 Prebiotics and Probiotics and Oral Health

J. H. Meurman

28.1 Introduction

The first part of this chapter describes the unique characteristics of the mouth with special emphasis on the oral microbiota. Next, the highly prevalent dental diseases are briefly described together with more rare but still important diseases and symptoms of the mouth. Prevention and treatment of oral and dental diseases are also discussed focusing on aspects considered important with respect to the potential application of prebiotics and probiotics. The second part of the chapter then concentrates on research data on prebiotics and probiotics in the oral health perspective, ending up with conclusions and visions for future research.

28.2 The Oral Cavity and Diseases of the Mouth

The mouth or oral cavity is the first part of the gastrointestinal tract. It is a unique organ in many respects due to its central role not only in nutrition but also in communication, such as speech. Mouth has a particularly important role early in life in organogenesis and in the psychic development of a human being. Anatomically the oral cavity is the only part of the human body where hard tissues directly penetrate soft tissue epithelium: teeth surrounded by the gingival epithelium and periodontal connective tissue extend from the alveolar bone to the oral cavity and thus may open the parenteral space to the outer environment.

The oral cavity is also an ideal habitat for microorganisms. Mouth temperature is optimal for bacteria to grow; there is adequate moisture, frequent serving of nutrients, and a variety of different surfaces for microbial attachment. These include mucosal surfaces of keratinized and non-keratinized epithelium, dental hard tissues (enamel, dentin and dental root cement), and often man-made appliances such as dental prostheses, and dental restorations. Hence, more than 700 microbial species have been identified in the mouth and it is estimated that

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each individual may carry 200–300 species at the same time (Paster et al., 2006). Microorganisms are found free in saliva, covering mucosal surfaces, and organized in supra- and sub-gingival dental plaque. In plaque the microorganisms form highly structured biofilms where bacteria appear to communicate with each other (Xie et al., 2007). 1 mg of plaque may contain up to 10^{11} bacteria. The *viridans*-group streptococci constitute the majority of the indigenous oral microbiota, but practically all human pathogenic bacteria known occasionally may also harbor in the mouth. In addition, yeasts are prevalent in the oral microbiota, and in particular in elderly individuals. *Candida albicans* is the predominant species of yeast in the oral cavity.

The tooth supporting tissues or periodontal tissues are highly liable for inflammation because dental plaque easily retains in the areas adjacent to teeth. Inflammation, in turn, causes destruction of the epithelial attachment to dental hard tissues and also disrupts the intra-epithelial cell-to-cell contacts. The pathological changes that follow then enable microorganisms to penetrate deeper tissues of the mouth and jaws with subsequent systemic spread of oral microorganisms. Similarly, severely decayed teeth offer direct access for oral microorganisms through necrotic dental pulp to deeper tissue in the periapical area and beyond. Diseased mouth mucosa may also be a portal of entry for systemic infections when the epithelial cell-to-cell contacts disrupt as is the case in mouth ulcers for example.

Oral and dental infections are among the most prevalent infections of man. Practically all people suffer from dental caries at some phase of their life span. Approximately 8–10% of adult populations suffer from periodontal diseases. Oral *Candida* infections are highly prevalent among diseased and the elderly. Up to 88% of elderly has been reported to carry *Candida* species in the mouth (Wilkieson et al., 1991). Hence, the mouth is indeed an important source of infections and poor oral health affects a variety of systemic diseases. Oral microorganisms may spread directly from the mouth via blood circulation causing metastatic infections, or they trigger cascades of pathogenic mechanisms with severe consequences (Meurman et al., 2004). The consequences of the spread of oral infections are particularly catastrophic for patients with reduced immune function (Meurman et al., 1997).

Man has probably always understood the meaning of good oral hygiene from the general health perspective. Toothbrushes have been found in the Egyptian pyramids dating up to 3,000 years before Christ. The Ancient Egyptian physicians also knew that infections of the mouth must be treated, as drill holes have been observed in the jaws of mummies with severely decayed teeth. Drilling holes to the jaws enabled the relief of pus from dental root tips.

28.2.1 Defensive Mechanisms of the Mouth

Healthy oral mucosa is the most essential defensive mechanism of the mouth. Disrupted epithelium opens up deeper tissue layers for oral microorganisms to invade with potential systemic complications. In immunosuppressed patients the mouth has been shown to be the source of septicaemia in 25–75% of cases undergoing treatment for cancer (Meurman et al., 1997), and among organ transplant patients (Rautemaa et al., 2007; Scully, 2008). Mouth ulcerations in mucositis and gingivitis are thought to be the major portals of entry of these severe infections. But also in healthy humans oral bacteraemia is highly prevalent and results from normal daily activities such as during chewing food and by tooth brushing. For example, it has been estimated that the cumulative one-month exposure to oral bacteraemia from routine daily activities is 5,370 min (Guntheroth, 1984). The examples demonstrate how important for health the well-functioning defensive mechanisms of the mouth are.

Oral surfaces are bathed in saliva which flushes down microorganisms which then will be swallowed and subsequently destroyed in the stomach. Normal salivary flow in adults, stimulated by chewing, is approximately 1–2 ml/min while flow rates below 0.7 ml/min are regarded as reduced flow. For unstimulated resting salivary flow the respective lower threshold value is 0.1 ml/min. Hence, hyposalivation is diagnosed if the patient's measured flow rate values are below these reference limits. However, subjective dry mouth or xerostomia does not necessarily follow clinical hyposalivation since the feeling of how much saliva is enough is indeed highly subjective. An individual with objectively measured satisfactory salivary flow may still report a feeling of dry mouth.

Saliva contains a variety of specific and non-specific defensive mechanisms of its own and can be regarded a "chemical cocktail". Saliva contains, for example, high concentrations of calcium and phosphates which are essential elements of the dental hard tissues. Saliva also contains lubricating mucopolysaccharides, proteolytic enzymes, immunoglobulins and other components of the defensive systems. Saliva also has buffering effects. In addition to saliva, "oral fluid" may contain detached epithelial cells, bacteria, and other microorganisms and food remnants (Edgar and O'Mullane, 1996).

Secretory immunoglubulins (Ig) originate from immune cells which are home to the salivary glands and are produced as response to antigenic stimulus, for example, by oral bacteria. Secretory IgA is the major immunoglobulin in saliva that specifically prevents microbial attachment to oral surfaces. The secretory component in the IgA molecule protects it from the innate proteolytic enzymes

of saliva. Saliva also contains lesser amounts of serum derived IgG and IgM. The Ig aggregate bacteria and may activate complement system in the gingival crevice, but do not cause bacterial lysis in saliva (Edgar and O'Mullane, 1996).

Of the non-specific defensive mechanisms, saliva contains lysozyme, which disrupts the peptidoglycan layer of bacterial cell walls and causes lysis and cell death. Salivary lysozyme also seems to link with systemic sugar metabolism (Janket et al., 2006). So this, partly from saliva and partly from white-blood cells derived enzyme, may have several functions. Of the several other non-specific salivary defensive systems the reader is advised to consult special texts beyond the topic of this review (Edgar and O'Mullane, 1996).

In general, however, normal salivary flow is essential for healthy mouth. Hence, reduced salivary flow directly affects the oral microbiota causing microbial shift towards colonization of more pathogenic species. This is particularly seen in medicated patients. There are hundreds of pharmacological agents which affect salivary glands causing reduction in saliva secretion. Drugs with anticholinergic effect are particularly harmful in this regard since salivary secretion is regulated by the autonomic nervous system. However, it has been shown that in practice the number of drugs taken daily is more important than the exact chemical nature of the medicine so that the more daily drugs a patient needs to take the less saliva in the mouth (Närhi et al., 1999). Consequently, in particular the elderly are patients-at-risk also in this regard and reduced salivary flow and function explains why these individuals often harbor oral *Candida*, for example. Further, it needs to be mentioned that an extremely problematic group of patients with dry mouth are those who have received radiotherapy to the head and neck. Irradiation may irreversibly damage salivary glands and thus render the patient highly liable to oral infections due to total lack of saliva.

To sum up, functioning defensive mechanisms of the oral cavity usually maintain homeostasis and prevent overgrowth of micro-organisms. If the balance is disturbed for one reason or another, the result may be colonization and emergence of potentially pathogenic microbiota which detrimentally affects not only the oral and dental health but also has systemic health consequences. Patients with hyposalivation are particularly at risk also in this regard.

28.2.2 Oral Hygiene Measures and Anti-Plaque Agents

As described in the first paragraph, mechanical cleaning of the teeth has a long history in mankind. Man has probably always sensed the need to pick out food

remnants and vegetable fibers from the teeth. Use of "chewing sticks" has been documented already from Babylonia dating back to 3,500 years BC. The chewing sticks then have evolved to tooth brushes and other cleaning aids of various designs. However, first in the nineteenth century systematic mechanical cleaning has been emphasized as an important health measure. The earliest known toothpaste was created by the Egyptians. It was said to contain a drachma of rock salt, two drachmas of mint, one drachma of dried iris flowers and 20 grains of pepper. This was then crushed and mixed together to form a powder (Emslie, 1980).

After the advent of bacteriology in the early nineteenth century, different chemicals have been introduced as "anti-plaque" agents mainly to ameliorate bad breath. In this context, however, it should be mentioned that already the Greek poet Homeros described about 700 BC that by chewing mastix-resin or betel-pepper bad breath could be reduced. Hence, bad breath in particular has been a nuisance to humans and probably only imagination has been the limit in the attempts to treat this problem. Modern times have then introduced more or less efficient chemicals targeted against dental plaque and oral biofilms. Today this is an area of intensive research with significant commercial interests. *Table 28.1* describes some currently used chemicals in oral hygiene products marketed as anti-plaque agents and/or for the prevention and treatment of bad breath. In this context,

Table 28.1

Chemical agents and other means for dental plaque control

Fluorides[a]
Chlohexidine
Povidone-iodine
Cetyl pyridinium chloride
Delmopinol
Zinc citrate
Triclosan
Sanguinarine
Essential oils
Olive oil
Tea tree oil
Other vegetable oil
Vitamin B
Sour milk products such as natural yoghurt

[a]Fluorides are used in dental caries prevention and their anti-plaque effect is minimal

it should be pointed out that scientific evidence is fairly weak about the efficacy of the different agents marketed for oral hygiene purposes, and placebo-controlled randomized trials with high numbers of patients are practically non-existent. Nevertheless, this is an area of increasing commercial volume and importance.

Chlorhexidine is the golden standard in chemical plaque control. It has been shown that combining frequent mechanical cleaning (tooth brushing and flossing) with chlorhexidine mouth rinses further decreases the number of bacteria in the mouth (Sekino et al., 2003). Chlorhexidine mouth rinses can also be used to control the number of *Candida* in patients with removable dentures. However, due to its potentially harmful side-effects such as allergic symptoms, continuous use of chlorhexidine is not advisable as a daily means of controlling oral hygiene unless the patient is at high risk of pneumonia or other systemic complications. Therefore, other chemical agents have also been investigated for controlling dental plaque. These include the daily use of a combination of amine fluoride and stannous fluoride, which also appears to have a slight antifungal effect, and povidone-iodine, cetyl pyridinium chloride, essential oils, zinc citrate, triclosan, tea tree oil, and sanguinarine. Local delivery of antibiotics, such as tetracycline derivates, has also been investigated in dental plaque control but for obvious reasons this is not a strategy for long-term population based preventive programs.

Finally, it must be reminded that there is no doubt that fluorides are effective in the prevention of dental caries (Twetman et al., 2003, 2004). The effect of fluorides is based on the fact that dental hard tissues are mainly composed of apatite minerals. The critical pH of the most prevalent, hydroxyapatite, is approximately 5.5. Subsequently, if the pH value of the fluid surrounding the tooth falls below this threshold, the crystalline tooth enamel structure dissolves. Fluoride is one of the most bone seeking elements known to mankind. Excessive amount of fluoride makes the bones brittle and the dental enamel more porous.

The actual chemical nature of fluoride used in mouth rinses or toothpastes does not seem to play a key role in the efficacy of a product as long as the concentration of fluoride is optimal (Twetman et al., 2003, 2004). Toothpastes for adults normally contain up to 4,000 ppm fluoride while professionally used topical fluoride preparations may contain 10,000–20,000 ppm fluoride. The recommended fluoride concentration in drinking water is 1 ppm and this must be taken into account when locally assessing the need for topical fluoride supplementation since especially domestic wells in certain areas may contain considerable amounts of this trace element. The maximum contaminant level for fluoride is 4 ppm. Hence, fluoride is a toxic element with a narrow therapeutic window and it is also considered toxic waste.

28.2.3 Dental Diseases and Their Treatment and Prevention

In the following, the principal dental diseases are briefly described in order to give the background for the discussion about probiotics and prebiotics in the oral health perspective.

Dental caries is an infectious disease affecting practically every individual in all countries at some phase of the person's life span. In the industrialized countries today caries is becoming highly polarized, as a minority of populations presents a high caries prevalence while the majority is not severely affected. Early in life caries infection is transmitted usually from mother to the child by the bacterium *Streptococcus mutans*. Studies have shown that if *S. mutans* infection is successfully prevented then caries does not follow as easily in the child (Newbrun, 1992).

These *viridans*-group streptococci have adopted to live on dental enamel by the help of highly developed extracellular polysaccharide metabolism, which results in glue-like substance that attaches the microorganism on the underlying surface. Caries then follows when dietary carbohydrates are being metabolized into organic acids that dissolve enamel apatite. Consequently, the more frequently one consumes carbohydrates, the more likely caries proceeds and hence the term "between-meal-snacks" has been introduced as a poor dietary habit for dental health. The nature and consistence of dietary carbohydrates is also important in this regard. Sticky, toffee-like sweets or food stuffs are more detrimental to teeth than liquids, because of their longer clearance time in the oral cavity. Sucrose is more cariogenic than lactose, for example, because sucrose is more rapidly metabolized by cariogenic streptococci. In this context it should be mentioned that the use of xylitol in dental caries prevention is based on the fact that this sugar alcohol is not metabolized by cariogenic streptococci and thus products sweetened with xylitol are not cariogenic, as is also the case with other non-fermentable sugar substitutes (Carlsson and Hamilton, 1996). However, as stated above, use of fluorides is the cornerstone of caries prevention, and on the market there are both systemically administered and topically used preparations to select.

Initial caries is symptomless, while toothache follows from dentin and pulpal infections. Caries lesions are treated mechanically by removing the diseased tissues with dental burs and other instruments, and by restoring the cavities using dental filling materials. Pulpal infections call for endodontal treatment when the necrotic tissue is mechanically removed and replaced by inert root filling materials, such as guttapercha.

Erosion. When dental plaque microorganisms ferment sugar into organic acids which then lower the oral fluid pH, dental caries follows as described above. Similarly, if one drinks or eats any low-pH items the acid dissolution of enamel takes place. In the absence of bacterial etiology such tooth dissolution is called dental erosion (Meurman and ten Cate, 1996).

Erosion has in recent years become highly prevalent because of increasing use of acid beverages particularly in young populations. Erosion is thus not a disease but symptom of poor dietary habits or underlying systemic disease. Namely, erosion may also follow if acid contents of the stomach are being propulsed into the mouth. This happens in vomiting among anorexia and bulimia patients who are particular patients-at-risk, or by regurgitation seen mainly in patients with reflux disease.

Periodontal disease affects approximately 8–10% of adult populations. It may take decades to manifest but also a juvenile form of the disease is known affecting young people. Periodontal disease is an infectious/inflammatory disease caused by chronic exposure of tooth supporting tissues to microorganisms of the oral biofilm. Mainly anaerobic bacteria such as *Porphyromonas gingivalis*, *Tannerella forsythia*, *Aggregatibacter actinomycetemcomitans* and *Treponema denticola* are considered as periopathogens, i.e., these bacteria have been associated with the prevalence and progression of the disease even though a direct causal link has not been verified. It is probable that the mere existence of dental plaque and particularly calcified plaque (dental calculus) triggers inflammatory reactions in the periodontal tissue. This then subsequently leads to tissue damage and loss of tooth support. Hence, periodontal disease ultimately leads to the loss of teeth after which the inflammation subsides. However, because the disease process usually takes years, the detrimental effect of periodontal disease on systemic health may be pronounced (Meurman et al., 2004).

Maintaining proper oral hygiene by mechanically removing the plaque on regular basis is the cornerstone in periodontal disease prevention. As stated above, there are also several chemicals on the market that are meant to control dental plaque. However, no mouth rinse or other preparation seems ever to compensate good tooth brushing and flossing. If the disease has progressed leading to deep periodontal pockets (usually >4 mm deep when measured with a periodontal probe) which further accumulate plaque, professional cleaning is needed and sometimes with help of periodontal surgery. However, severely affected teeth need to be extracted since periodontal infection understandably causes a continuous threat of oral microorganisms spreading into deeper tissues and finally all over the body.

Bacteraemia of oral origin has indeed been estimated to follow from normal daily activities such as chewing food stuffs more easily if periodontal tissue is inflamed (Hartzell et al., 2005). For example, Forner et al. (2006) observed in patients with periodontitis, bacteraemia during 30 min after onset of blood sampling at chewing, while no bacteria were detected in blood of periodontally healthy subjects. Consequently, periodontal disease has been associated with a variety of systemic diseases where, the pathogenic mechanisms involve up-regulating inflammatory cytokines and other inflammatory mediators by the bacterial burden. In recent years research data have shown that periodontal disease significantly associates with atherosclerosis with its life-threatening consequences (Meurman et al., 2004).

Dental caries and periodontal disease together take the majority of patient treatment time at dental offices. The profession of dentists has in fact evolved from the need to treat carious teeth. Today dental hygienists also help patients by providing professional tooth cleaning services. From a health care costs, caries and periodontal disease account for a significant proportion, since it has been estimated that the share of oral health care is about 7% of the total costs. These are huge sums of money. For example, in the European Union, with a total population of 456 million and an oral health workforce of 900,000 (some 300,000 of whom were dentists), the cost of oral health care in the year 2000 was about 54,000,000,000 euros (Widström and Eaton, 2004). This should be placed in perspective of the total health care costs which vary between nations from approximately 7% (Ireland) up to +15% (The United States) of the gross domestic product (www.kff.org/insurance/snapshot/chcm010307th.cfm). Consequently, every means that might reduce the costs of oral health care amount to significant sums. In this context, novel strategies such as those based on the application of probiotics for control of the oral microbiota are very welcome.

28.2.4 Diseases of Mouth Mucosa

Yeast infections. Oral mucosal infections are mainly caused by *Candida albicans* (Richardson and Warnock, 2003). Concomitant use of several drugs, including antimicrobial agents, causes selective suppression of resident bacteria in the oral cavity leading to yeast overgrowth. Particularly elderly individuals and patients in long-term-care facilities harbor *Candida* species in the mouth. Wearing dentures has long been known to contribute to yeast infection and good oral hygiene has

been shown to decrease the colonization of *Candida* (Grimoud et al., 2005). Systemic, invasive yeast infections are rare and mainly encountered in high-risk patients. Mortality in *Candida* blood-stream infections is of the magnitude of 40%, however. These potentially fatal infections are seen in intensive care units and in patients with severe and prolonged neutropenia, and often with multi-organ failure (Richardson and Warnock, 2003).

Yeast infections are treated with special antifungal drugs. For oral health purposes there are both topical and systemic preparations on the market. Usually, locally applied preparations are prescribed for milder infections while severe infections call for administration of systemic drugs. The emergence of antibacterial and antifungal drug resistance is a growing global problem and there is a reason for concern also in oral health care in this respect. In a study on cancer patients receiving palliative care, oral colonization with non-*albicans* yeasts was observed in more than 40% of the isolates with a high percentage of resistance to both fluconazole and itraconazole (Bagg et al., 2003). Consequently, new means are also needed for controlling oral yeast infections in the future. There are preliminary data showing that probiotics may help in controlling oral yeast infections (see later).

Oral lichen planus is a disease of unknown etiology. It affects approximately 1–2% of the adult population (Sugerman and Porter, 2008). The symptoms are white striations and papules, erythema, and erosions or blisters. The lesions are often bilateral and seen on the buccal mucosa. The patients experience mucosal sensitivity and pain particularly when eating spicy food. Oral mucosa is often very sensitive to oral hygiene products, too, and then the patients cannot use toothpaste or mouthwash preparations. Corticosteroids are of help in the treatment and the preparations are used either topically or in severe cases systemically. However, there are no evidence based data for the best treatment of oral lichen planus. Ameliorating symptoms is often possible by simply abstaining from all irritants. Regular use of sour milk products may also help subjectively the patient but no randomized trials have been published in this area.

In general, several systemic diseases and skin diseases in particular may also manifest in the mouth with highly non-specific symptoms and signs. These are, however, beyond the scope of this chapter and the interested reader is advised to textbooks of oral medicine. Diabetes and rheumatic diseases may be mentioned as merely two examples. Very little evidence based data exist on the topic of treating oral symptoms connected to systemic diseases, however. Nevertheless, clinical practice has shown that maintaining good oral hygiene and use of saline as mouth rinse may help the patients by ameliorating the symptoms. Regardless

of the underlying disease the patients with oral lesions often suffer from mucosal pain and burning sensation in the mouth. Controlling the underlying systemic disease is of course essential in the treatment of patients with symptoms and signs of the mouth, too. So far, there are no data of use of probiotics in this area. Theoretically, controlling oral microbiota by probiotics might also beneficially affect mucosal diseases of the mouth.

28.2.5 Non-Specific Symptoms of the Mouth

Xerostomia or subjective feeling of dry mouth is highly prevalent in elderly populations (Nederfors, 2000). The estimates of the percentage of older individuals with xerostomia range from 10 to 40%. Medications are believed to be responsible for a significant proportion of cases with xerostomia and the list of drugs that are believed to affect saliva secretion includes more than 400 agents (Närhi et al., 1999). Several studies indicate that the risk of xerostomia increases with increasing numbers of medications used. As discussed earlier, reduced saliva flow inevitably also reduces the defensive mechanisms in the oral cavity. Consequently, dry mouth should be treated. But apart from drinking water and using local oral gels and other preparations for dry mouth, pharmacological means for ameliorating xerostomia are sparse. Pilocarpine (5 mg tablets taken several times daily) has been used in cases with severe hyposalivation in patients with no systemic contraindications for using cholinergic drugs. In this context, it must be re-emphasized that subjective xerostomia does not necessarily reflect reduced salivary flow rates because the feeling of "how much saliva is enough" is highly subjective.

Burning mouth syndrome and *glossodynia.* These symptom entities comprise dull pain or feeling of burn in mouth mucosa and tongue among patients where no clinical pathology can be seen in the symptomatic areas. Burning mouth can be a mere nuisance to the patient, while in severe cases the symptom is intolerable. The prevalence of the sensation of burning mouth is estimated to be of the magnitude of 10% in elderly (+60 year-old) populations (Bergdahl and Bergdahl, 1999). Women are more often affected than men, and the symptom is particularly prevalent at menopause. Burning mouth mostly affects the tongue, hence the name glossodynia. The symptom very often presents itself simultaneously with dry mouth. The etiology is not known but psychological factors play an important role as is the case with all patients with chronic pain. In women, hormonal changes have been thought to be associated with the symptom but the data are

controversial in this regard (Tarkkila et al., 2001). Because the cause for burning mouth is unknown there are no specific treatments available. Many patients subjectively benefit from using soothing oral gels and mouth rinses but severe cases of burning mouth need antidepressants such as amitriptylin or antiepileptic drugs targeted for chronic pain. These include preparations containing carbamazepine or newer drugs such as pregabaline. Hence, pharmacological therapy of the symptom calls for specialist treatment.

28.3 Prebiotics and Probiotics and the Mouth

28.3.1 Definitions and Mechanisms of Action of Prebiotics and Probiotics

Here, the basic facts about probiotics and prebiotics are only briefly summarized. Man has used fermented milk for over 10,000 years (Tamime, 2002). However, first early in 1900 Ukrainian-born bacteriologist and later Nobel laureate Ilya Metchnikoff introduced the concept that consumption of fermented milk affects positively the longevity of humans. In 2002 the World Health Organization defined the concept of probiotics as follows: "A supplement of living microorganisms that bring a health benefit by improving the balance of the intestinal microbiota" (Joint FAO/WHO, 2002 Working Group Report on Drafting Guidelines for the Evaluation of Probiotics in Food). Probiotics exert their effect as viable microorganisms; but the concept is applicable independent of the site of action and route of administration. Probiotic effect therefore may include sites such as the oral cavity, the intestine, the vagina, and the skin (Schrezenmeir and de Vrese, 2001). Probiotics are given as functional foods or dietary supplements, and function activating the mucosal immune system, preventing pathogen colonization and translocation by strengthening the mucosal barrier, interfering with pathogen colonization and, in some instances, by producing secretory antibacterial substances. There are a variety of microorganisms with anticipated probiotic effects. Strains investigated from an oral health perspective are given in *Table 28.2*.

The mechanisms of probiotic action are still unclear, but may indeed include the strengthening of the non-immunological mucosal barrier, the interference with pathogen adhesion and growth inhibition, and the enhancement of the local mucosal immune system in the gut, as well as of the systemic immune response (Hatakka and Saxelin, 2008). Probiotic effects on the microecology and pathology

Table 28.2

Examples of bacterial strains investigated in the oral health perspective for their putative probiotic effect

Lactobacilli	**Bifidobacteria**
L. bulgaricus	*B. breve*
L. rhamnosus	*B. lactis*
L. casei	*B. longum*
L. dellbrueckii subsp. *bulgaricus*	*B. adolescentis*
L. casei strain Shirota	*B. infantis*
L. paracasei	**Weissella**
L. johnsonii	*W. cibaria*
L. reuteri	**Propionibacterium**
L. acidophilus	*P. freudenreichii* subsp. shermanii
L. plantarum	**Streptococcus**
L. helveticus L. fermentum	*S. salivarius*
Lactococcus	*S. thermophilus*
Lactococcus lactis	*S. sanguinis*

of microbiota of the mouth, stomach, and vaginal tract have been observed. The beneficial effects may be mediated through immune influences, systemic effects such as reduced severity of colds, or other respiratory conditions, impact on allergy incidence and symptoms; reduced absences from work or daycare have also been noted (Lenoir-Wijnkoop et al., 2007).

Prebiotics on the other hand are a category of functional food ingredients, defined as non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improve host health (Gibson and Roberfroid, 1995). Prebiotics are non-digestible carbohydrates, principally oligosoccharides that are fermented by colonic commensals, stimulating their proliferation and producing short-chain fatty acids. They have been shown to reduce the incidence and severity of infantile diarrhea, particularly rotavirus gastroenteritis, prevent antibiotic-induced diarrhea, and prevent and treat intestinal food allergy (Chen and Walker, 2005).

As recently reviewed by Guarner (2007) it has been suggested that the symbiosis between host and commensal microbiota can be optimized by prebiotics. Inulin-type fructans have been shown to improve the microbial balance of the intestinal ecosystem by stimulating the growth of bifidobacteria and

lactobacilli. These changes have been associated with several health benefits, including the prevention of gastrointestinal and systemic infections. Inulin-type fructans induce changes of the intestinal mucosa characterized by higher villi, deeper crypts, increased number of goblet cells, and a thicker mucus layer on the colonic epithelium. Bacterial antagonism and competition of bifidobacteria and lactobacilli with pathogens, as well as the trophic effects on the intestinal epithelium, may explain the protective role of inulin against enteric infections. For example, a study on mice where dietary cellulose was replaced by non-digestible oligosaccharides, oligofructose and inulin, and then infected with *Listeria monocytogenes* and *Salmonella typhimurium*, showed that the inulin diet significantly protected the animals from fatal infections (Buddington et al., 2002). In addition to what has been observed in animal models, inulin and oligofructose have proven useful in preventing mucosal inflammatory disorders in patients with inflammatory bowel disease (Guarner, 2007).

Probiotics and prebiotics have also been investigated as supplements to infant cereals. Such preparations have shown a preventive effect on diarrhea, and a recent study has suggested that a milk fat globule membrane protein fraction added to an infant cereal reduces the risk of diarrhea. There are some promising results suggesting that infant cereals supplemented with probiotics or prebiotics may also prevent atopic eczema (Domellöf and West, 2007).

28.3.2 Prebiotics and Oral Health

There are no controlled studies on the effect of prebiotics on oral health. However, there is every reason to believe that at least some of the putative mechanisms of action of prebiotics discussed above might also function in the oral cavity. Such effects might be mediated by two mechanisms. First, a direct effect of oligosaccharides, for example, on the development and metabolisms of oral biofilms could be anticipated. These compounds have shown to increase the number of bifidobacteria and lactobacilli in mucosal biofilms of the colon and they may thus prevent the colonization of pathogenic bacteria (for review, see Guarner, 2007). Similar effect could be hypothesized also with respect to oral biofilms.

Secondly, a systemic immunomodulatory effect could be expected in the mouth, similar to that mediated by the colonic microbiota in the presence of oligosaccharides and other types of prebiotics, such as inulin, lactitol and

lactulose, and lactose not absorbed in the small intestine. This affects the composition of the bacteria in the colon, thus influencing the gut-associated immune function (Batista de Morais and Jacob, 2007). However, up to now there are no data on the effect of prebiotics on oral and dental diseases. Considering the short clearance time of food items in the mouth (before swallowing), it is probable that if a prebiotic effect exists or can be locally administered in the oral cavity, any beneficial changes in the oral microbiota are unlikely. However, systemic immunomodulatory effects may naturally also affect the mouth.

28.3.3 Probiotics and Oral Diseases

Based on the experience from using probiotic preparations mainly in connection with gastrointestinal diseases, partly the same mechanisms of function can be anticipated to affect also the oral cavity after probiotic administration. These putative effects are summarized in *Table 28.3*.

Several different bacterial strains have been investigated experimentally and in clinical trials for their anticipated probiotic effects on oral and dental diseases.

Table 28.3

Anticipated effects of probiotics on the oral cavity

Mechanism of action	Putative function in oral cavity
• Effect on pH of the microbiota	• Reduction in harmful microorganisms and modifying effect on oral biofilm
• Antimicrobial substances	• Inhibition and control of oral microorganisms
• Competition for nutrients	• Selection pressure on oral microbiota
• Effect on microbial adhesion	• Competition for adhesion sites on mucosa and dental hard tissues with subsequent effect on colonization
• Interference with bacterial metabolism	• Reduction in harmful bacterial end products
• Immunomodulatory effect	• Control of oral microbiota
• Effect on mucosal permeability	• Control of oral biofilm
• Effect on epithelial cell metabolism	• Control of oral biofilm

As said earlier, *Table 28.2* gives examples of such strains. In the following discussion the experiences from oral health-related investigations with these strains are being summarized.

28.3.3.1 Lactobacilli

Briefly, strains of the genus *Lactobacillus* have been used in fermented foods since the early history of mankind, and later extensively in the dairy industry. However, their potential probiotic activities were first suggested by Metchinkoff who worked at the Pasteur Institute in Paris (Metchnikoff, 1907). Today, lactobacilli have been intensively studied for their probiotic properties. Early in 1990s these bacteria were also investigated in the oral health perspective. *L. rhamnosus* strain GG was first found to inhibit cariogenic streptococci (Meurman et al., 1994). It was then administered in yoghurt for an oral colonization study, which however, that permanent colonization was highly improbable (Meurman et al., 1995; Yli-Knuuttila et al., 2006). Even today *L. rhamnosus* strain GG is one of the most extensively studied probiotic and it is of particular interest for oral biology since the bacterium does not readily ferment sucrose and might hence be more "safe-for-teeth" than other lactic acid producing bacteria. Later controlled clinical studies have indeed shown its efficacy in caries prevention in children and in controlling oral *Candida* in the elderly.

In the 7-month kindergarten study by Näse et al. (2001), 594 children, 1–6 years old, received probiotic *L. rhamnosus* strain GG versus normal milk. The caries risk was calculated based on clinical and microbiological data, the latter consisting of *Streptococcus mutans* levels from dental plaque and saliva. The results showed less dental caries in the probiotic milk group and lower *Streptococcus mutans* counts at the end of the study. The probiotic milk was found to reduce the risk of caries significantly (OR = 0.51, p = 0.004). The effect was particularly clear in the 3- to 4-year-olds.

Secondly, in the elderly, Hatakka et al. (2007) conducted a 16-week, randomized, double-blind, placebo-controlled study on 276 people, 70–100 years old, who consumed daily 50 g of either probiotic or control cheese. High salivary yeast count was the primary outcome measure. The probiotic cheese, contained *Lactococcus lactis* and *L. helveticus* starter cultures with added *L. rhamnosus* GG, *L. rhamnosus* LC705, and *Propionibacterium freudenreichii* subsp. *shermanii* JS, while the control cheese only contained the starter culture of *Lactococcus lactis* with no probiotic strains added. The results showed that the prevalence of high

yeast count decreased in the probiotic group from 30 to 21% (32% reduction), and increased in the control group from 28 to 34%. Probiotic intervention reduced the risk of high yeast counts by 75% (OR = 0.25, p = 0.004).

Also, other *Lactobacillus* strains have been investigated with respect to their effect on oral microorganisms, in particular with dental caries prevention in mind. For example, *L. reuteri*, *L. casei* and *L. acidophilus* have been found to inhibit cariogenic streptococci both in experimental and clinical studies (Busscher et al., 1999; Caglar et al., 2006). The latter group, studied in 120 young adults, 20–24 years old, the effect of delivering *L. reuteri* ATCC 55730 versus placebo, using either specifically made straws or tablets for 3 weeks. They observed a statistically significant reduction of the *Streptococcus mutans* levels after ingestion of the probiotic bacteria via the straw ($p < 0.05$) and the tablets ($p < 0.01$) when compared with the placebo controls (Caglar et al., 2006). Hence, even though more randomized controlled trials are needed for further evidence, data cumulated so far already indicate that there is potential of probiotic therapy for oral pathogens, especially in children (Twetman and Stecksen-Blicks, 2008).

In addition to targeting dental caries probiotic lactobacilli have also been successfully investigated with respect to periodontal pathogens. A decrease in gum bleeding and reduced gingivitis has been observed by Krasse et al. (2006) with the application of *L. reuteri*. Kõll-Klais et al. (2005) have reported that the resident *Lactobacillus* flora inhibits the growth of *Porphyromonas gingivalis* and *Prevotella intermedia* by 82 and 65%, respectively. Kõll et al. (2008) later observed that *L. plantarum*, *L. paracasei*, *L. rhamnosus* and *L. salivarius* expressed a high antimicrobial activity against periodontal pathogens *A. actinomycetemcomitans* and *Porphyromonas gingivalis* and against the periodontal bacterium *P. intermedia*. However, the antimicrobial activity of *Lactobacillus* species varies *in vitro* when examined together with several oral microorganisms including the periodontal pathogens *A. actinomycetemcomitans* and *P. gingivalis* (Stamatova et al., 2008). Consequently, specific probiotic species are probably needed in combating different microorganisms.

Adhesion capacity to oral surfaces and antimicrobial effect of the putative probiotic species are key characteristics needed for successful effect on oral microbiota. Hence, systematic investigations are needed in this regard when screening potential probiotic bacteria. Research data accumulated so far have shown huge differences in the attachment of putative probiotic strains onto surfaces in test models that mimic oral tissues (Haukioja et al., 2006). These model systems mainly comprise adhesion experiments with radio-labeled bacteria

to hydroxyapatite beads with or without salivary components. Van Hoogmoed et al. (2008) have recently observed in a flow chamber study that streptococci may also inhibit periodontal pathogen adhesion. This observation may open new possibilities for developing products with antagonistic properties.

The *Lactobacillus* strains that are effective against oral streptococci and periodontal microorganisms do not necessarily inhibit *Candida*. The results by Kõll et al. (2008) confirmed earlier results indicating that particularly *L. salivarius* and *L. gasseri* might be good candidates as potential probiotic strains against dental infections but not for oral yeast infections (Strahinic et al., 2007). However, there are data indicating that many other *Lactobacillus* strains in addition to *L. rhamnosus* GG, inhibit *Candida* in various parts of the gastrointestinal tract, the vagina and the oral cavity (Elahi et al., 2005; Manzoni et al., 2006; Paraje et al., 2000; Strus et al., 2005; Wagner et al., 2000). Subsequently, it is indeed apparent that specific probiotic species need to be identified for each particular indication. There also seems to be intra-species variation among lactobacilli in their inhibitory effect against different pathogens.

28.3.3.2 Lactococci

Lactococci are Gram-positive bacteria, and particularly *Lactococcus lactis*, has been extensively used in the production of buttermilk, yoghurt and cheese. The bacterium is homo-fermentative and produces exclusively lactic acid (Akerberg et al., 1998). Consequently, from the dental health point of view this characteristic may be considered detrimental to teeth. Nevertheless, an *in vitro* study has shown that a lantibiotic named lacticin isolated from *Lactococcus lactis* inhibits cariogenic streptococci (O'Connor, 2006). The authors suggested that the preparation might be considered as an anti-cariogenic and could be used in the development of functional foods. More studies are called for, however.

28.3.3.3 Bifidobacteria

Bifidobacteria aid in digestion, and they have been shown to be associated with a lower incidence of allergies (Björkstén et al., 2001; He et al., 2001a) and may also affect some forms of tumor growth (Guarner and Malagelada, 2003). Some bifidobacteria are being used as probiotics because they have good adhesion capacity onto intestinal mucosa (He et al., 2001b), and have proved to be safe

for human consumption (Mäkeläinen et al., 2003). The latter aspect is particularly important (Apostolou et al., 2001). The safety aspects in general will be discussed in more detail later in this chapter. When bifidobacteria were added in yoghurt, a significant reduction was observed in salivary *S. mutans* counts, which supports the hypothesis that these bacteria might also be used as probiotics for caries prevention (Caglar et al., 2005b).

28.3.3.4 Weissella

Weissella cibaria has been investigated for its effect on *Fusobacterium nucleatum*, which is one of the major "bridging" microorganisms in the oral biofilm development (Kang et al., 2006). Furthermore, *W. cibaria* was also found to inhibit volatile sulfur compounds produced by dental plaque microorganisms (Kang et al., 2005). This characteristic may offer possibilities for the development of products, such as mouth washes, that might inhibit bad breath, as the volatile sulfur compounds are the principal culprits in halitosis (bad breath). Members of the genus *Weissella* are important bacteria in various kinds of foods, such as fresh vegetables, meat and fish products (Björkroth et al., 2002). A bacteriocin from *W. cibrata* has recently been characterized (Srionnual et al., 2007).

28.3.3.5 Propionibacteria

Propionibacteria are slow-growing, non-sporeforming, Gram-positive, anaerobic bacteria, which produce lactic acid, propionic acid, and acetic acid from glucose. The species *P. freudenreichii* is used in Swiss cheese manufacturing and hence included in food preparations with probiotic properties. In the study by Hatakka et al. (2007) on oral *Candida* infections among elderly patients, propionibacteria were included in the specially manufactured cheese, which was used as a vehicle for probiotic administration (see later for the results of this study). However, more studies are needed on the specific effects of propionibacteria on oral microbiota.

28.3.3.6 Streptococci

Teughels and co-workers have investigated the inhibitory effect of streptococci against periodontal bacteria and found that repeated application of *S. sanguinis*,

S. salivarius and *S. mitis* after root planning, successfully reduced and maintained low levels of anaerobic species and black-pigmented bacteria (Teughels et al., 2007a). The same group also observed that streptococci inhibited the recovery and colonization of *A. actinomycetemcomitans*, thus supporting the concept that oral streptococci may interfere with periodontal infections (Teughels et al., 2007b).

28.3.4 Clinical and Experimental Studies

Table 28.4 summarizes the studies where probiotic bacterial strains have been investigated regarding their oral health attributes (Caglar et al., 2005a; Meurman, 2005; Meurman and Stamatova, 2007). The majority of experimental studies and clinical trials have concentrated in dental caries, by investigating eventual probiotic effects on the caries-inducing properties of *S. mutans*. In general, the results have been positive showing an inhibitory effect. However, more data are called for, in particular from properly controlled randomized trials with good statistical power and long-enough follow-up time. Up to today, only one long-term randomized placebo controlled trial has been published (Näse et al., 2001). In this 7-month study conducted on 594 kindergarten children, the administration of probiotic milk containing *L. rhamnosus* GG reduced caries risk with an odds ratio of 0.51 ($p = 0.004$) when controlled for age and gender. Nevertheless, although these results were positive, the conclusion whether or not probiotic therapy might be recommended for prevention and control of dental caries cannot not be drawn based on the present research data.

Oral *Candida* infection has also been the focus in some studies but here the evidence is even weaker than in the dental caries investigations. As seen in *Table 28.4*, only one clinical trial has been published. In this study by Hatakka et al. (2007), an experimental probiotic cheese preparation containing *L. rhamnosus* GG and *P. freudenreichii* subsp. *shermanii* was tested against placebo (cheese without the probiotics) in 276 elderly people who consumed daily 50 g of cheese for 16 weeks. The results showed that the prevalence of high salivary yeast counts ($>10^4$ cfu/ml) decreased in the probiotic group from 30 to 21% (32% reduction), and increased in the control group from 28 to 34%. Probiotic intervention reduced the risk of high yeast counts by 75% (OR = 0.25, 95% CI 0.10–0.65, $p = 0.004$). Interestingly, the risk of hyposalivation also decreased in the probiotic group by 56% (OR = 0.44, $p = 0.05$); this effect might be mediated by enhanced

Table 28.4

Recent studies on probiotics in the oral health perspective (*Cont'd* p. 1088)

Strain investigated	Preparation used	Effect	Reference
Clinical trials			
L. reuteri	Lozenge	Reduction in salivary *S. mutans*	Caglar et al. (2008)
L. rhamnosus	Cheese	Reduction in salivary *S. mutans*	Ahola et al. (2002)
L. rhamnosus	Milk	Reduction in salivary *S. mutans*	Näse et al. (2001)
Bifidobacterium DN-173 010	Yoghurt	Reduction in salivary *S. mutans*	Caglar et al. (2005b)
L. rhamnosus	Cheese	Reduction in salivary *Candida*	Hatakka et al. (2007)
L. brevis	Lozenge	Anti-inflammatory effect	Riccia et al. (2007)
In vivo studies			
L. reuteri	Straw and tablet	Reduction in *S. mutans*	Caglar et al. (2006)
S. salivarius	Mouthwash	Inhibition of black pigmented salivary bacteria	Burton et al. (2006)
L. reuteri	Experimental formulation	Reduction in dental plaque and gingivitis	Krasse et al. (2006)
L. casei	Periodontal dressing	Improved remission after periodontal therapy	Volozhin et al. (2004)
L. acidophilus	Live bacteria fed to mice by gastric intubation	Clearance of *Candida*	Elahi et al. (2005)
In vitro studies			
Mixture of lactobacilli	–	Inhibition of *S. mutans* growth	Köll-Klais et al. (2005)
W. cibaria	–	Inhibition of *S. mutans* in biofilm	Kang et al. (2006)
Lactoccoccus lactis	–	Inhibition of *S. mutans*	O'Connor et al. (2002)
Lactococcus lactis	–	Effect on colonization and growth of oral micro-organisms	Comelli et al. (2002)
W. cibaria	Mouthwash	Inhibition of biofilm formation	Kang et al. 2006
L. casei	–	Inhibition of interleukin-8 production by dental pulp cells	Thaweboon et al. (2006)

Table 28.4

Strain investigated	Preparation used	Effect	Reference
S. sanguinis, S. mitis, S. salivarius	–	Inhibition of *A. actinomycetemcomitans* colonization	Teughels et al. (2007)
L. bulgaricus	–	Inhibition of streptococci and *A. actinomycetemcomitans*	Stamatova et al. (2008)

immune function. However, similar to the case with dental caries, more studies are definitely needed before final conclusions.

Regarding the periodontal disease, the probiotic concept has not been investigated in a randomized controlled clinical trial setting. As seen in *Table 28.4*, there are very few data from laboratory studies showing that probiotics might affect periodontal microorganisms, such as *A. actinomycetemcomitans* (Teughels et al., 2007). Recently, unpublished data from our laboratory have shown that some putative probiotic *L. delbrueckii* subsp. *bulgaricus* (*L. bulgaricus*) strains, in addition to the established probiotic *L. rhamnosus* GG, inhibited *P. gingivalis* and *F. nucleatum*. Also, *A. actinomycetemcomitans* appeared to be the most sensitive to the probiotic effect among the periodontal bacteria investigated. However, it needs to be emphasized that the whole concept of probiotic therapy versus periodontal disease is still in its cradle.

28.3.5 Vehicles for Probiotic Administration

An additional issue that needs to be discussed in this context is the vehicle for probiotic administration for oral health purposes. Dairy products supplemented with probiotics have been investigated and they might be the easiest way for oral administration, as a daily dietary regime. Hence, milk, yoghurt, ice cream, cheese, bio-drinks and juice preparations have been studied but the evidence for best means of administration is still lacking (Meurman and Stamatova, 2007). However, in order to maximize the topical effect in the mouth some low-release probiotic preparations might be needed. Therefore, some investigators have introduced chewing gums, tablets, sucking tablets, lozenges and specially made straws to which probiotic strains have been added. Marketed products often contain single bacterial strains commonly derived from dairy industry (Twetman and Stecksen-Blicks, 2008). But as discussed above, these preparations have not

been investigated in randomized controlled trials, and therefore there is no clear evidence of their efficacy.

Montalto et al. (2004) administered probiotic bacterial mixes in capsules and in liquid form without observing statistically significant difference in the *S. mutans* counts between the two test groups consuming the different preparations. Comparing the effect of a specially made straw (BioGaia®, Stockholm, Sweden) and a lozenge containing *L. reuteri*, a pronounced reduction of *S. mutans* counts in saliva was achieved with both means of delivery after three weeks of administration (Çaglar et al., 2006). A chewing gum containing *L. reuteri* Prodentis has also been marketed, and it is meant to be consumed twice daily for controlling of *S. mutans* growth (www.biogaia.se). However, no placebo-controlled studies have been published. The average content of *L. reuteri* in this probiotic delivery product was 10^8 cfu/ml which, in general, has been considered necessary in probiotic dairy products, where the minimum bacterial concentrations should be at least 10^6 cfu/ml (Kurmann and Rasic, 1991; Shah, 2000; Tamime et al., 1995). To sum up, however, the best vehicle for probiotic administration for oral health purposes has yet to be defined.

28.3.6 Safety Aspects of Probiotics in the Oral Health Perspective

As discussed earlier, most lactobacilli used in dairy products readily ferment dietary carbohydrates to organic acids. If such a bacterium is in close contact with tooth enamel, tissue damage may occur. The critical pH of hydroxyapatite is 5.5 and subsequently any substance with pH value lower than this may cause enamel dissolution. Therefore, as an example, if a probiotic is administered in a milk product it can be anticipated to be more safe-for-teeth than if given in juice without added calcium and phosphates. Thus, care must be taken not to promote caries or erosion by using acid-producing probiotic bacteria or preparations with low pH value. This fact leads to one of the principal safety issues for putative oral probiotic strains, i.e., that they should exhibit a low fermentation capacity for dietary carbohydrates. It seems that in this regard the metabolic activity differs between probiotic species as recently shown by Hedberg et al. (2008).

Whether or not probiotic interference in oral micro-ecology can cause or trigger super-infections is not known. In this context, it is interesting that some *Lactobacillus* strains inhibit *Candida* (see *Table 28.4*). The possible transfer of

antimicrobial resistance genes between probiotic strains and microorganisms of the resident flora has also been discussed but so far the research data do not indicate that this would be a problem (Meurman and Stamatova, 2007). It should be reminded again that dairy products in particular have been used since the early history of mankind and it is thus improbable that a dairy-derived probiotic strain would be detrimental to the host in general. The effect of selected lactobacilli on human tissue modulating enzymes was recently investigated to see if the studied *Lactobacillus* strains are able to degrade host tissue components. The results showed very low gelatinolytic activity indicating tissue friendliness of the studied *L. bulgaricus* strains (Stamatova et al., 2007).

28.3.7 Resident Lactic Acid Bacteria in the Mouth

Lactobacilli make approximately 1% of the cultivable oral microbiota (Marsh and Martin, 1999). The most common *Lactobacillus* species recovered from saliva in a study by Teanpaisan and Dahlen (2006) were *L. fermentum, L. rhamnosus, L. salivarius, L. casei, L. acidophilus* and *L. plantarum*. Apart from *L. fermentum* and *L. salivarius* these are probiotic species used in dairy products. A similar diversity in the oral *Lactobacillus* flora was observed by Colloca et al. (2000) who found *L. fermentum, L. plantarum, L. salivarius* and *L. rhamnosus* to be the predominant species in a healthy human mouth. Kõll-Klais et al. (2005) found no differences in salivary lactobacilli counts between chronic periodontitis and healthy patients, *L. gasseri* and *L. fermentum* being the predominant species among other isolates, i.e., *L. oris, L. plantarum, L. paracasei, L. rhamnosus, L. gasseri, L. acidophilus* and *L. cispatus*. In a later study, the same authors observed a higher prevalence of homofermentative lactobacilli in healthy mouths compared to samples from patients with chronic periodontitis (Kõll-Klais et al., 2006). These findings indicate that lactobacilli, as members of resident oral microbiota, could play an important role in the ecological balance in the oral cavity. These studies further demonstrated that *Lactobacillus* strains with potential probiotic properties may indeed be found in the oral cavity. However, it is not known if these lactobacilli were detected due to the frequent consumption of dairy products leading to temporary colonization only, or if the oral environment was their permanent habitat. There are no long-term follow-up studies published to answer this question. Short-term colonization studies indicate that permanent colonization is rare (Meurman et al., 1994; Yli-Knuuttila et al., 2006). There is only one study published, where one single case was found where the mouth of the subject

was thought to be permanently colonized by *L. rhamnosus* GG. This woman had received probiotic preparations against allergy since her early childhood (Yli-Knuuttila et al., 2006). Nevertheless, more systematic studies are needed to screen the oral resident microbiota for microbial species with probiotic properties.

28.3.8 Future Perspectives

There are hardly any data on prebiotics and oral health. It may be assumed, however, that the modification of resident microbiota by prebiotics also influences the type of microorganisms present in the oral cavity. However, we need to learn more about the complex development and metabolism of oral biofilms in particular, before experiments with prebiotics can be conducted. Similarly, more data are needed to answer the question whether the prebiotic-triggered immunomodulatory effects observed in the gut also favorably extend to the oral cavity.

Regarding probiotics, data are accumulating and the first randomized controlled trials have given promising results. Emphasis has been given on dental caries and oral yeast infections but there are also preliminary experimental data showing that probiotics might affect periodontal microorganisms. The vision that probiotic preparations could be developed to support therapy for oral symptoms, such as bad breath, is also interesting. For example, Burton et al. (2005) reported that *S. salivarius* strain K12 produced two lantibiotic bacteriocins, compounds that are inhibitory to strains of several species of Gram-positive bacteria implicated in halitosis (bad breath).

Understanding the mechanisms whereby probiotic species modulate oral immunity is of pertinent importance, however. In this respect, probiotic therapy may have a role in the treatment of oral mucosal diseases, such as lichen planus, and in manifestations of other diseases, such as skin diseases. However, there are no data on the eventual effect of probiotics on oral manifestations of autoimmune diseases. In this regard, it might indeed be interesting to conduct studies on patients with lichen planus, pemphigus vulgaris, cicatricial pemphigoid or aphthous stomatitis. In addition, the role of probiotics in soothing burning mouth and relieving the feeling of dry mouth, provides a lot of scope for research.

Finally, new means of probiotic administration should be developed and systematically investigated in order to find out the best delivery means and strategies for oral and dental diseases. Based on the current knowledge, it appears realistic to assume that different probiotic strains are needed for different

Table 28.5

Oral diseases where probiotics might or might not be used in the future

Disease or condition	Evidence for probiotic use
• Dental caries	• Clinical and experimental evidence showing positive effects
• Oral yeast infections	• Weak experimental evidence
• Oral manifestations of systemic diseases	• Probiotic therapy hardly applicable
	• Clinical and experimental evidence showing positive effects
• Burning mouth syndrome	• No evidence but potential exists
	• No evidence but potential exists
	• Weak evidence
	• No evidence

indications. Hence, no single bacterial strain is expected to be suitable for all the problems, whether symptoms or disease states, where probiotic therapy is being considered. Because dental diseases in particular pose such a big socio-economic burden to mankind, not to mention about individual suffering, there is great potential in the future for oral probiotics (*Table 28.5*).

28.4 Summary

- Oral cavity harbors billions of microorganisms in biofilms covering the teeth (dental plaque) and mucosal surfaces, and in saliva.
- Pathogenic microorganisms reside among the resident microbiota, in particular in the elderly and in medically compromised patients.
- Dental diseases affect the majority of populations with paramount socio-economic consequences and great suffering to the patients – at worst with life-threatening systemic infection complications.
- Traditional oral health preventive measures, such as mechanical cleaning of the teeth and use of fluorides, only partly control the diseases of the oral cavity.
- Probiotics have been successfully administered for controlling dental caries in children and oral yeast infections in elderly patients.
- Scientific research data are still sparse about probiotics and oral health.
- Oral and dental diseases are anticipated to be good targets for future probiotic therapy.
- There are no data so far regarding the effect of prebiotics on the oral microbiota.

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29 Development of Mucosal Vaccines Based on Lactic Acid Bacteria

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29.1 Introduction

Today, sufficient data are available to support the use of lactic acid bacteria (LAB), notably lactococci and lactobacilli, as delivery vehicles for the development of new mucosal vaccines. These non-pathogenic Gram-positive bacteria have been safely consumed by humans for centuries in fermented foods. They thus constitute an attractive alternative to the attenuated pathogens (most popular live vectors actually studied) which could recover their pathogenic potential and are thus not totally safe for use in humans. This chapter reviews the current research and advances in the use of LAB as live delivery vectors of proteins of interest for the development of new safe mucosal vaccines. The use of LAB as DNA vaccine vehicles to deliver DNA directly to antigen-presenting cells of the immune system is also discussed.

29.2 Potential Applications of Mucosal Immunisation

Mucosal surfaces are the primary interaction sites between an organism and its environment and they thus represent the major portal of entry for pathogens. In the last 10 years, there have been several reports of successful immunisation with a variety of mucosal vector vaccines (Holmgren and Czerkinsky, 2005). The choice of this route of immunisation is governed by the efficiency of vaccines at different Mucosa-Associated Lymphoid Tissue (MALT): lymphoid structures associated with the nasopharynx, tonsils, salivary glands, and upper respiratory tract, termed Nasal-Associated Lymphoid Tissues (NALT), the Bronchoepithelium and Lower respiratory Tract (BALT), Gastrointestinal tract (GIT), and

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male and female genital tracts (Cesta, 2006; Corr et al., 2008). Each MALT is covered by epithelium containing specialized cells known as follicle-associated epithelium or microfold (M) cells and plays an important role in the maintenance of the mucosal surface barrier and initiation of mucosal immune reactions (Corr et al., 2008). M cells transport soluble and particulate matter across the mucosal epithelium and perform sampling of luminal antigens; they thus constitute potential inductive sites to stimulate immune responses. Furthermore, mucosal immunisation also induces efficient systemic immune responses and presents less collateral side effects than systemic vaccines. Finally, mucosal immunisation is more easily performed without the need of needles and syringes and thus trained personnel (important feature for mass vaccination programs).

29.3 Brief Description of the Various Delivery Systems for Mucosal Administration

Recent advances in biotechnology and in the understanding of the immune system have now rendered possible the design of new mucosal delivery systems. Such vehicles include inert systems in which purified antigens or naked DNA are associated in microspheres, liposomes, nanoparticules, immunostimulating complexes as well as live bacterial or viral vector systems (Christensen et al., 2007; Daudel et al., 2007; Hu et al., 2001; Illum and Davis, 2001; Jennings and Bachmann, 2008; Mielcarek et al., 2001; Singh et al., 2008). Live bacteria and viruses are more immunogenic than inert vectors and thus represent better candidates to induce both mucosal and systemic immune responses against infectious agents.

Vaccinia virus and its derivatives are the most frequently used virus vaccines (Moss, 1991; Ulaeto and Hruby, 1994); however in the last years, these vectors have been progressively replaced by other poxviruses, such as canary and fowl pox viruses, and by adenoviruses (Beukema et al., 2006; Karkhanis and Ross, 2007; Patterson and Robert-Guroff, 2008). The live bacterial vectors are either based on attenuated pathogens or on non-pathogenic bacteria (Daudel et al., 2007; Wells and Mercenier, 2008) (*Table 29.1*). Compared to viruses genomes which are limited in their capacity to encapsulate several foreign DNA, the genomes of live bacterial vectors can harbor many such heterologous genes. Recombinant bacteria can thus produce many different heterologous antigens which may allow the development of multivalent vaccines.

Table 29.1

Bacterial vectors used as live vaccines

Bacterial vector	Reference
Attenuated pathogens	
Mycobacterium bovis BCG	Stover et al. (1992)
Listeria monocytogenes	Jensen et al. (1997)
Salmonella spp.	Curtiss et al. (1994)
Vibrio cholera	Killeen et al. (1999)
Shigella spp.	Brahmbhatt et al. (1992)
Bordetella spp.	Stevenson and Roberts (2003)
Non-pathogenic bacteria	
Streptococcus gordonii	Lee (2003)
Lactococcus lactis	Bermúdez-Humarán et al. (2004a)
Lactobacillus spp.	Seegers (2002)
Staphylococcus spp.	Ståhl et al. (1997)

29.4 Lactic Acid Bacteria as Carrier Systems

The immunogenicity of soluble proteins orally and intranasally administered is low and it can be significantly enhanced by either coupling the protein to a bacterial carrier or by genetic engineering of bacteria resulting in the production of the desired antigen. Attenuated pathogen bacteria such as derivatives of *Mycobacterium, Salmonella and Bordetella* spp. are particularly well adapted to interact with mucosal surfaces that most of them use to initiate the infection process. Unfortunately, these organisms could recover their pathogenic potential and are not totally safe for use in humans, especially in children and immunosuppressed patients (Alexandersen, 1996). Gram-positive food-grade or commensal bacteria (belonging to commensal flora of the human MALT) constitute an attractive alternative to attenuated pathogenic bacteria (Wells and Mercenier, 2008). In particular, the food-grade lactic acid bacteria (LAB) such as *Lactococcus lactis* and certain species of lactobacilli possess a number of properties which make them attractive candidates for the development of mucosal vaccines (Bermúdez-Humarán et al., 2004a). Indeed, LAB have been used for centuries in the fermentation and preservation of food and they are considered as safe organisms with a GRAS (Generally Recognized As Safe) status. Moreover, several antigens and/or cytokines have been successfully expressed in LAB, and mucosal

administration of these genetic engineered LAB has been shown to elicit both systemic and mucosal immunity (◉ *Table 29.2*).

The production of a desired antigen by LAB can, in theory, occur in three different cellular locations: (1) intracellular, this location allows the protein to escape the drastic environmental conditions (such as gastric juices in the stomach after oral administration of the recombinant strain) but it requires cellular lysis for protein delivery; (2) extracellular, this location allows the release of the protein in external medium and thus a direct interaction with environment (food product or the digestive tract); and (3) cell surface-attached, a cellular location that combines the advantages of the first ones, i.e., interaction between the cell wall-anchored protein and the environment, and protection from proteolysis degradation. In this context, several studies have compared the production of different antigens in LAB using these three localisations and evaluated the subsequent immunological effects (reviewed in Bermúdez-Humarán et al., 2004a; Wells and Mercenier, 2008). These studies have shown that most of the highest immune responses are obtained with antigens exposed to the surface of LAB. Therefore, most of recent studies have selected surface exposure of the antigen of interest, rather than intra- or extracellular production.

29.5 *Lactococcus lactis* as Live Vaccine Delivery Vector

Lactococcus lactis is the most widely used LAB in the production of fermented milk products and is considered as the model LAB because many genetic tools have been developed and its complete genome is sequenced (Bolotin et al., 2001). *Lo. lactis* is considered as a good candidate for heterologous proteins production because it secretes relatively few proteins (van Asseldonk et al., 1993). In addition, the most commonly used laboratory strain (*L. lactis* MG1363) is plasmid-free and does not produce extracellular proteases (Gasson, 1983). However, the major advantage of the use of *L. lactis* as live vector for mucosal delivery of therapeutic proteins resides in its extraordinary safety profile since this bacterium is catalogued as a non-invasive and non-pathogenic organism with a GRAS status. Finally, the capacity of *L. lactis* to produce antigens has been clearly demonstrated in the last 2 decades (◉ *Table 29.2*). These features make *L. lactis* a potential candidate for the development of new safe mucosal vaccines.

Table 29.2

Antigens and cytokines (adjuvants) successfully expressed in lactic acid bacteria (*Cont'd* p. 1104)

Antigens	Source	Vector	Indication/ Potential use	Reference
Bacterial				
PA	*Bacillus anthracis*	*Lb. casei*	Anthrax vaccine	Zegers et al. (1999)
		L. lactis	Anthrax vaccine	Unpublished data
LpA	*Borrelia burgdorferi*	*Lb. plantarum*	Lyme disease vaccine	del Rio et al. (2008)
L7/L12	*Brucella abortus*	*L. lactis*	Brucellosis vaccine	Ribero et al. (2002)
GroEL	*Brucella abortus*	*L. lactis*	Brucellosis vaccine	Miyoshi et al. (2006)
TTFC	*Clostridium tetani*	*L. lactis*	Tetanus vaccine	Wells et al. (1993)
		Lb. casei	Tetanus vaccine	Maassen et al. (1999)
		Lb. plantarum	Tetanus vaccine	Grangette et al. (2001)
β-toxin	*Clostridium perfringens*	*L. lactis*	*C. perfringens* type B and C vaccine	Nijland et al. (2007)
K99	Enterotoxigenic *Escherichia coli* (ETEC)	*Lb. acidophilus*	Enteric colibacillosis treatment	Chu et al. (2005)
SpaA	*Erysipelothrix rhusiopathiae*	*L. lactis*	Swine erysipelas vaccine	Cheun et al. (2004)
UreB	*Helicobacter pylori*	*L. lactis*	Helicobacter vaccine	Lee et al. (2001)
		Lb. plantarum	Helicobacter vaccine	Corthésy et al. (2005)
Cag12	*Helicobacter pylori*	*L. lactis*	Helicobacter vaccine	Kim et al. (2006)
FliC	*Salmonella enterica* serovar Enteritidis (SE)	*Lb. casei*	SE vaccine	Kajikawa et al. (2007)
PAc	*Streptococcus mutans*	*L. lactis*	Dental caries vaccine	Iwaki et al. (1990)
M6	*Streptococcus pyogenes*	*L. lactis*	Dental caries vaccine	Mannam et al. (2004)
PsaA	*Streptococcus pneumoniae*	*L. lactis*	Pneumococcal vaccine	Hanniffy et al. (2007)

Table 29.2 (*Cont'd* p. 1105)

Antigens	Source	Vector	Indication/ Potential use	Reference
		Lb. plantarum	Pneumococcal vaccine	Oliveira et al. (2006)
		Lb. helveticus	Pneumococcal vaccine	Oliveira et al. (2006)
Pili	*Streptococcus agalactiae* GBS	*L. lactis*	Streptococcal vaccine	Buccato et al. (2006)
Viral				
NSP4	Bovine coronavirus	*L. lactis*	Coronavirus vaccine	Enouf et al. (2001)
SARS	Coronavirus	*Lb. casei*	SARS-CoV vaccine	Lee et al. (2006)
Spike glycoprotein S	Coronavirus	*Lb. casei*	Gastroenteritis coronavirus vaccine	Ho et al. (2005)
EDIII	Dengue virus serotype 2	*L. lactis*	Dengue vaccine	Sim et al. (2008)
V3	Human immunodefficiency virus (HIV-1)	*L. lactis*	HIV vaccine	Xin et al. (2003)
E7	Human papillomavirus type-16 (HPV-16)	*L. lactis*	Cervical cancer therapeutic vaccine	(Bermúdez-Humarán et al., 2002)
		Lb. casei	Cervical cancer therapeutic vaccine	Poo et al. (2006)
		Lb. plantarum	Cervical cancer therapeutic vaccine	Cortes-Perez et al. (2007)
L1	HPV-16	*L. lactis*	Cervical cancer prophylactic vaccine	Cho et al. (2007) and Cortes-Perez et al. (Unpublished data)
		Lb. casei	Cervical cancer prophylactic vaccine	Aires et al. (2006)
VP2 and VP3	Infectious bursal disease virus (IBDV)	*L. lactis*	Coronavirus vaccine	Dieye et al. (2003)
Cap	Porcine circovirus type 2 (PCV2)	*L. lactis*	PCV2 vaccine	Wang et al. (2008)
VP2	Porcine parvovirus	*Lb. casei*	Parvovirus vaccine	Xu and Li (2007)
VP7	Rotavirus	*L. lactis*	Rotavirus vaccine	Perez et al. (2005)

Table 29.2 (*Cont'd* p. 1106)

Antigens	Source	Vector	Indication/ Potential use	Reference
Others				
MSP-1	*Plasmodium yoelii*	*L. lactis*	Malaria vaccine	Zhang et al. (2005)
MSA2	*Plasmodium falciparum*	*L. lactis*	Malaria vaccine	Ramasamy et al. (2006)
Sm28	*Schistosoma mansoni*	*L. lactis*	Schistosomiasis vaccine	Wells et al. (1995)
beta-lacto-globulin	Bovine blacto-globulin	*L. lactis*	Allergy modulations	Chatel et al. (2001)
		Lb. casei	Allergy modulations	Hazebrouck et al. (2006)
Der p 5 allergen	*Dermatophagoides pteronyssinus*	*Lb. acidophilus*	Allergy treatment	Charng et al. (2006)
CWP2	*Giardia lamblia*	*L. lactis*	Giardiasis vaccine	Lee and Faubert. (2006)
Cytokines				
IL-2	*Mus musculus*	*L. lactis*	TTFC vaccine adjuvant	Steidler et al. (1995)
IL-6	*Mus musculus*	*L. lactis*	TTFC vaccine adjuvant	Steidler et al. (1998)
IL-10	*Mus musculus*	*L. lactis*	Colitis treatment	Steidler et al. (2000)
	Homo sapiens	*L. lactis*	Crohn's disease treatment	Steidler et al. (2003)
IL-12	*Mus musculus*	*L. lactis*	E7 vaccine adjuvant	Bermúdez-Humarán et al. (2003a)
		Lb. plantarum	E7 vaccine adjuvant	Bermudez-Humaran et al. Unpublished data
IFN-ω	*Mus musculus*	*L. lactis*	Antiviral treatment	Bermúdez-Humarán et al. (2003b)
IFN-γ	*Mus musculus*	*L. lactis*	Antiviral/ antitumoral treatment	Bermúdez-Humarán et al. (2008)
	Sus scrofa	*L. lactis*	Antiviral/ antitumoral treatment	Rupa et al. (2008)

Table 29.2

Antigens	Source	Vector	Indication/ Potential use	Reference
IFN-β	*Homo sapiens*	*L. lactis*	Antiviral/anti-inflammatory treatment	Zhuang et al. (2008)
MIG/IP-10	*Mus musculus*	*L. lactis*	Novel vaccine adjuvant	Cortes-Perez et al. (2008)
Leptin	*Homo sapiens*	*L. lactis*	Novel vaccine adjuvant	Bermúdez-Humarán et al. (2007)

29.6 Immune Response to Antigens Delivered by *Lactococcus lactis*

Today, a number of studies support the use of recombinant *L. lactis* to induce mucosal and systemic immune response against a desired antigen (Bermúdez-Humarán et al., 2004a). The first attempt to analyze the potential of *L. lactis* as mucosal vaccine was performed with killed recombinant lactococci producing a cell wall-attached form of a *Streptococcus mutans* protective antigen (PAc). Mice immunized orally with this recombinant strain developed PAc-specific serum IgG and mucosal IgA antibodies (Iwaki et al., 1990). These results showed for the first time that *L. lactis* can be used as a delivery vector to present an antigen to the immune system. However, Wells et al. (1993) reported for the first time the use of live recombinant *L. lactis,* producing the tetanus fragment C (TTFC), to protect mice via subcutaneous injection against a lethal challenge with tetanus toxin. Later, the same group evaluated the effect of oral or intranasal administration with live recombinant lactococci producing TTFC in mice (Norton et al., 1997; Robinson et al., 1997). Oral immunization in mice resulted in a lower serum IgG and mucosal IgA antibodies response than nasal immunization, whereas the protective efficacy (i.e., challenge with tetanus toxin) was the same.

Several studies were conducted to analyze the expression of many viral, bacterial or eukaryotic heterologous proteins in *L. lactis* (Bermúdez-Humarán et al., 2004a and *Table 29.2*). The immunogenicity of the resulting recombinant strains has been evaluated in some cases in mouse models with very promising results. Among them, one of the best documented projects is based on the use of recombinant *L. lactis* producing Human Papillomavirus type-16 (HPV-16) E7 antigen.

This viral protein is considered as a major candidate antigen for vaccines against HPV-related cervical cancer, the second cause of cancer death in women. The intracellular production of E7 antigen model led to its rapid degradation in the cytoplasm of *L. lactis* even when produced in a protease-free strain (Bermúdez-Humarán et al., 2002). In contrast, secreted and cell wall-anchored forms are rescued from proteolysis and produced a higher level of E7 in *L. lactis* (Bermúdez-Humarán et al., 2002, 2004b). Antigen-specific humoral (production of E7 antibodies) and cellular (secretion of IL-2 and IFN-γ cytokines) responses were observed after intranasal administrations to mice of recombinant lactococci expressing E7 antigen at different levels and cellular locations. They were significantly higher in mice immunized with *L. lactis* expressing E7 as a cell wall-anchored form (Bermúdez-Humarán et al., 2004b). These first reports of E7 production in a food-grade LAB represent one more step towards the development of a therapy against HPV-related cervical cancer. Indeed, the protective effects of mucosally co-administered live *L. lactis* strains expressing cell wall-anchored E7 and a secreted form of interleukin-12 to treat HPV-16-induced tumors in a murine model were then evaluated (Bermúdez-Humarán et al., 2005). When challenged with lethal levels of tumor cell line TC-1 expressing E7, 50% of pre-treated mice showed full prevention of TC-1-induced tumors. Therapeutic immunization with these recombinant strains, i.e., 7 days after TC-1 injection, induced regression of palpable tumors in 35% of treated mice. These preclinical results suggest the feasibility of mucosal vaccination and/or immunotherapy against HPV-related cervical cancer using genetically engineered lactococci.

Although most immunological studies have been performed with *L. lactis* producing TTFC and E7 antigen, the reports supporting recombinant lactococci as mucosal vaccine continue to grow, and today, approximately 50 peer-reviewed publications validated this potential (*Table 29.2*).

29.7 Lactobacilli as Live Vaccine Delivery Vector

In contrast to lactococci, some lactobacilli species can persist longer in the GIT and sometimes colonize certain regions of the mucosa and induce a local immune response. A second benefit of the use of lactobacilli is that some strains are considered probiotics (i.e., show health-promoting activities for humans and animals) (Seegers, 2002). Indeed, this genus is widespread and contains over 60 species differing in biochemical, ecological and immunological properties. This biodiversity

rendered the use of *Lactobacillus* spp. as vaccine vehicles more complex compared to *L. lactis,* for which only one single strain (MG1363) was used. However, the capacity of the genus *Lactobacillus* to produce antigens has also been demonstrated.

29.8 Immune Response to Antigens Delivered by *Lactobacillus* spp

The use of genetically modified lactobacilli (i.e., *L. fermentum, L. acidophilus, L. casei* and *L. plantarum*) to produce heterologous proteins and to develop a new generation of mucosal vaccines was first proposed in the 90s decade (Pouwels et al., 1996; Rush et al., 1995). By the end of the 1990s and early 2000s, several laboratories used recombinant strains of *Lb. casei* and *Lb. plantarum* as vehicles for medical proteins delivery at mucosal surfaces; both stimulated strong local immune responses (reviewed in Seegers, 2002; Wells and Mercenier, 2008). Approximately 30 peer-reviewed publications have been published confirming the advantages of the genus *Lactobacillus* as a live mucosal vaccine.

As for *L. lactis*, several studies were also conducted to analyze the expression of a variety of viral, bacterial or eukaryotic origins in *Lb. plantarum* and *Lb. casei* (› *Table 29.2*). The immunogenicity of recombinant *Lb. plantarum* producing E7 antigen has been evaluated in mouse models with promising results (Cortes-Perez et al., 2007).

29.9 Recombinant Lactic Acid Bacteria as DNA Delivery Vehicles

In contrast to bacteria-mediated delivery of protein antigens, bacteria-mediated delivery of DNA vaccines leads to the expression of post-translationally modified antigens by the host cells and therefore to the presentation of conformationally restricted epitopes (Fouts et al., 2003). As for protein delivery, the use of food-grade LAB as DNA delivery vehicles is a promising alternative to attenuated pathogens as DNA vaccines carriers.

L. lactis strains have been used to deliver an expression cassette encoding for bovine β-lactoglobulin (BLG) cDNA, one of the major cow's milk allergen, under the transcriptional control of the viral promoter CMV into the epithelial cell line Caco-2. The expression cassette was inserted in one *L. lactis* replicating plasmid.

Production and secretion of BLG was observed in Caco-2 cells after incubation with *L. lactis* carrying the expression plasmid, demonstrating that non invasive *L. lactis* is able to deliver fully functional plasmid into epithelial cells. Interestingly, no production of BLG was observed when Caco-2 cells were co-incubated with purified plasmid alone or mixed with *L. lactis*, suggesting that the plasmid should be inside the bacterium to achieve transfer and subsequent BLG production into epithelial cells (Guimarães et al., 2006).

After oral administration of *L. lactis* carrying the eukaryotic expression cassette encoding for BLG, BLG cDNA and protein were detected in the small intestine 72 h after the last administration. No BLG was detected 6 days after the last oral administration. The mice developed a BLG specific Th1 primary immune response characterized by a weak and transitory IgG2a response in serum. In sensitized pre-treated mice, IgE and IL-5 concentrations decreased 70 and 40% respectively compared to sensitized naive mice. Moreover, only splenocytes from pre-treated mice secreted IFN-γ after BLG specific re-activation (Chatel et al., 2008). Mice were effectively protected against further sensitization by a specific Th1 response.

Immune response to *L. acidophilus* carrying a DNA vaccine against VP1 antigen of food-and-mouth-diseases virus (FMDV) was investigated after administration by systemic and mucosal routes. The route of administration had a significant impact on the magnitude of the systemic immure response. Indeed, strong immune response to the vaccine antigen was detected only for injected routes of administration although mucosal administration could prime a specific immune response. The intramuscular administration generated the highest level of FMDV VP1 antibodies followed by the intraperitoneal, intranasal and oral routes (Li et al., 2007).

29.10 Recombinant Invasive Lactic Acid Bacteria as DNA Delivery Vehicles

As demonstrated with recombinant *E. coli*, invasion of the host cell is a limiting step to achieve an efficient DNA vaccine delivery (Grillot-Courvalin et al., 1998). To increase LAB DNA vaccine delivery efficiency, *L. lactis* has been modified in order to become invasive by expression of the *InlA* gene of *Listeria monocytogenes InlA* gene coding for the Internalin A surface protein, which mediates the invasion of non phagocytic cells by *Listeria monocytogenes* (Gaillard et al., 1991; Mengaud et al., 1996). InlA binds to an extracellular domain of E-cadherin,

a transmembrane cell-to-cell adhesion molecule (Mengaud et al., 1996). InlA is necessary for invasion of epithelial cells and is sufficient to reconstitute invasion when expressed in non-pathogenic and non-invasive species of *Listeria innocua* (Mengaud et al., 1996). Moreover, when InlA is expressed in *L. lactis*, it can promote the internalization of lactococci into the human epithelial line Caco-2 *in vitro* and into enterocytes *in vivo* after oral administration to guinea pigs. In addition, *L. lactis InlA+ is able to deliver a functional plasmid coding for GFP and about 1% of Caco-2 cells express GFP after co-culture with this strain (Guimarães et al., 2005).*

29.11 Methodologies and Techniques for Genetic Manipulations of Lactic Acid Bacteria

29.11.1 Genetic Engineering of LAB to Produce Heterologous Proteins

The expression system used (based on constitutive or inducible promoters) is an important feature that must be considered for *in vivo* delivery by live bacterial vectors. High production level of heterologous proteins in *L. lactis* can be achieved using constitutive promoters. However, continuous high-level production of certain proteins, such as cytokines (our observations) could lead to intracellular accumulation or degradation which could be deleterious to the cell. Thereby, to prevent possible negative effects caused by high production, inducible promoters have been developed. In these systems, gene expression can be controlled by an inductor, a repressor or by environmental factors, such as pH, temperature or ion concentrations (Morello et al., 2008).

Today, one of the best characterized expression systems is the NICE (Nisin-Controlled Expression) system (Mierau and Kleerebezem, 2005). NICE is a system that allows controlled gene expression by addition of nisin, an antimicrobial peptide used as a natural preservative in the food industry. With this versatile system, the level of gene expression can be controlled by the amount of nisin used for the induction and can be up-regulated more than 1,000-fold (Mierau and Kleerebezem, 2005). As *L. lactis* is a non-colonizing bacterium, therefore, a system that allows preload of the organism with the antigen before *in vivo* application is highly desirable. Induction with NICE system can be considered as a good strategy to obtain high levels of heterologous antigen production *in vitro*. In addition, even after an *in vitro* nisin-pulse, recombinant *L. lactis*

continue to produce heterologous proteins and to evoke an antigen-specific response when administered in mice (Bermúdez-Humarán et al., 2003b, 2004b). These observations support the use of NICE system for the expression of heterologous proteins in *L. lactis*.

Several delivery systems have been developed to target heterologous proteins at different levels and cellular locations within *L. lactis* (Bermúdez-Humarán et al., 2004a; Wells and Mercenier, 2008; Wells et al., 1995). In this context, a family of new vectors which allow heterologous antigens expression in *L. lactis* either intracellularly, extracellularly or cell wall-attached were designed (Bermúdez-Humarán et al., 2003b; Cortes-Perez et al., 2003). These small vectors, based on the broad-host-range pGK plasmid (Kok et al., 1984), are composed of cassettes that allow easy exchange of different expression signals and/or genes. Moreover, as these vectors have the capacity to replicate in Gram-positive bacteria (including *Bacillus subtilis*, *L. lactis* and *Lactobacillus* spp.) and *Escherichia coli*, the procedure of DNA cloning (sometimes laborious in Gram-positive organisms) can be performed in *E. coli*. Once the recombinant vector is established in this bacterium, it can be transferred to the desired Gram-positive bacterium. Most importantly, these vectors have been used to produce successfully different heterologous antigens in *L. lactis* (› *Table 29.2*). A well illustrated example of the efficiency of our system to produce heterologous antigens is the expression of E7 antigen from human papillomavirus type-16 (HPV-16). Initially, DNA plasmid constructions were performed in *E. coli* for E7 expression at different levels and cellular locations using the family of vectors described above (i.e., pCYT:E7, pSEC:E7 and pCWA:E7). After confirmation of the sequence of these recombinant vectors, they were transferred successfully to *L. lactis* and E7 production was evaluated by western blot analysis.

29.11.2 Transformation of LAB

The following protocol to prepare electrocompetent cells of *L. lactis* (1×10^7 CFU/μg DNA, approximately) is recommended: the strain is grown overnight in 5 ml of M17 medium (Difco) supplemented with 0.5% of glucose (GM17), 0.5 M sucrose and 2% glycine (GM17SG) at 30°C without agitation. Then 1/200 from this culture is inoculated in 200 ml of the same medium and incubated at 30°C until optical density (DO_{600}) at 0.5–0.8 is reached. The culture is then incubated immediately in ice for 15 min and stirred every 5 min before the cells are pelleted by centrifugation at 7,000 rpm for 10 min at 4°C. Cellular pellet is washed twice in

100 ml of washed buffer (0.5 M sucrose, 10% glycerol). A third washing is performed with 20 ml of the same buffer. The final pellet is resuspended in 1 ml of PEG-Gly (polyethylene-glycol 3000, 10% glycerol). Aliquots of 100 µl are made in 1.5 ml microcentrifuge tubes, frozen immediately in liquid nitrogen and stored at −80°C until further use. For the transformation, 100 µl of electrocompetents cells are mixed with 1 µl of DNA (10 µl in the case of ligation), transferred to chilled electroporation cuves (2 mm), and exposed to a single electric pulse (Gene-Pulser, BIORAD Laboratories), 25 µF, 200 Ω, 2.4 kV. Immediately after electric discharge, 900 µl of medium GM17S (GM17, 0.5 M sucrose) are added and incubated for 1 h to plasmid expression. Finally, different dilutions are plated in GM17 (1% agar) plus the antibiotic marker. Recombinant colonies are selected after 48 h of incubation at 30°C.

For *Lactobacillus* spp., we have adapted the protocol used for *L. lactis*: briefly, an overnight culture of the strain of *Lactobacillus* (we have tested successfully three different *Lactobacillus* species) are grown in MRS medium (DIFCO) supplemented with 1% of glycine (MRSG) at 37°C without agitation. Then 1/20 of the culture is inoculated in 200 ml of the same medium and culture continued at 37°C until $DO_{600=}$ 0.6–0.7. The culture is then incubated immediately in ice for 15 min and stirred every 5 min before pelleting the cells by centrifugation at 7,000 rpm for 8 min at 4°C. The pellet is washed once with 1 volume of 1 mM of cold magnesium chloride (MgCl2) and once with 1 volume of cold polyethylene glycol 3000, glycerol 10% (PEG-Gly). The final pellet is suspended in 1/100 of the initial volume with PEG-Gly. Aliquots of 50 µl are immediately frozen in liquid nitrogen and stored at −80°C until use. The optimal conditions of the electrotransformation are: a single electric pulse (Gene-Pulser, BIORAD Laboratories), 25 µF, 400 Ω, 1.5 kV with a chilled electroporation cuve (1 mm). Immediately after the electric shock, 500 µl of MRSSM medium (MRS, plus 500 mM sucrose, 100 mM MgCl2) are added and incubated for 3 h for plasmid expression. Finally, different dilutions are plated in MRS (agar 1%) plus the antibiotic. Recombinant clones are selected after 48–72 h of incubation at 37°C.

29.11.3 Nisin Induction, Protein Samples Preparation and Immunoblotting for LAB

Expression of a desired antigen in *L. lactis* using NICE system is recommended as follows: heterologous protein expression can be performed using either 1

or 10 ng/ml of nisin (SIGMA) for a 1- or 3-h period as previously described (Bermúdez-Humarán et al., 2002, 2003c). Protein samples are then prepared from 2 ml of induced cultures. Cell pellet and supernatant are treated separately. To inhibit proteolysis in supernatant samples, 1 mM phenylmethylsulfonyl fluoride and 10 mM dithiothreitol are added. Proteins are precipitated by addition of 100 µl of 100% trichloroacetic acid, incubated 10 min on ice, and centrifuged 10 min at 13,000 rpm at 4°C. For the cell fraction, TES- Lys buffer (25% sucrose, 1 mM EDTA, 50 mM Tris-HCl [pH 8.0], lysozyme [10 mg/ml]) is complemented with 1 mM phenylmethylsulfonyl fluoride and 10 mM dithiothreitol. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis, western blotting, and immunorevelation with antibodies can be then performed.

For lactobacilli, induction is essentially performed as follows: an overnight culture is diluted 1/20 and after 1 h of growth, nisin is added at 25 ng/ml and the culture continued for 5 h. Protein extractions and immunoblotting assays with antibodies are performed as previously described.

29.11.4 Immunofluorescence Microscopy (IFM)

To corroborate protein surface expression in lactococci and lactobacilli, recombinant strains are grown and induced as described above and analysed by immunofluorescent microscopy (IFM). For this, at the end of the induction phase, 2 ml of culture are harvested and suspended in 1 ml of sterile PBS-BSA (bovine serum albumin 3%) containing the corresponding antibody (1/500) and incubated overnight at room temperature. After three washes with PBS-T (PBS-Tween 0.05%), the cell-antibody complex is incubated for 5 h at room temperature (avoiding light exposure) with a solution (1/50 dilution in PBS-BSA) of goat-derived anti-mouse immunoglobulin G (IgG, H + L), conjugated to Alexa Fluor 546 dye (Molecular Probes, Europe BV). Cells are washed three times in PBS-T and the pellet is suspended in 100X µl of PBS from final DO_{600}. Afterwards, different dilutions are performed to determine the optimal quantities to obtain a clear field in the microscopy, usually 2 µl from a dilution 1:10. To visualize the entire cell population, bacteria are stained with 40,6-diamidino-2-phenylindole (DAPI, 2.5 mg/ml; SIGMA) laid on a glass slide, air dried and heat fixed. Pictures of cells are taken with an IFM equipped with a three band filter set for emission light (Nikon, Tokyo, Japan) and Sensia 400 film (Fuji, Tokyo, Japan). Filters appropriate for red excitation light are used to visualize cells, which

are stained with an Alexa Fluor 546 fluorophore. In addition, bacterial images are taken without a filter for excitation light; this allows cells stained with DAPI and Alexa Fluor 546 to be compared simultaneously. Hybridized cells are counted on images captured with the image analysis system Visiolab 1000 (Biocom, Les Ulis, France).

29.11.5 Preparation of Live Bacterial Inoculum and Immunization Protocol

Bacterial cultures are induced as described above and cell pellets are harvested and washed three times with sterile PBS. The pellets are suspended in 10 µl of PBS to obtain a final concentration of 1×10^9 colony-forming units (CFU). Three mice (6–8 weeks) are immunized intranasally with 1×10^9 CFU of induced recombinant LAB strains (5 µl are administered with a micropipette into each nostril) on days 0, 14 and 28. Mice are partially anesthetized by intra-peritoneal injection of a combination of xylazine and ketamine (0.40 ml for 10 kg of weight). Plate counts are performed to check the amount of CFU administered. The control mice received identical quantities of wild-type LAB strain.

29.11.6 Invasiveness Assays of Bacteria into Human Epithelial Cells

Bacterial entry into human epithelial cells was assayed using the human colon carcinoma cell line Caco-2 (ATCC number HTB37), as described by Dramsi et al. (1995). Eukaryotic cells were cultured in RPMI supplemented with 2 mM L-glutamine (BioWhittaker, Cambrex Bio Science, Verviers, Belgium) and 20% fetal calf serum (FCS). The gentamicin survival assay was used to estimate bacteria survival: *L. lactis* strains were grown to an OD_{600} of 0.9–1.0, washed in PBS, and diluted such that the multiplicity of infection (MOI) was about 1,000 bacteria per cell. The bacterial suspension was added to mammalian cells grown in P-24 plates (Corning Glass Works). 2×10^5 cells were seeded in each well the day before the experiment. After 1 h of contact (internalization), gentamicin (20 mg/l) was added to the culture medium. After 2 h of incubation, the cells were washed, then lyzed in 0.2% Triton-X100, and serial dilutions of the lysate were plated for bacterial counting. Gentamicin invasiveness assays were done in triplicate.

29.12 Conclusion

Therapeutic applications of LAB have progressed rapidly in the last years, and following the demonstration that IL-10-producing LAB (i.e., *L. lactis*) could treat colitis in mouse models (Steidler et al., 2000) a successful phase I clinical trial was recently conducted in patients with Crohn's disease (Braat et al., 2006). However, before the approval of this clinical study the development of a containment system for the genetically modified *L. lactis* was necessary. To address safety concerns with the use of IL-10-secreting *L. lactis* in humans, the chromosomal thymidylate synthase (*thyA*) gene was replaced by the gene encoding for IL-10 to generate a thymine auxotroph phenotype. Viability of the *thyA* hIL-10$^+$ strain was reduced by several orders of magnitude in the absence of thymidine or thymine and containment was validated *in vivo* in pigs (Steidler et al., 2003). Strikingly, the phase I clinical trial conducted with the *thyA* hIL10$^+$ strain in patients with Crohn's disease showed that the containment strategy was effective (Braat et al., 2006). These studies open new doors for the use of recombinant LAB as delivery vehicles in the future.

Other exciting applications on the horizon concern the delivery of DNA vaccines using LAB (Chatel et al., 2008), allergen-specific immunotherapy of allergic diseases (Huibregtse et al., 2007) and anti-infectives molecules such as scFv antibodies and microbiocides (Chang et al., 2003; Chancey et al., 2006; Krüger et al., 2002; Liu et al., 2006). With the possibility to express factors such as ScFv antibodies, host targeting molecules and immunomodulators in LAB, we can hope to see more applications and progress towards studies in humans.

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30 Application of Prebiotics and Probiotics in Livestock

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30.1 Introduction

30.1.1 Replacing the Use of Antibiotics

The advent of antibiotics and their use for treatment of clinical manifestations of infections has had a profound impact on animal health and welfare. In addition to direct application in the control of infection, low concentrations of antibiotics given in animal feed has been shown to correlate with higher health status and improved performance in terms of feed conversion (productive weight gain). Thus it is that antibiotics have been used as "growth promoters" in feed for livestock since the 1940s (Cromwell, 2001). Since the inception of this growth promotion concept there has been a debate on precisely how low level antibiotics mediate their action and whether or not this contributes to the acquisition of resistance in the bacterial flora of livestock.

The emergence of resistance to antibiotics in zoonotic bacteria has been well charted for five decades and currently resistance to "antibiotics of last resort" for human health is recognized as a significant veterinary public health issue. Use of antibiotics in animals is cited as a prime factor in the emergence of resistance. However, this should be put into context with the unregulated liberal use of self medication in many parts of the developing world from which are emerging novel resistances such as the wide range of resistances to extended spectrum β-lactams. The key issue is that resistance is emerging through a number of drivers and the organisms carrying those resistances are circulating in a number of different ecological cycles, with food production being but one cycle. In recent years, the concern regarding antibiotic mediated growth promotion in livestock has led to the withdrawal of many prophylactic and therapeutic compounds. In 2006 antimicrobial growth promoters were banned from animal feed destined for use

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in the European Union (EU). There is evidence that there have been increased endemic problems since the introduction of this ban, engendering increased interest in the development of alternative intervention strategies for common livestock pathogens. For example, slaughter of affected animals has been applied in certain limited circumstances. Vaccines are used widely with, for example, diarrhea in neonates and post weaning animals mediated by enterotoxigenic *Escherichia coli* controlled by appropriate whole cell and sub-unit vaccines. Interestingly, vaccination of poultry against *Salmonella enterica* serotype Enteritidis is more to do with protecting human health than protecting the health status of the bird. Stringent biosecurity measures which include barrier methods of animal husbandry, redesign of animal housing, limited access to livestock, control of vermin, modifying air flow, high level disinfection regimes and so on have also been applied. Acidification of feed has also been shown to reduce pathogen intake. Also, suppression of pathogens through the action of the gut flora itself, often enhanced by pre and/or probiotics, is an area of intense scientific activity and has been for at least four decades. Originally, it was described as the Nurmi effect (described in more detail elsewhere in this treatise), enhancing the natural protection afforded by mature gut flora on reducing the pathological impact of gastrointestinal pathogens on younger susceptible animals.

Enteric disease issues in man are increasingly at the forefront as governmental and public concerns regarding preharvest food safety, with organisms such as *E. coli* O157, the non-typhoidal *Salmonella enterica* species, *Campylobacter jejuni*, *Listeria monocytogenes* and many others frequently associated with significant incidences and outbreaks, and all associated with food producing animals as the primary source of infections. Add to this the rise of multiple antibiotic resistance with for example *Salmonella enterica* serotype Typhimurium found in food producing animals, and the problem is exacerbated. Whilst the ban on the use of growth promoting antibiotics is a major recent driver designed to reduce the emergence of antibiotic resistance in animals, there is a pressing need to develop and deploy alternative strategies to achieve this goal, such as feed additives/modifications, which are cost effective.

30.1.2 Current Legislation

Animal feed additives (including yeasts and bacteria) are strictly regulated within the EU legislative framework. Since the decision was made to phase out antimicrobial feed supplements, there has been a great deal of interest in alternative feed supplements for animals and consequently, the procedures and legislation

governing this area have been undergoing significant change in recent years. Until May 2003, the risk assessment of animal feed additives for use in Europe was the responsibility of the Scientific Committee of Animal Nutrition (SCAN) (Anadon et al., 2006; von Wright, 2005). After this date, the European Food Safety Authority (EFSA) took over the functions of SCAN. While EFSA provide expert scientific advice to the European Commission (EC), ultimately, the approval and risk management of a probiotic product is the responsibility of the EC and its constituent member states. In the United States, a microorganism which is used as a feed additive is subject to approval by the Food and Drug Administration (FDA).

In order for a novel probiotic product to fulfill the current EU regulations on animal feed additives the component(s) of the product must be clearly identified and characterized to species level, efficacy data must be provided in support of any claims made for the product, the product must be tolerated by the target animal species (i.e., have no adverse effects on the health or performance), it must be safe for the operator (i.e., have no adverse effects upon exposure) and it must not pose a risk to the safety of the end-consumer (SCAN, 2001). In addition, a novel probiotic product must not harbor any acquired antimicrobial resistance determinants which may be transferred to other bacteria (EFSA, 2005; SCAN, 2001, 2003).

30.1.3 Scope of Use of Pre- and Probiotics to Control Gastrointestinal Diseases in Livestock

Traditionally, antimicrobial feed supplements have been used to control many bacterial and parasitic diseases in farm animal species. However, as described above, the use of antimicrobial feed supplements was banned in the EU in 2006 with the result that intensively farmed livestock in particular are at increased risk of contracting gastrointestinal diseases if prophylactic antimicrobial feed supplements are not utilized. Of the intensively reared animal sector, poultry have been most affected with increasing incidences of avian intestinal spirochaetosis, avian colibacillosis and avian necrotic enteritis amongst other generalized disbacterioses. Similarly, the incidence of enterotoxigenic *E. coli* (ETEC) in pigs has increased. Consequently, there is a requirement for alternative control/prophylactic measures to be developed.

Historically, growth promoters have also been used to increase feed:weight conversions in farm animals and, although the mechanisms involved have not been fully elucidated, a reduction in pathogen carriage and subsequent clinical

disease is one possible mechanism. Certainly, the removal of growth promoters has led to a significant increase in the incidence of diseases as mentioned above, but with increases in feed costs, the reduced feed:weight conversion is also a major concern. Also, many of the other interventions described above are potentially very expensive, especially the full range of biosecurity measures that were discussed.

30.1.4 Use of Probiotics in Animals

Probiotics are one form of alternative feed supplement, or "functional food" which may be used for prophylaxis in animals and humans. Probiotics can be defined as "live microorganisms which, when administered in adequate amounts, confer a health benefit on the host" (FAO/WHO, 2002). There are numerous probiotic products commercially available for livestock.

Commercial livestock probiotic products available today can be separated into two categories, competitive exclusion products that are defined and those that are undefined. In defined competitive exclusion products, the microorganisms that compose the product have been identified and may contain individual or combinations of probiotics. In contrast, undefined competitive exclusion products, are products where the bacterial cultures are either partially or completely undefined (Carita, 1992; Nakamura et al., 2002). Particular problems arise when trying to evaluate the effectiveness of undefined competitive exclusion products. The dose and administration of commercial probiotics are important factors in their effective use (Carita, 1992). The recommended dose of each microorganism varies between products due to the potential strength of probiotic action and industrial production limitations. Recommended doses usually fall within the range of $1x10^8$–$1x10^{10}$ cfu of bacteria per kg of feed.

Nurmi and Rantala developed the first competitive exclusion product to be administered to chickens (Nurmi and Rantala, 1973). This was administered by oral gavage directly into the crop of the chicks. This method was particularly crude and extremely impractical for broiler farmers who would have to administer the product to thousands of birds. Over the years other methods have been developed to administer probiotic supplements into animal feed including pellets, capsules, pastes, powders and granules (Fuller, 1992). The form in which the probiotic is administered depends on the intended use of the product, for example as a prophylactic, the frequency of administration, and also on the

type of animal being dosed (Fuller, 1992). The preferred method of dosing chickens with probiotic products has been via drinking water, although problems have arisen due to the refusal of chicks to drink the water with the probiotic product (Carita, 1992). Droplet application systems have been developed to improve the administration of probiotics to chicks (Carita, 1992). These systems range from the use of simple hand-held garden sprayers to modified bronchitis vaccination apparatus (Carita, 1992).

Knowledge of how probiotics may function as prophylactic agents in the host gastrointestinal (GI) tract is largely conjecture at present. Mechanisms proposed include the secretion of antimicrobial compounds, competition for host cell binding receptors, competition for essential nutrients and stimulation of the host immune system. Studies by Cartman et al. (2008) have demonstrated that orally administered spores germinate in the chicken GI tract, and that it is feasible that spore probiotics produce antimicrobial compounds, following germination in the host GI tract. However, few studies, if any, have attempted to address the relevance of antimicrobial compounds produced by probiotics *in vitro*, to the *in vivo* situation (Cartman and La Ragione, 2004; Cartman et al., 2007).

The limited understanding of how probiotics function as prophylactic agents means that desirable specific traits remain elusive at present. Moreover, empirical studies are an absolute necessity to identify probiotics which are efficacious prophylactic agents. If it is assumed that a probiotic must be present in the host GI tract to exert its probiotic effect, then it follows that for long term protection, persistence is a prerequisite.

Probiotics are used widely in farm livestock, companion animals and in aquaculture, as they are principly associated with reducing clinical disease and increasing growth rates. Numerous probiotic formulations exist for use in animals, however, the majority are based on lactic acid producing bacteria, mainly enterococci and lactobacilli, although *Bacillus* and *Streptococcus* form the active ingredients of some products.

Although it is well documented that pathogens that attach to epithelial cells conscript the regulatory control of that cell, more recent studies indicate that at least some species of the symbiotic microbiota that are in close association with the epithelium also influence activities of epithelial cells (Hooper et al., 2002; Xu and Gordon, 2003). These changes in epithelial cell metabolism enhance colonization by those bacterial species and potentially enhance protection of the epithelial surface from colonization by pathogens. Thus, it is becoming increasingly clear that there is extensive cross-talk between the intestinal microbiota and epithelial cells.

The mucosal epithelium is heavily innervated, with sympathetic and parasympathetic nerves influencing normal function of the intestinal system and mucosal tissues are exposed to hormonal action. When the animal is stressed, the hypothalamic-pituitary axis (HPA) responds by secreting corticosteroids and through direct neuronal stimulation of the mucosal tissues (Matteri et al., 2000; Petrovsky, 2001). Thus, stressors including the environment, nutrition, weaning, transportation and commingling, suppress the animal's ability to resist pathogens and increases the incidence of subclinical or clinical infection. Furthermore, the growth of some pathogenic bacteria is stimulated by addition of the stress hormones such as norepinephrine and epinephrine. Lyte (2004) noted increases in Gram negative (*E. coli*) bacterial populations in the intestinal tracts of animals stimulated to release norepinephrine. In order to further understand this phenomenon more information is required on the interactions between the neuroendocrine response to stressors and the regulation of pathogenic and symbiotic microbiota in the intestine.

30.1.5 Use of Prebiotics in Animals

Prebiotics are defined as non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon (Gibson and Roberfroid, 1995). They are resistant to digestion in the small bowel, through being resistant to gastric acid and hydrolytic enzymes, so pass unhydrolyzed to the colon where members of the resident microbiota, such as bifidobacteria and lactobacilli, can ferment them. Thus, prebiotics can be used in both human and animal hosts to stimulate specific members of the resident microbiota to proliferate and subsequently confer potential health benefits to the host. Currently numerous compounds have been shown to have potential prebiotic activity and these include: lactulose, fructooligosaccharides (FOS), mannan-oligosaccharides (MOS), galactooligosaccharides (GOS), soybean oligosaccharide, lactosucrose, isomalto-oligosaccharides (IMOS), glucooligosaccharides, xylo-oligosaccharides (XOS) and palatinose.

In the last decade there have been increasing attempts to include prebiotics in feed for horses (Respondek et al., 2008), dairy cattle (Franklin et al., 2005), pigs (Loh et al., 2006; Smiricky-Tjardes et al., 2003; Tzortzis et al., 2005), poultry (Fernandez et al., 2002; Spring et al., 2000) and other livestock. In the last two decades the Japanese pioneered the use of oligosaccharides in food products. For example, the commercially available prebiotic Neosugar®, a short

chain fructooligosaccharide (scFOS) containing prebiotic, has been included in more than 500 products (Macfarlane et al., 2008), and prebiotics are included in more than 90% of infant formulas (Ghisolfi, 2003). Prebiotics are found in a range of natural sources; GOS can be obtained from human breast milk and soybean, FOS and fructans from chichory root, banana, Jerusalem artichoke, asparagus, onions and garlic.

Macfarlane et al. (2008) extensively reviewed the prebiotic GOS and stated that the advantages of using prebiotics, such as GOS, are that they are viscous with good moisture retention, they are a third of the sweetness of sucrose, and are stable at high temperatures and in acidic environments. Thus, prebiotics can be included in a wide range of products such as animal feed, water, and human products such as infant formula, dairy products, snack bars and sugar replacements. Additionally, the authors pointed out that they have a low calorific value, specifically, 1.7 kcal/g and 1.5 kcal/g for GOS and FOS, respectively (Macfarlane et al., 2008).

In animals, prebiotics have a long history, with many ancient farmers guiding animals to certain pastures in order to obtain the desired prebiotic from the pasture. In recent years prebiotics have been commonly utilized in both farm and companion animal nutrition. Gibson and Roberfroid, originally classified prebiotics, as defined previously, this definition, however, was based on the use of prebiotics in humans and their use in animals and particularly in ruminants and birds may be more complex.

In recent years the use of prebiotics in the prevention of infections has become a popular area of research. Monosaccharides, disaccharides, oligosaccharides and polysaccharides have been used in poultry as prebiotics. These may help prevent pathogenic infections by binding to the target pathogen directly, or alternatively through their utilization by the intestinal flora they may produce antimicrobial metabolites such as bacteriocins. Mannose is a monosaccharide which is often used to prevent bacterial infections. Type 1 (F1) fimbriae of *Salmonella* and *E. coli* have been shown to bind to mannose residues on the host epithelial glycoproteins and thus free mannose and preparations of yeast mannan-oligosaccharide are thought to interfere with *Salmonella* binding to host cells. Furthermore, prebiotics are associated with stimulating numbers of desired beneficial bacteria to increase in the colon. Specifically, FOS have been shown to promote the growth of *Enterococcus faecium, Lactobacillus lactis* and *Pediococcus* species *in vitro*. Subsequently, prebiotics have been associated with modulating the metabolic activity of the intestinal flora to consequently have a beneficial effect on the host. Specifically, the fermentation of saccharides is associated with

altering concentrations of volatile fatty acids, lactate, carbon dioxide, methane and hydrogen (van Immerseel et al., 2002). Recent studies by Tzortzis et al. (2005) elegantly demonstrated the activity of these two mechanisms by a galactooligosaccharide mixture *in vitro* and *in vivo*. The GOS mixture promoted the growth of *Bifidobacteria* spp. in a continuous culture model and in pigs *in vivo* (Tzortzis et al., 2005). Furthermore, the GOS mixture inhibited *Salmonella* binding to HT29 epithelial cells, presumably by the saturation of *Salmonella* cell binding receptors. Recent studies utilizing a murine model of salmonellosis have demonstrated that a GOS mixture reduces colonization and subsequent clinical signs and pathology (Searle et al., 2009). Furthermore, recent reports have demonstrated the use of IMOS in poultry to promote the growth of bifidobacteria *ex vivo* and have also demonstrated the ability of this oligosaccharide to inhibit *Salmonella* growth *in vitro* (Chung and Day, 2004). Recent research into the use of prebiotics to enhance clearance of *Salmonella* in poultry opens new possibilities for the effective clearance of these zoonotic enteropathogens. The use of prebiotics and probiotics offers another tool for the control of *S.* Enteritidis in poultry and may one day become an integrated part of pathogen control in commercial poultry production.

As previously discussed, probiotics have been utilized to increase the number of probiotic organisms in the GI tract and mitigate against pathogens. However, a limitation of this is that the protection conferred is limited to those times when the probiotic organisms are present in the GI tract, and therefore multiple doses of probiotic organisms are required to maintain protection. A further limitation of probiotics is that they require the delivery of viable microorganisms, such as the obligate anaerobe bifidobacteria, which may reduce in number on exposure to oxygen, and thus storage and administration become complex. An alternative method to increase the number of lactobacilli and bifidobacteria in the colon is to incorporate prebiotics into animal feed to stimulate the resident microbiota to proliferate. Prebiotics are extremely stable and therefore the inclusion into animal feed or water seems a feasible method to increase and sustain the levels of beneficial bacteria such as bifidobacteria and lactobacilli resident in the host. This assumes, however, that the animal currently possesses a "beneficial" microbiota, which if stimulated will confer health benefits to the host. Using chicks as an example, there is often no maternal contact due to intensive rearing methods, and it is suggested that the microbiota does not develop fully, thus making young animals susceptible to enteric disease. In this instance prebiotics alone may not be able to beneficially alter an immature microbiota and furthermore, chicks are often heavily colonized by coliforms and streptococci at a young age.

This advocates synbiotic preparations containing both prebiotics and probiotics as a viable alternative. Additionally, prebiotics act by selectively stimulating the resident microbiota to proliferate and thus, a disadvantage of prebiotics is that if, for any reason, the microbiota is disturbed, that is through the use of antibiotics, they are unlikely to be effective.

In humans, prebiotics have been implicated in a range of physiological benefits to the host, specifically, in reducing the incidence of colorectal carcinoma, increasing mineral absorption, improving gastrointestinal barrier function, improving host immune responses, aiding fat metabolism, reducing inflammation of the bowel and reducing the incidence and severity of pathogenic infections. Furthermore, through stimulating members of the microbiota to proliferate, prebiotics can increase the concentration of short chain fatty acids (SCFA's) present in the host and improve colonic health. These factors will be discussed later in this chapter.

30.2 Probiotics

30.2.1 Probiotic Products

The use of probiotics in farm animals is based upon the central principle that a healthy gut microbiota confers resistance to disease (Fuller, 1992). It is common practice during intensive farming to remove chicks, piglets and calves into isolated "clean" environments, which limits contact with the mother with a number of consequences with regard to acquiring passive immunity, and probable restriction of the development of a normal healthy microbiota. These animals are subsequently more susceptible to infections than their conventionally reared counterparts. Additionally, as mentioned previously, young animals are subjected to a multitude of stresses including transport, nutrition, temperature fluctuations and de-horning. Such stressors have been shown to induce imbalances of the host microbiota (Tannock and Savage, 1974). Specific nutritional stresses include the change from milk to solid feed, from high fiber to high protein diets in young animals, and are associated with imbalances in the microbiota, which can lead to increased disease susceptibility, for example, increased incidences in diarrhea in post weaning piglets and ruminal acidosis in cattle (Krehbiel et al., 2003).

The use of probiotics in farm animals seeks to restore or beneficially alter the microbiota present in young, stressed or antibiotic treated animals so that they can better resist infectious disease. The work of Rantala and Nurmi (1973)

highlights the importance of a "normal" microbiota and its role in the resistance to enteric disease. The reputed health benefits of probiotics are listed in *Table 30.1*.

Antimicrobials are efficacious at clearing target microorganisms causing infection but are also associated with side effects due to altering the native microbiota often drastically. Specifically, antibiotic induced diarrhea results from such imbalances in the microbiota and is often a consequence of bacterial overgrowth of species already present within the GI tract. Probiotics may be administered in conjunction with antimicrobials in feed to prevent infections, restore the microbiota and thus suppress the incidence of antibiotic associated diarrhea (Surawicz, 2008).

The range of products containing probiotic bacteria available for use in food producing animals varies greatly; typically they are developed for use in monogastric animals, including poultry. The use of probiotics in ruminants is more complicated and often depends on whether the target is to combat acidosis, alter the feed:weight conversion, reduce the incidence of disease or decrease methane production (Krehbiel et al., 2003). In ruminants yeasts and fungi have been shown to be particularly effective; furthermore, the bacterial probiotics lactobacilli and *Enterococcus faecium* have been shown to be efficacious (Krehbiel et al., 2003; Wallace, 1994). Typical probiotics which are commercially available in the UK are listed in *Table 30.2* and those commercially available veterinary probiotics deemed as safe by the Scientific Committee for Animal Nutrition within

Table 30.1

A list of health benefits observed in food producing animals administered probiotics

Health benefits associated with probiotics
Improved digestion
Improved feed conversion ratios
Increased resistance to infectious disease
Reduced carcass contamination
Increased growth rate
Control of ruminal acidosis (cattle)
Increased milk yield (dairy cattle)
Increased egg production (layer hens)
Improved egg quality (layer hens)
Improved digestion
Reduced mortality/morbidity

Table 30.2

Veterinary probiotics available for use in food producing animals in the UK (*Cont'd* p. 1134)

Producer	Product name	Probiotic organism(s)	Target organism(s)	Other additives	Method of administration
Protexin	Protexin Concentrate®	*Enterococcus faecium* (NCIMB 10415) EC No. 13	Cattle, pigs, lambs	–	–
	Protexin Lifestart®	Unspecified multi-strain	Cattle, pigs, lambs	MCFA, vitamins, colostrum, garlic powder	–
	Protexin Pro-soluble®	*Enterococcus faecium* (NCIMB 10415) EC No. 13	Cattle, poultry, pigs, lambs	–	Water or milk
Christian Hansen	Bioplus 2B®	*Bacillus licheniformis, Bacillus subtilis*	Pigs, Sow, piglets, grower, finisher	–	–
	Probio precise®	*Enterococcus faecium, Saccharomyces cerevisiae*	Ruminants	–	–
	Gallipro®	*Bacillus subtilis*	Poultry	–	–
Medipharm (Chr Hansen group)	Lactiferm caps®	Unknown	Cattle, poultry, pigs	–	Capsules
	Lactiferm Basic®	Unknown	Cattle, poultry, pigs	–	–
	Lactiferm Spray®	Unknown strain M74	Cattle, poultry, pigs	–	Applied to feed
	Lactiferm DW (Drinking Water) ®	Unknown strain M74	Cattle, poultry, pigs	–	–
	Lactiferm Synergy CPS®	Unknown	Pigs and cattle	–	Milk and feed
	Lactiferm paste®	Unknown			

Table 30.2

Producer	Product name	Probiotic organism(s)	Target organism(s)	Other additives	Method of administration
Provita Eurotech	Provita lacteal®	*Lactobacillus acidophilus, Enterococcus faecium, Bacillus subtilis*	–	*Salmonella* and rotaviral antibodies, vitamins	–
	Provita New Born piglet®	*Lactobacillus acidophilus* LA101 & 107, *Enterococcus faecium* SF 101	Pigs	Vitamins	–
	Provita Protect®	*Lactobacillus acidophilus* LA101 & 107, *Enterococcus faecium* SF 101	Cattle	–	–
	Provita Response®	Unknown	–	Polyethene-glycol base	
	Provita Biogrow®	*Bacillus subtilis, Bacillus licheniformis*	Poultry	–	–

Table 30.3

List of commercially available veterinary probiotics deemed as safe by the Scientific Committee for Animal Nutrition within the EU (*Cont'd* p. 1136)

Product name	Probiotic organism	Culture collection	Target organism
Adjulact 2000®	*Streptococcus infantarius*	CNCM I-841	Calves
	Lactobacillus plantarum	CNCM I-840	
Bactocell®	*Pediococcus acidilacti*	CNCM MA 18/5 M	Broilers
	Pediococcus acidilactici	CNCM MA 18/5 M	Broilers
Biacton®	*Lactobacillus farciminis*	CNCM MA 67/4 R	Piglets
Bioplus 2B®	*Bacillus licheniformis*	DSM 5749	Piglets, sows, piglets for fattening, broilers, turkeys and calves
	Bacillus subtilis	DSM 5750	
Biosprint®	*Saccharomyces cerevisiae*	BCCM/ MUCL 39885	Beef cattle, piglets and pigs for fattening
Bonvital®	*Enterococcus faecium*	DSM 7134	Pigs for fattening and calves
	Lactobacillus rhamnosus	DSM 7133	
Biosaf SC 47®	*Saccharomyces cerevisiae*	NCYC Sc 47	Piglets, sows, beef and dairy cattle
Cylactin LBC®	*Enterococcus faecium*	NCIMB 10415	Piglets, pigs for fattening, calves and broilers
Fecinor plus®	*Enterococcus faecium*	CECT 4515	Piglets, pigs for fattening, calves and beef cattle
Gardion®	*Enterococcus faecium*	NCIMB 30096	Calves
		NCIMB 30098	
Kluyten®	*Kluyveromyces marxiamus*	MUCL 39434	Dairy cattle
Lactiferm ®	*Enterococcus faecium*	NCIMB 11181	Calves, piglets
Lactobacillus acidophilus D2/CSL®	*Lactobacillus acidophilus*	CECT 4529	Broilers and laying hens
Levucell SB20®	*Saccharomyces cerevisiae*	CNCM I-1079	Piglets and sows
Levucell SC20®	*Saccharomyces cerevisiae*	CNCM I-1077	Beef and dairy cattle

Table 30.3

Product name	Probiotic organism	Culture collection	Target organism
Microferm®	*Enterococcus faecium*	DSM 5464	Piglets, calves and broilers
Mirimil-Biomin®	*Enterococcus faecium*	DSM 3520	Calves
Oralin®	*Enterococcus faecium*	NCIMB 10415	Pigs for fattening, calves and broilers
Primver Pro ®	*Enterococcus mundtii*	CNCM MA 27/4E	Lambs
Probios PDFM Granular®	*Enterococcus faecium*	DSM 4788/ ATCC 53519	Broilers
	Enterococcus faecium	DSM 4789/ ATCC 55593	
Yea-Sacc®	*Saccharomyces cerevisiae* 1026	CBS 493 94	Calves, beef and dairy

Adapted from the SCAN Opinion on the use of certain microorganisms as additives in feeding stuffs 26th September 1997. http://ec.europa.eu/food/fs/sc/scan/out93_en.pdf 19/12/09

the EU are listed in *Table 30.3*. This table is adapted from the SCAN Opinion on the use of certain microorganisms as additives in feeding stuffs 26th September 1997 (http://ec.europa.eu/food/fs/sc/scan/out93_en.pdf 19/12/09).

30.2.2 Selection Criteria for Veterinary Probiotics

There is little evidence linking the efficacy of probiotics *in vivo* to specific *in vitro* phenotypes. Typically, probiotics are chosen based upon a number of defined selection criteria. For example, *in vitro* adherence to epithelial cells has been used but whether adhesion to epithelial cells *in vitro* provides adequate evidence of the ability of a probiotic to adhere to the intestinal epithelium and persist in the gastrointestinal tract in vivo is open to question. Other selection characteristics focus on the ability of a particular probiotic strain to survive multiple environmental stresses, typically acid, heat and dessication to reflect manufacture, storage and *in vivo* environments with, for example, *in vitro* models which simulate porcine gastric fluid proving effective for selection (De Angelis et al., 2006). Another additional selection criterion is that candidate probiotics should, ideally, exhibit antimicrobial activity against target food borne pathogens or some degree

of antagonism (De Angelis et al., 2006; Dunne et al., 2001). There are other factors unrelated to possible mode of action, such as the origin being the same host species as for the intended treatment, and freedom from transmissible antibiotic resistance genes.

Other factors which affect the choice of probiotic include the intended host and whether single strain or multiple strain probiotic products are required. Specifically, many probiotic strains are host adapted, for example *Lactobacillus fermentum* strain 104R has been shown to secrete bridging proteins which facilitate the adhesion to porcine small intestinal mucus and gastric mucin. Single strain probiotics are defined as probiotics containing one strain of a certain species and multiple strain probiotics contain more than one strain of the same species or closely related species. Multiple species probiotics, unlike multiple strain probiotics contain strains of different probiotic species that belong to one or more genera, for example *L. plantarum*, *Bifidobacterium breve* and *Pediococcus acidilacti* (Timmerman et al., 2004). Recent studies have advocated multigenera probiotics as a viable and efficacious alternative to single strain probiotics for the competitive exclusion (CE) of pathogens from pigs (Casey et al., 2007). There are advantages and disadvantages to single, multiple strain and multispecies probiotics (Timmerman et al., 2004), however, little peer-reviewed evidence is available that directly compares their efficacy in food producing animals. It is known that different genera/species of probiotics can work synergistically to enhance the metabolic function of not only the host but other bacteria. Cross-feeding between different microbial genera/species is well documented with the production of 1,4-dihydroxy-2-napthoic acid by propionibacteria, having a bifidogenic effect, being a well defined example (Kaneko et al., 1994; Martin et al., 2008).

30.2.3 Safety Considerations in Probiotic Selection

Lactobacilli are generally regarded as safe (GRAS) and it is mostly accepted that they do not cause disease in humans or food producing animals. However, with the increasing consumption of lactobacilli, due to its presence in probiotic food products, it is essential that legislation accurately reflects the risk posed by consumption of these live microbial feed supplements to the consumer. Concerns over the safety of probiotics include deleterious metabolic activation, excessive immune stimulation and gene transfer of virulence and antimicrobial resistance genes amongst microorganisms (Agostoni et al., 2004). These health

considerations have led to the publication of guidelines for the selection of probiotics which are listed below (87/153 EEC):

Safety has to be assessed in accordance with the test set out in the Directive guidelines.

- Probiotic strains that produce toxins are not permitted.
- Probiotic strains that produce virulence factors are not permitted.
- Probiotic strains that produce antibiotic substances of clinical or veterinary significance are not permitted.
- Probiotic strains that carry transmissible antibiotic resistance determinants are not permitted.

30.2.4 Carriage of Antimicrobial Resistance Genes in Lactic Acid Bacteria

If probiotics are to be administered to food producing animals, they must not carry transferable antimicrobial resistance genes. There is a large body of evidence suggesting that antimicrobial resistance genes can be transferred between resistant and sensitive species of enterococci within the GI tract of mammals. Furthermore, there is increasing evidence detailing the carriage of transferable antimicrobial resistance genes amongst probiotic *Lactobacillus* and *Enterococcus* strains.

Antimicrobial resistance occurs naturally within bacterial genera/species, through permeability barriers or efflux for example, and this resistance to antimicrobial compounds is described as intrinsic or innate. For example, some lactobacilli are naturally resistant to the "last resort" antibiotic vancomycin. This antibiotic targets the peptidoglycan cell wall by binding to the peptidoglycan precursors D-alanine D-alanine termini and preventing cross-linking between peptidoglycan chains. Resistant lactobacilli possess a D-alanine D-lactate termini on their peptidoglycan precursors, which exhibits a 1,000 fold reduction in vancomycin binding (Roper et al., 2000). In contrast, acquired resistance relies on the acquisition of antimicrobial resistance genes via mobile genetic elements, such as plasmids and transposons. The risk of antimicrobial resistance genes transferring to human pathogens, either directly or indirectly through the commensal bacteria is a concern.

Alarmingly there is also evidence that *Lactobacillus* spp. can act as an environmental reservoir for antimicrobial resistance genes in broilers. In a survey

of 20 different Belgium farms in 2006 a substantial pool of mobile tetracycline resistance genes were discovered in broilers including; tet (K), tet(L), tet(W) and tet(Z) (Cauwerts et al., 2006a, b). This is of biological significance as tetracycline is an important antibiotic used in the treatment of broiler infections. Additionally, tetracycline resistance plasmids have also been found in *L. plantarum* 5057, which contained lactobacilli specific insertion sequences (Danielsen, 2002). Collectively, the evidence presented here suggests a need for monitoring the carriage of antimicrobial resistance genes in potential probiotics, but also for evaluating the effects of prophylactic antibiotic use on "beneficial bacteria" whose growth may be augmented by the addition of probiotic cultures (Cauwerts et al., 2006b). It is useful to consult the recommended minimum inhibitory concentration (MIC) of clinically important antibiotics in potential probiotic isolates (› *Table 30.4*) when selecting for potential probiotic candidates. It is important to note that bacteria expressing mobile or transferable (acquired) resistance genes should not be used.

Table 30.4

SCAN Recommended MIC values for commonly used probiotic bacteria. R-MIC determination not necessary for species designated as inherently resistant to the antibiotic

Antibiotic	*Enterococcus faecium*	*Enterococcus faecalis*	*Pediococcus*	*Lactobacillus*	*Bacillus*
Ampicillin	8	8	2	2[a]	2[a]
Streptomycin	1,024	1,024	32	16	64
Kanamycin/ neomycin	1,024	1,024	32	32	64
Gentamicin	500	500	4	1	8
Chloramphenicol	16	16	16	16	16
Tetracycline	16	16	16	16	16
Erythromycin	4	4	4	4	4
Quinupristin/ dalfopristin	4	R	4	4	4
Vancomycin	8	8	R	4[a]	4
Trimethoprim	8	8	16	32	8
Ciprofloxacin/ enrofloxacin	4	2	16	4[a]	1
Linezolid	4	4	4	4	4
Rifampin	4	4	8	32	4

Adapted from SCAN (http://ec.europa.eu/food/fs/sc/scan/out64_en.pdf 15/12/08)

[a]Certain species are inherently resistant

30.3 The Use of Probiotics in Poultry

Probiotics have been used extensively in poultry since the 1970s, particularly in newly hatched chicks. The seminal work of Nurmi and Rantala first demonstrated Competitive Exclusion (CE) (Rantala and Nurmi, 1973) which is described as the exclusion of a pathogen from an ecological niche by outcompetion by a probiotic. Nurmi and Rantala illustrated that crude cecal extracts from mature birds administered orally to newly hatched chicks could confer resistance to *Salmonella enterica* serotype Enteritidis (Rantala and Nurmi, 1973). To date a number of studies have demonstrated that the CE effects of probiotics can protect hosts against pathogens such as *S.* Typhimurium, *Campylobacter jejuni*, *Yersinia Enterocolitica* and *Escherichia coli* O157:H7 (Casey et al., 2007; Schoeni and Wong, 1994; Soerjadi-Liem et al., 1984b; Weinack et al., 1981, 1982; Zhang et al., 2007).

Incubator hatched chicks are particularly amenable to probiotic interventions because they are deprived of the protective microbiota, which they would normally acquire from the hen and the environment. Interestingly, in conventionally reared chicks the caecal microbiota does not stabilize until the birds are 4–6 weeks old (Mead, 1989), suggesting that probiotics are best administered to newly hatched chicks, however, they may still be beneficial up to 6 weeks post hatching.

Single strain probiotic preparations have been used to control *S.* Typhimurium and *S.* Enteritidis infections in broilers (Carter, 2008). Furthermore, recently a spray application has been used to dose chicks with a *Lactobacillus* based probiotic (FM-Probiotic) (Higgins et al., 2008).

Probiotics have been shown to be an effective CE agent for *Salmonella* spp. in poultry and their use was extensively reviewed by Revolledo et al. (2006). The effect of probiotics on broilers and layer hens were critically reviewed by Fuller (1992) who found that a large proportion of published studies reported administration of probiotics produced a substantial effect on the parameters listed in *Table 30.1*, including vitamin and serum triglyceride levels. However, Fuller also concluded that little significant evidence existed, even when the claims made for a probiotic product were substantial. In the years since the Fuller review there is still limited evidence regarding the efficacy of probiotics in poultry other than for the CE of foodborne pathogens. Many of the studies conducted today still remain poorly designed, with either too few animals or insufficient statistical analysis and validation. However, there seems to be a consensus of opinion that probiotics are efficacious in poultry when they are kept under sub-optimal conditions (Fuller, 1992). In the text below, the focus will be on the application of probiotics to control foodborne pathogens in poultry.

30.3.1 Recent Advances in the CE of *Salmonella, E. coli* and *C. perfringens* in Poultry

The CE effect against *Salmonella* spp. and avian pathogenic *E. coli* has been studied extensively (Fuller, 1992; Schoeni and Wong, 1994; Zhang et al., 2007). The most effective form of CE demonstrated to date has been from Nurmi type cultures which consist of undefined caecal bacterial cultures. In the early 1990s two commercially available probiotic supplements, BROILACT® and AVIGUARD®, which consisted of undefined competitive exclusion preparations were sold commercially in the UK. BROILACT® demonstrated a strong CE effect on *S.* Enteritidis PT4 with a significant reduction in *Salmonella* recovered from cecal contents (Nuotio et al., 1992; Schneitz et al., 1992). AVIGUARD® has also been shown to protect 1-day old chicks from colonization with high titers of *S.* Enteritidis and *S.* Typhimurium which are the two most commonly isolated *Salmonella* serotypes causing human infection (Nakamura et al., 2002).

Although many of the CE treatments are either defined or undefined cecal contents or multiple bacterial species derived from said contents. Single strain probiotics, particularly *Lactobacillus reuteri*, was shown to decrease the colonization of chicks and turkey poults by *Salmonella* and *Escherichia coli* (Edens et al., 1997). Recently, reports where lactobacilli have been used to control *S.* Enteritidis in poultry have been described (Higgins et al., 2007; La Ragione and Woodward, 2003; La Ragione et al., 2004; Vicente et al., 2008). Vicente and Higgins both reported the use of commercial *Lactobacillus* preparation FM-B11 to inhibit *S.* Enteritidis in commercial broilers (Higgins et al., 2007; Vicente et al., 2008). Both studies showed an overall reduction in *Salmonella* recovery when the probiotic was administered prophylactically (Higgins et al., 2007; Vicente et al., 2008). However, the use of probiotics as prophylactic treatments is controversial especially when antibiotics are readily available and effective. It is suggested that while these studies show some promise, it is questioned whether there is a need for probiotic prophylaxis in the commercial market and if so how effective would probiotics need to be to be used preferentially? Are they ever likely, when administered as a prophylactic, to totally exclude a pathogen? To date, even *in vivo* studies utilizing potent bacteriocin, producing strains still leave between 10^3–10^4 cfu/g feces of viable pathogens (Corr et al., 2007). Finally, *S.* Enteritidis has been excluded from the 1-day old chick model using both *L. johnsonii* and *Bacillus subtilis* in a probiotic protection assay (La Ragione and Woodward, 2003; La Ragione et al., 2004). Whilst showing considerable promise, neither probiotic completely eliminated the pathogenic strain.

The majority of poultry competitive exclusion experiments have focused on the two main zoonoses in which poultry are a major reservoir; *Salmonella* spp. and *Campylobacter* spp. However, recent work has focused on emerging or opportunistic pathogens found in poultry with for example recent studies with *Clostridium perfringens*, the causative agent of necrotic enteritis in poultry, and *E. coli* O78:K80, the cause of avian colibacillosis. La Ragione et al. (2004) showed a significant reduction in *C. perfringens* and *E. coli* O78:K80 when dosing specific pathogen free chicks with *L. johnsonii* F19785. Interestingly, *Bacillus subtilis* spores have also been shown to competitively exclude *C. perfringens* from a 1-day chick model but the effect was extremely delayed leading to suggestions that the spores may need to germinate within the GI tract or that immunomodulation was occurring (La Ragione and Woodward, 2003). The protective effect of the adult chicken microbiota against pathogenic *E. coli* is well documented (Weinack et al., 1981, 1982, 1984). Other undefined CE preparations have also shown some efficacy against the human pathogen *E. coli* O157:H7 (Stavric et al., 1993) and some small studies conducted over a short time period have evaluated the effects of BROILACT® on *E. coli* O20:K-:H8.

30.3.2 Recent Advances in the CE of *Campylobacter* in Poultry

The efficacy of CE with cecal contents has also been demonstrated against *Campylobacter jejuni*, although the cecal contents required to protect against *C. jejuni* infections instead of *Salmonella* infections were found to be different (Fuller, 1992; Zhang et al., 2007). As a consequence, studies are now focusing on the use of defined CE cecal preparations which are active against not only *C. jejuni* but also *S.* Typhimurium. Zhang et al. (2007) carefully selected adult chickens from family farms, commercial farms and broiler research centers with the purpose of selecting donor *C. jejuni* free birds. They subsequently identified the CE effect of cecal preparations from these adult birds on *C. jejuni* and *Salmonella* spp. (multiple serotypes). Zhang and colleagues identified 636 isolates inhibitory to *C. jejuni* and 194 extremely inhibitory isolates (Zhang et al., 2007). Interestingly, of the 194 extremely inhibitory isolates 145 were isolated from the ceca and 117 were facultatively anaerobic bacteria (Zhang et al., 2007). Schoeni and Wong (1994) tested defined CE mixtures against *C. jejuni* colonization in chicks. A defined CE mixture, which by the definition of Timmerman et al. (2004) is a multi-species probiotic; contained *Citrobacter diversus* 22, *Klebsiella*

pneumoniae 23 and *Escherichia coli* 25 (CE3) was extremely effective at reducing *C. jejuni* colonization in 1-day old chicks (Schoeni and Wong, 1994). It should be pointed out that this is a very artificial model as chicks are usually colonized by *C. jejuni* much latter in life than at 1 day of age. This CE3 mixture when tested on groups of chicks reduced the colonization by *C. jejuni* from 80% of control birds to between 11% and 17% (Schoeni and Wong, 1994). Timmerman suggested that a multispecies probiotic would be more efficacious than single strain preparations and studies like that of Zhang pave the way to making defined multispecies probiotic preparations which are efficacious against more than one foodborne pathogen. The efficacy of undefined CE agents for *C. jejuni* have shown varying degrees of effectiveness, however, the more recent studies indicated above, using defined CE cultures still how efficacy but are much more likely to meet with future legislative changes (Mulder and Bolder, 1991; Shanker et al., 1988, 1990; Soerjadi et al., 1982; Soerjadi-liem et al., 1984a; Stern et al., 1988).

Some other approaches have also been undertaken to competitively exclude *Campylobacter* from poultry. Chen and Stern (2001) used commensal chicken *C. jejuni* isolates to competitively exclude human isolates from day-old broiler chicks. This story is similar to that of *E. coli* Nissle, where a non-pathogenic strain of a common foodborne pathogen is used to displace a pathogenic species. However, there is little data to define *Campylobacter* as non-pathogenic, so, as a control strategy this is highly flawed. Also, it is important to note that these potential probiotics have a much greater chance of acquiring antimicrobial resistance genes and/or virulence traits from similar pathogenic bacteria present within the GI tract and in the authors' opinion should be avoided unless they are proven to be safe by well controlled peer-reviewed studies.

30.3.3 Future of CE Strategies and Probiotics in Poultry

The seminal work of Nurmi and Rantala highlighted the importance of a "normal" adult microbiota in the protection of chicks against enteric disease (Rantala and Nurmi, 1973). CE is an accepted mechanism by which probiotics confer a protective effect against foodborne pathogens (Fuller, 1992). Currently the mechanisms behind the CE effect are still largely not understood and will be discussed further in this review (below). CE cultures show varying efficacy across *Salmonella*, *E. coli*, *Campylobacter* and other foodborne pathogens. This is most likely due to the inadequate selection of cecal contents as demonstrated by Zhang et al. (2007). Defined CE cultures as a whole are being developed, and

the work of Zhang et al. (2007) is the bridge between CE cultures and extremely effective multi-species probiotics, which Timmerman has advocated as having the best potential in clinical and *in vivo* trials to exert beneficial effects (Timmerman et al., 2004; Zhang et al., 2007). Treatment with undefined CE cultures presents two major concerns. Firstly, human or animal pathogens may be present within the mixtures, presenting a considerable health risk, and secondly regulatory bodies are becoming stricter with their policy and the days of undefined CE cultures are coming to an end. It is clear that the future for probiotics in poultry requires the need for studies advocating multi-species probiotics against more than one foodborne pathogen, with *Salmonella* and *Campylobacter* being the most obvious choices. All animal studies would benefit from a consensus of opinion from experts in the field with regards to how they should be carried out, allowing maximal biological significance to be obtained from every experiment; this would also have the effect of making meta analysis feasible.

30.4 The Use of Probiotics in Ruminants

Probiotics are employed in cattle production to reduce the use of antibiotics in neonatal and stressed calves, enhance milk production, prevent ruminal acidosis, improve the feed conversion ratio, enhance the CE of enteropathogens, and to rapidly establish a stable microbiota in neonatal calves (Krehbiel et al., 2003). The use of probiotics in beef production can be separated into two categories; protection of stressed calves and the continuous dosing of feedlot cattle. Calves undergo a variety of stresses when entering the feedlot, which can drastically alter the microbiota in the rumen and lower GI tract. Such stressors include weaning, transport, vaccination, castration, and dehorning.

Historically, CE has not been considered as a viable alternative to antimicrobials in cattle because of the complexity of rumen microbiology and the relatively long production cycles in the cattle industry. Very few studies have looked at the CE of foodborne pathogens from feedlot cattle (Tkalcic et al., 2003; Zhao et al., 1998, 2003). However, non-pathogenic *E. coli* were evaluated by Zhao for their ability to CE pathogenic *E. coli* O157:H7 from feedlot cattle and enterohemorrhagic *E. coli* (EHEC) in neonatal calves, respectively (Zhao et al., 1998, 2003). Tkalcic et al. (2003) also used non-pathogenic *E. coli* to CE EHEC in weaned calves. Interestingly, Brashears et al. (2003a) demonstrated a significant reduction in the fecal shedding and carcass contamination of feedlot cattle with *E. coli* O157:H7 using a mono-strain probiotic (*Lactobacillus acidophilus* NPC 747, Brashears et al., 2003a, b).

30.4.1 Probiotics and Their Use to Control Ruminal Acidosis

Ruminal acidosis can be defined as a low ruminal pH of below 5.6 and high ruminal volatile fatty acid (VFA) concentrations. High ruminal VFA concentrations can be divided into subacute acidosis, where elevated levels of short chain or branched chain fatty acids but not lactate accumulate in the rumen, or into acute acidosis which consists of high levels of lactic acid in the rumen. Initial approaches adopted to control and prevent acute acidosis in cattle concentrated on the use of lactate utilizers to reduce lactate levels in the rumen. *Megasphaera elsdenii*, a lactate utilizer, has been successfully used to modify lactate levels in cases of acute acidosis in cattle, during the transition from low to high concentrate diets (Greening et al., 1991). However, *M. elsdenii* can cause inefficient metabolism in the rumen, which can be detrimental to levels of propionate and subsequently methane production. Surprisingly, the addition of lactobacilli, specifically *L. acidophilus,* has been shown to reduce total D/L-lactate levels and sustain the ruminal pH around pH 6 (Huffman et al., 1992; Krehbiel et al., 2003). Thus, lactobacilli may be useful in reducing ruminal acidosis.

Other intervention strategies using probiotic fungi and yeasts have also proved highly effective in controlling ruminal acidosis in cattle. Specifically, *Aspergillus oryzae* and *Saccharomyces cerevisiae* are regularly used as direct feed microbials in cattle. It is believed that the delivery of live yeast to cattle exerts health benefits on the host by removing oxygen in the rumen and boosting the anaerobic environment. This has the knock on effect of increasing the viability of anaerobes in the rumen by 50–100% and subsequently increases the number of lactate utilizing anaerobic bacteria that are believed to control the acidosis. Furthermore, levels of dicarboxylic acids also rise which stimulate lactate uptake by *Selenomas ruminatium* and other potent lactate utilizers (please refer to a review by Wallace, 1994). The yeasts must be metabolically active to elicit their stimulatory effect, as indicated by studies utilizing *S. cerevisiae* respiratory mutants (Newbold et al., 1991, 1993a).

30.4.2 Probiotics and Their Use to Control Methane Production in Ruminants

Rumen microbial ecosystems produce methane from anaerobic fermentation and methane production by ruminants is estimated to account for 15% of all

environmental methane (Takahashi et al., 2005). Methanogens in the rumen convert carbon dioxide into methane by reduction with hydrogen, and methanogens play an important role in the rumen by actively scavenging hydrogen which is detrimental to ruminal digestion (Wollin, 1979). To understand methane production in the rumen it is important to understand the role that different nutritional components and specific ruminal microorganisms have on ruminal digestion. *Table 30.5* illustrates some of the major culturable ruminal microorganisms along with their metabolic end products and the role of these metabolites in methane production will be discussed later. The major methane pre-cursors in the rumen are acetate, carbon dioxide and hydrogen; these metabolites are produced mainly by the breakdown of carbohydrates, in particular cellulose. Therefore, rumen metabolism and subsequent methane production is expressed as the sum of all the different metabolisms depending on the level of carbohydrates provided. For an extensive review of methane production in ruminants and the detailed biochemistry behind methane production see (Mitsumori, 2008; Wollin, 1979).

At least 20% of all rumen methanogenic bacteria are endosymbionts and are found attached to the surface of protozoal strains (Stumm et al., 1982; Vogels

Table 30.5

Examples of ruminal microorganisms and their metabolic end products

Rumen microorganism	Final metabolic product
Fibrobacter succinogenes	Acetate and succinate
Ruminococcus albus	Acetate
Ruminococcus flavefacience	Acetate and succinate
Prevotella ruminicola	Succinate[a]
Ruminobacter amylophilus	Succinate[a]
Fibrobacter succinogenes	Succinate[a]
Ruminococcus flavefaciens	Succinate[a]
Succinivibrio dextrinosolvens	Succinate[a]
Succinomonas amylolyitca	Succinate[a]
Selomonas ruminantium	Propionate
Veillonella alcalescens	Propionate[b]
Succiniclasticum ruminis	Propionate[b]

Adapted from Wollin, (1979)

[a]Succinate is a propionate generating intermediate and is believed to be converted to propionate via cross feeding to *Selonomas ruminantium, Veillonella alcalescens and Succiniclasticum ruminis,* as well as other succinate degrading bacteria

[b]Succinate is decarboxylated to produce propionate, which is a major energy source in cattle

Table 30.6

Examples of methanogens commonly found in ruminant animals and the substrates they require to form methane

Ruminal microorganism[a]	Metabolites required to form methane[b]
Methanobrevibacter ruminatum	Hydrogen, carbon dioxide and acetate
Methanomicrobium mobile	Hydrogen, carbon dioxide and acetate
Methanosarcina mazei	Acetate, methanol, methylamines
Methanosarcina barkeri	Acetate, methanol, methylamines
Methanobacterium formicium	Hydrogen, carbon dioxide and acetate

Note: The majority of hydrogen and carbon dioxide is provided by carbohydrate degradation in the rumen

[a]These data were obtained from Mitsumori et al. (2002)

[b]These data were obtained from Jarvis et al. (2000)

et al., 1980). *Table 30.6* shows examples of culturable methanogens found in ruminants, and it accentuates the fact that hydrogen and carbon dioxide are essential in the production of methane, and therefore the metabolism of carbohydrates in the rumen is essential for methane production (Mitsumori, 2008; Mitsumori et al., 2002). Therefore, initial strategies aimed at reducing methane production have focused on targeting protozoal species present within the rumen and also by altering the ruminant diet, although this will not be discussed further here. Traditionally, protozoal and in some cases methanogen levels were controlled using the antibiotic ionophores monensin and salinomycin, which also have the benefit of increasing beneficial propionate levels (Newbold et al., 1993a). However, it is important to note that the prolonged use of monensin in steers led to loss of its methane suppressing activity and rumen bacteria readily develop resistance (McCaughey et al., 1997; Newbold et al., 1993b). To date there is little peer-reviewed evidence to suggest the efficacy of probiotics to control the production of methane in cattle. However, some contrasting data is available which demonstrates some efficacy with fungal direct feed antimicrobials. *Saccharomyces cerevisiae* has been shown to reduce methane production in feedlot cattle (Lila et al., 2004; Martin et al., 1990; Takahashi et al., 2004). However, the feed given to the cattle must be taken into account, especially its methanogenic potential. Multiple types of corn feed were shown to have modest methanogenic potential when compared to high protein diets or feed with a high cellulose content. *In vitro* data using twin strains of *Saccharomyces cerevisiae* demonstrated a small reduction in methane production after 24 hour incubation of the *Saccharomyces* in a mixed ruminal fermentation vessel (Lila et al., 2004). Limited *in vitro* data using orchard grass hay, lucerne hay cube or concentrate, which are rich in

cellulose (giving a high methane yield), demonstrated that the prebiotic galactooligosaccharide (GOS) and a synbiotic containing GOS and *Aspergillus oryzae* were efficacious in reducing methane production in replacement dairy cows. Supplementation of dairy cows with GOS resulted in an 11% reduction in methane production, whereas supplementation with the synbiotic increased methane production (Takahashi et al., 2004). In contrast, *Aspergillus oryzae* alone has been shown to increase overall methanogenesis and hydrogen production, although again the feed used was different, thus making comparisons difficult (Matrin et al., 1990).

30.5 The Application of Probiotics in Pigs

Although probiotics are used widely in monogastric food producing animals, this section will concentrate on their application in pigs. Currently, probiotics are only administered to pigs orally and can be administered by direct addition to the water or feed, which is either pelleted or ground. There are also some examples where probiotics are administered as fermentation products in wet, frozen, freeze-dried preparations and pastes.

Lactic acid bacteria (LAB), in particular lactobacilli, are ideal probiotic candidates for use in pigs. Pigs are naturally heavily colonized with LAB that have been shown to associate with the non-secreting *pars oesophage* area of the stomach (Fuller et al., 1978). As a consequence of the continual sloughing of *pars oesophegeal* cells, with attached LAB, it has been suggested that this area of the stomach is continuously re-inoculated with LAB (Fuller, 1992). The survival of LAB in the stomach is enhanced by the relatively low pH at the top of the porcine stomach and the production of high levels of lactate to reduce the pH in the stomach, which in turn contributes to the control of the target enteropathogens (Fuller, 1992).

Probiotics are utilized to establish a stable microbiota in neonatal piglets, control diarrhea disease, improve feed:weight conversion, improve the efficiency of digestion during the crucial fattening stage and control swine dysentery. Stressing an animal has been shown to affect detrimentally the microbiota of mice, humans and piglets. Disruption of the microbiota, especially during weaning of piglets, allows for the colonization of pathogenic microorganisms such as *S.* Typhimurium, enterotoxigenic *E. coli* and *Brachyspira hyodysenteriae.* These are the causative agents of the majority of recorded cases of porcine enteritis in the UK.

Competitive exclusion products for pigs are not nearly as developed or as extensively researched as in poultry. Although, some promising studies have shown that CE can be effective against *S.* Typhimurium and *E. coli* in swine (Anderson and Foulks, 1976; Casey et al., 2007; Fedorka-Cray et al., 1999; Genovese et al., 2000; Harvey et al., 2002). Genovese et al. (2003) used an undefined CE culture, PCF1, to CE *Salmonella enterica serotype* Choleraesuis from pre and post weaning piglets. *S.* Choleraesuis is a heavily adapted porcine specific *Salmonella* serovar and causes ulcerative colitis, diarrhea, muscle wasting and in some cases mortality in pigs. The PCF1 CE culture significantly reduced the systemic dissemination of *S.* Choleraesuis and also dramatically reduced fecal shedding. Interestingly, the authors monitored horizontal transfer of *Salmonella* between pen mates for 14 days post weaning and the PCF1 again proved efficacious (Genovese et al., 2003). Recently, Casey et al. (2007) designed a multi-genera probiotic based upon defined *in vitro* criteria; the CE agent consisted of *L. murinus, L. pentosus, L. salivarus* and *Pediococcus pentosus.* This probiotic preparation was found to reduce the incidence and duration of the diarrhea, and the pigs also gained weight faster than the controls. Fecal shedding of *S.* Typhimurium was reduced 15 days post infection (Casey et al., 2007). Enterotoxigenic *E. coli* is a common cause of neonatal scours in piglets. Genovese et al. (2000) demonstrated that the CE agent PCF1 also caused significant reductions in mortality from 12 and 24 hours after birth, reducing the overall mortality by 17.5% and significantly reduced fecal shedding of *E. coli* at 1 and 3 days post challenge (Genovese et al., 2000). Other less successful approaches to controlling *Yersinia enterocolitica* in pigs have been demonstrated, Hussein et al. (2003) attempted to use a non-pathogenic *Y. enterocolitica* biotype 1A, serotype O:6,30 to CE the pathogenic biotype 4, serotype O:3. However, this CE approach worked *in vitro* but not *in vivo* (Hussein et al., 2003). The use of "non-virulent" pathogens for CE is highly controversial, particularly due to the potential ease of transfer of resistance genes and virulence determinants between pathogenic and "non-pathogenic" strains.

30.6 The Mechanisms behind the Efficacy of Probiotics in Reducing Pathogenic Infection

Probiotics are commonly lactic acid producing bacteria such as lactobacilli, bifidobacteria or enterococci. The lactic acid producing genera has been shown to exert strong anti-pathogenic effects on a range of pathogens such as *E. coli* spp.,

Salmonella spp., *Pseudomonas* spp., and *Campylobacter* spp. (Johnson-Henry et al., 2008; Stern et al., 2006). Other genera of bacteria can be used as probiotics, for example, *Bacillus subtilis* strains are used either as a live vegetative cell preparation or in the form of spores to reduce *Salmonella* colonization in chickens (Cartman et al., 2008; La Ragione and Woodward 2003). In a comprehensive study evaluating the probiotic potential of a variety of microorganisms Gram positive cocci such as *E. faecium*, certain *Streptococcus* strains and yeasts were evaluated and were shown to have less of an anti-pathogenic effect compared to lactic acid producing bacteria (Fuller, 1992).

The exact mechanisms of probiotic efficacy are yet to be fully elucidated, although it is accepted that the production of organic acids by probiotics can exert a strong antimicrobial effect against food borne pathogens (De Keersmaecker et al., 2006). Probiotics may also mitigate against foodborne pathogens by enhancing epithelial barrier function, the antagonism of receptor sites on the host epithelium, producing antimicrobial peptides, producing low molecular weight antimicrobials, competing for nutrients, inhibiting quorum sensing systems and through the production of organic acids (Bernet-Camard et al., 1997; Chen et al., 2007; Fayol-Messaoudi et al., 2005; Makras et al., 2006; Medellin-Pena et al., 2007; Mikolajczyk and Radkowski, 2002).

30.6.1 Production of Organic Acids

Organic acids have a long history of use in the food industry as food preservatives and more recently as possible replacements for in-feed antibiotics (Skrivanova and Marounek, 2007; Skrivanova et al., 2004). A wide variety of low molecular weight organic acids have been added to porcine, bovine and avian feed to reduce colonization of *Enterobacteriacae* (Dibner and Buttin, 2002; Van Immerseel et al., 2006). Organic acids have been used to control *Enterobacteriacae* colonization in chickens (Van Immerseel et al., 2006) and in the UK formic, propionic and lactic acids are regularly used to acidify feed. Fermentative bacteria present within the GI tract produce a wide spectrum of organic acids during anaerobic growth, although the production of organic acids varies greatly, depending on the bacterial species. Lactobacilli are grouped according to the Orla-Jensen subgenera, which is devised of three groups; homofermentative, facultatively heterofermentative and obligatively heterofermentative, according to their carbohydrate fermentation patterns.

- **Homofermentative** lactobacilli ferment hexoses, such as glucose, primarily into lactic acid via the Embden-Meyerhoff pathway (Glycolysis). These homolactic fermentors are incapable of fermenting pentoses and gluconate (Weiss, 1986).
- **Facultatively Heterofermentive** lactobacilli possess an inducible phosphoketolase and can ferment pentoses. Hexoses are metabolized as above, but the terminal electron acceptor, and thus metabolic end product, can be acetic acid, ethanol and formic acid in addition to lactic acid (Weiss, 1986).
- **Obligatively Heterofermentative** lactobacilli ferment hexoses to lactic acid, acetic acid, ethanol and carbon dioxide whereas pentoses are fermented to lactic acid and acetic acid. Any lactobacilli which produces a gas from carbohydrate breakdown is also included in this group or lactobacilli that produce "exotic" metabolites, for example 1,2 propanediol (Weiss, 1986).

The simplest form of fermentation is the conversion of sugar to lactate and this type of fermentation is indicative of homofermentative LAB. Many bacterial species, however, when under glucose limiting conditions are capable of heterolactic fermentation in which the main fermentation products are lactate, acetate and a very small amount of ethanol. These organic acids can exert an antimicrobial effect on pathogens, whilst others which are not produced by LAB, for example, butyrate, propionate and formate can modulate bacterial pathogenicity (Gantois et al., 2006; Van Immerseel et al., 2006). Some SCFA's may act as indicators of population density in a manner indicative of quorum sensing (Gantois et al., 2006; Huang et al., 2008; Johnson-Henry et al., 2008; Van Immerseel et al., 2006).

Some pathogens appear to use volatile fatty acids as an indicator for "preferential" invasion conditions. Using *S.* Typhimurium as an example; the BarA/SirA two-component regulator is believed to sense the SCFA environment of the gut and subsequently regulate the expression of the Type Three Secretion System 1 (TTSS-1). This secretion system encodes the machinery, chaperones and effector proteins required for *Salmonella* to invade into non-phagocytic epithelial cells (Gantois et al., 2006; Huang et al., 2008; Van Immerseel et al., 2006). Acetate and formate appear to act as inducers of invasion and facilitate an invasive profile in *S.* Typhimurium. In contrast, butyrate and propionate cause down regulation of the *Salmonella* pathogenicity island 1 (SPI-1) which encodes the TTSS-1. There is great potential for these environmental switches to be exploited to reduce the invasion and dissemination of *Salmonella* in food producing animals.

Probiotics can modulate the production of SCFA's within the gastrointestinal tract of the host in two ways; firstly, by the production of organic acids and secondly by direct modulation of the microbial community that results in an altered SCFA profile. Modulation of the microbial community is often achieved by a combination of competitive exclusion of competing species or by direct cross-feeding *in vivo*. Cross-feeding is defined as the production of a secondary metabolite which confers a growth advantage to a specific subset of bacteria (Kaneko et al., 1994). Production of SCFA's has been linked to the reduction of *Enterobacteriacae* in the crop of broiler chickens, and subsequent *in vitro* data has shown that varying concentrations of organic acids decreased the levels of *Enterobacteriacae* (Van Immerseel et al., 2006).

The pH of the GI tract is believed to exert a strong antimicrobial effect mediated by organic acids (Skrivanova and Marounek, 2007; Skrivanova et al., 2004). At a low pH organic acids exhibit a high level of dissociation which is directly dependant on the pKa (weak acid dissociation constant) of the organic acid. The pH directly affects the amount of un-dissociated acid available to diffuse across the bacterial membrane. High levels of disassociated organic anions and protons within the cell deplete the proton motive force across the bacterial membrane. This impairs the ability of the bacterium to produce ATP because there is no proton gradient available to power the F1/F0 ATPase. Organic acids deplete intracellular ATP, cause membrane permeabilization, LPS release from the bacterial membrane, and sensitize the membrane to other agents such as bacteriocins, detergents and bile (Alakomi et al., 2003, 2005; Coconnier-Polter et al., 2005; De Keersmacecker et al., 2006).

30.6.2 Lactic Acid

The major fermentation product of LAB is L-lactate, it has been used as a preservative for meat products and to control pathogens on such products and meat preparation surfaces. Studies using microarrays have also shown that exposure of *S.* Typhimurium to lactic acid induces a unique acid tolerance response which is independent from a standard acid tolerance response induced by exposure to inorganic acids (Greenacre et al., 2006). Much of the antimicrobial activity exhibited by probiotics can be attributed to the production of lactic acid (Bernet-Camard et al., 1997; Fayol-Messaoudi et al., 2005; Makras et al., 2006; Mikolajczyk and Radkowski, 2002). Lactic acid has been shown to be a potent membrane permeabilizer, causing sub-lethal membrane damage in *E. coli*

(Alakomi et al., 2000; Fayol-Messaoudi et al., 2005). The mechanism behind the antimicrobial effects of lactate are attributed to lowering the internal pH of a bacterial cell through the dissociation of organic acids and the leaching of divalent cations which stabilize nascent LPS chains (Alakomi et al., 2000).

30.6.3 Production of Antimicrobial Compounds

LAB compete with other microorganisms within their specific niche environment by producing antagonistic compounds and by modifying their environment via the production of a wide variety of secondary metabolites. Lactobacilli have been reported to produce a range of antimicrobial compounds (*Table 30.7*), many of which have been characterized and their mode of action determined (Alakomi et al., 2000; Fayol-Messaoudi et al., 2005; Makras et al., 2006; Mikolajczyk and Radkowski, 2002; Rodiguez et al., 2003). Typically these antimicrobial compounds are divided into two groups; low molecular weight compounds with a molecular weight $<$1,000 Daltons (Da) and bacteriocins which have a molecular weight of $>$1,000 Da. High molecular weight compounds such as bacteriocins are proteinaceous in nature and will be discussed at length in subsequent sections along with the low molecular weight metabolites, such as organic acids, reuterin, reutericyclin, hydrogen peroxide, acetoin and diacyl.

30.6.4 Reuterin and Reutericyclin

Other than bacteriocins and lactic acid, reuterin is the best characterized antimicrobial compound produced by *Lactobacillus* spp. Reuterin, or β-hydroxypropionaldehyde, is a broad spectrum antimicrobial produced by some strains of *L. reuteri* (Rodriguez et al., 2003) and it is a very good example of how varying the source of carbon for a specific type of lactobacilli can drastically alter its antimicrobial activity and metabolism. Specifically, reuterin production requires glycerol as a carbon source, and production is optimal under conditions of anaerobiosis. Production of reuterin is dependant upon the presence of a co-enzyme B12 dependant glycerol dehydratase, which has been used as an indicator of potential reuterin producing lactobacilli. Reuterin, a water soluble aldehyde, exists in solution as a complex mixture of monomeric, hydrated monomeric and cyclic dimeric forms of β-hydroxypropionaldehyde. They are active across a wide pH range, are resistant to proteases and lipases, and exhibit antimicrobial activity

Table 30.7

Summary of Antimicrobial compounds produced by *Lactobacillus* species

Compound type	Compound	Synonym/trade name	Typical source organism	Applications
Organic acids	Lactic acid[a]	–	*Lactobacillus plantarum*	Preservative, antimicrobial, food additive
	Acetic acid[a]	–	–	–
	3-Phenyllactic acid[b]	–	*Lactobacillus plantarum* (MiLAB 393)	–
	4-hydroxyphenyllactic acid and Tetrameric acid derivative[c]	Reutericyclin®	*Lactobacillus plantarum* (MiLAB 393)	–
Aldehydes	Acetaldehyde[d]	–	–	–
	3-hydroxypropionaldehyde (3-HPA)[e]	Reuterin®	*Lactobacillus reuteri*	–
Bacteriocins[f]	Nicin[g]	–	–	Preservative
	Plantiricin[g]	–	–	–

Cyclic dipeptides	L-Phe-L-Pro[b]	–	*Lactobacillus plantarum*	Broad spectrum antifungal
	Cyclo-(Gly-L-Leu)[b]	–	*Lactobacillus pentosus*	Antifungal
	L-Phe-trans-4-OH-L-Pro[b]	–	*Lactobacillus plantarum* (MiLAB 393)	Antifungal
Others	Hydrogen peroxide[h]	–	–	Broadspectrum antimicrobial
	Tetrahydro-4-hydroxy-4-methyl-2H-pyran-2-one[b]	Mevalonolactone®	*Lactobacillus plantarum*	Antifungal
	5-methyl-2,4-imidazolidinedione[b]	Methylhydantoin®	*Lactobacillus plantarum*	–
	Diacyl[d]	–	–	–

[a]These data were obtained from Makras et al. (2006)

[b]These data were obtained from Strom et al. (2002 and references contained within)

[c]These data were obtained from Ganzle and Vogel (2003)

[d]These data were obtained from Jyoti et al. (2003)

[e]These data were obtained from Rodiguez et al. (2003)

[f]The list of bacteriocins is by no means comprehensive and is purely to illustrate the full range of antimicrobial molecules produced by *Lactobacillus* spp.

[g]These data were obtained from Cotter et al. (2005a)

[h]These data were obtained from Pridmore et al. (2008)

against a wide range of food borne pathogens, including *Listeria monocytogenes*, *E. coli* O157:H7 and *S.* Typhimurium (Rodriguez et al., 2003).

Reutericyclin, unlike reuterin, is a tetrameric acid derivative produced by strains of *L. reuteri.* It consists of a hydrophilic negatively charged head group and two hydrophobic side chains. Reutericyclin was thought to form pores in the bacterial membrane, however, extensive studies utilizing fluorescent dyes, measuring trans-membrane proton potential, pore forming ability and trans-membrane potassium potential, showed that reutericyclin does not form pores but selectively dissipates the trans-membrane proton potential causing a dissipation in proton motive force (PMF) (Ganzle and Vogel, 2003).

30.6.5 Hydrogen Peroxide

Some species of *Lactobacillus* can produce hydrogen peroxide if cultured in the presence of oxygen. Typically, hydrogen peroxide producing strains are *L. johnsonii, L. reuteri, L. acidophilus or L. gasseri* (Martin et al., 2005; Pridmore et al., 2008; Strus et al., 2004). Hydrogen peroxide is an important regulatory molecule in the vaginal microbiota and is also cytotoxic to a wide range of pathogens including *S.* Typhimurium, against which hydrogen peroxide producing lactobacilli have demonstrated strong antimicrobial activity both *in vitro* and *in vivo* (Eschenbach et al., 1989). It is believed that the antimicrobial properties of hydrogen peroxide are caused by its ability to dissociate into a highly reactive oxygen species (hydroxyl radical) (Halliwell, 1978). It is highly likely that certain species of lactobacilli can produce hydrogen peroxide within the GI tract due to the small amount of oxygen being present, especially in the small intestine.

30.6.6 Other Secondary Metabolites

Many lactobacilli produce fragrant flavor compounds as fermentation by-products. The two main fragrant compounds are diacetyl and acetoin; these can only be produced by heterofermentative lactobacilli. Diacetyl is formed when a cell creates active acetaldehyde from pyruvate and thiamine pyrophosphate, the active acetaldehyde then condenses with a molecule of pyruvate forming α-acetolactate and it is finally converted to diacetyl by α-acetolactate synthases (Jyoti et al., 2003). Subsequently, acetoin is formed either by the

decarboxylation of α-acetolactate, or by the reduction of diacetyl by diacetyl reductase (Jyoti et al., 2003).

30.6.7 Antimicrobial Peptides

Antimicrobial peptides are produced by a diverse range of organisms including insects, humans and plants as part of their innate immune response. The production of antimicrobial peptides is not exclusive to multicellular organisms, for example, bacteriocins are ribosomally synthesized antimicrobial peptides produced by bacteria. Bacteriocins are active against susceptible competitors present within their niche environment and usually target against the same species (narrow spectrum) or across genera (broad spectrum). Bacteriocins are highly active, and thus, producer organisms rely on immunity proteins to protect themselves from their own bacteriocins.

The large majority of bacteriocins characterized to date, are produced by LAB. Interestingly it has been suggested that 30–99% of bacteria and archaea can synthesize at least one bacteriocin (Klaenhammer, 1988; Riley, 1998). Although bacteriocins can be both broad and narrow spectrum they are normally most active against Gram positive bacteria and in particular other LAB. However, there are examples of bacteriocins which are active against Gram negative bacteria (Stern et al., 2006). Bacteriocins which are active against gram negative *Enterobacteriacae* are an important area for future probiotic research and would be ideal for the control of pathogens in food-producing animals. Bacteriocins may be useful in targeting undesirable bacterial species, for example, pathogens present in food, and in the case of probiotics they could be used to competitively exclude pathogens from the GI tract of the host.

30.6.8 Classification of Bacteriocins

Bacteriocin classification is constantly changing due to improved knowledge regarding how bacteriocins exert their antimicrobial effects. A comprehensive review by Cotter et al. (2005b) discusses the key role that genome sequencing has played in the identification of bacteriocins and the further elucidation of structure function relationships which is crucial if the aim is to engineer novel antimicrobial peptides (AMP's). It has been suggested that bacteriocins can be divided into three main groups; Class I lanthionine containing bacteriocins

deemed lantibiotics, Class II non-lanthionine containing bacteriocins and bacteriolysins (non-bacteriocin lytic proteins).

- **Class I Lantibiotics:** Lantibiotics possess the non-proteogenic amino acid residues lanthionine and β–methyllanthionine, which form covalent bridges between amino acids resulting in internal rings, which are characteristic motifs present within the class I bacteriocins. It has been suggested that the lantibiotics can be divided into subgroups based upon the sequences of the unmodified pro-peptides (Cotter et al., 2005a). However, their complex structures and multiple modes of action make them a difficult group to sub-classify (Cotter et al., 2005b). Two well known examples of lantibiotics are nisin and mersacidin. Nisin is a standard Class I bacteriocin which consists of an elongated amphiphilic cationic peptide, which forms pores in the membrane of bacteria. This dissipates PMF and causes non-specific effluxes of small metabolites from the cell. The attachment of nisin is mediated by lipid II, which may also allow for a dual mode of action.
- **Class II:** Non-lanthionine containing bacteriocins are much more common than the lantibiotics, possibly because they require less extensive post-translational modifications. Class II bacteriocins are small (<10kDa) heat-stable peptides. Their mechanism of action is similar to the lantibiotics by forming pores in bacterial membranes and causing the non-specific leakage of metabolites from target bacteria. Class II bacteriocins are grouped into four sub-classes; IIa pediocin like, class IIb two-peptide bacteriocins, class IIc cyclic bacteriocins and class IId non-pediocin single linear peptides. Class IIa bacteriocins are narrow spectrum bacteriocins which exert a strong antimicrobial activity against *Listeria monocytogenes* due to the presence of an N-terminal "pediocin box" (Heng et al., 2007). The class IIC bacteriocins have a cyclic structure due to a linked N and C terminus. Further sub-divisions within this group have been proposed, however, very few class IIc bacteriocins have been identified to date. Class IId contains the remaining class II bacteriocins; typical examples include lactococcin A and divergicin A (Cotter et al., 2005b).
- **Bacteriolysins:** It has been suggested that Class III bacteriocins should form a new group termed bacteriolysins (Cotter et al., 2005b). These antimicrobial peptides differ from Class I to II bacteriocins because they cause cell wall hydrolysis in sensitive cells. They show some homology to endopeptidases and it has been suggested that their C-terminal domain may play a role in attachment and host specificity (Cotter et al., 2005b. Lysostaphin, a highly potent bacteriolysin kills *Staphylococcus aureus* strains within minutes by its glycylglycine endopeptidase action which specifically targets and cleaves the pentaglycine cross-bridges in *Staphylococcal* peptidoglycan (Wu et al., 2003).

30.6.9 Inhibition of Quorum Sensing Mechanisms

Probiotic bacteria secrete a variety of secondary metabolites into the environment that they are cultured in. Many of these compounds exhibit antimicrobial effects against a wide variety of other micro-organisms. However, recently *L. plantarum* was shown to secrete an as yet unidentified compound which has been shown to strongly interfere with *Pseudomonas aeruginosa* quorum sensing signals and significantly reduced *P. aeruginosa* colonization in the burnt mouse model (Valdez et al., 2005).

30.6.10 Protection or Enhancement of the Epithelial "Barrier Function"

A key requirement for pathogens to colonize the host GI tract is to adhere or associate with the host epithelium. Many *in vitro* studies focus on the ability of probiotics to affect the association of pathogens to epithelial cells, or for the affinity of the probiotic to host epithelial cells/tissues. Numerous studies *in vitro* show that some probiotics, in particular lactobacilli and bifidobacteria, affect the association of pathogens to intestinal epithelial cells (Coconnier et al., 1993a, b, 1997; Hudault et al., 1997; Larsen et al., 2007; Lehto and Salminen, 1997). For a comprehensive review on the adhesive properties of LAB and their use to antagonize pathogens see Servin and Coconnier (2003) and Servin (2004). The inhibition of *S.* Typhimurium and EHEC by a variety of probiotics to adhere and invade host cells have been studied extensively *in vitro*. Many probiotics have the potential to competitively exclude these pathogens by interfering with the initial intimate association of pathogens to epithelial cells, some by physical interference and others by mechanisms that are as yet unclear (Johnson-Henry et al., 2008; Sherman et al., 2005). Interestingly, *L. acidophilus* La-5 has been shown to down regulate the expression of EHEC O157 genes that are essential for bacterial colonization (Medellin-Pena et al., 2007). Furthermore, as yet unidentified compound/s present in the supernatant of *L. acidophilus*, have been shown to act as quorum sensing inhibitors or directly effect the locus of enterocyte effacement (LEE) by direct transcriptional regulation (Medellin-Pena et al., 2007).

Food borne pathogens need to intimately associate to, or penetrate the host intestinal epithelium. These processes are often active and require either the type three or type IV secretion systems. EspF, a type three secretion system effector molecule secreted by enteropathogenic *E. coli* causes rearrangement of the actin

cytoskeleton and compromises the epithelial barrier function (Sallee et al., 2008). This can be determined visually via actin staining or by assessing the trans-epithelial resistance (TEER) of the cells/tissue. *L. acidophilus* and *L. rhamnosus* have been shown to reduce the adhesion of *E. coli* O157:H7 and *E. coli* O127 to intestinal epithelial cells (Sherman et al., 2005). Sherman et al. (2005) also documented reductions in alpha-actin re-arrangements, reduced TEER in a probiotic protection assay; they also determined that viable bacteria were required for the probiotic effect (Sherman et al., 2005). It is highly likely that the reduced drop in TEER was due to the probiotic reducing the number of *E. coli* bound to the cells and therefore reducing the number of type three effector molecules available to compromise barrier function.

30.6.11 Immunomodulation

The immunomodulatory effects of probiotics will not be discussed in this chapter due to the sheer scope of the subject and the excellent reviews already available. The comprehensive review by Cummings et al. (2004) addresses the normal function of the gut and immune system of healthy individuals, paying particular attention to the systemic and mucosal immunity. A review by Delcenserie et al. (2008) concentrates on the interactions between the probiotics and the intestinal epithelium and their subsequent effect on innate immunity. The authors also accentuate the fact that *in vitro* cytokine profiling of the probiotic host cell interaction may be a useful selection criterion. To truly understand the immunomodulatory effects of probiotics randomized double blind clinical studies are required. One exciting facet of the immunostimulatory effects of probiotics not mentioned in the above review articles, is the potential use of genetically modified (GM) probiotics with enhanced immunostimulatory effects. Grangette et al. (2005) constructed a Dlt- mutant in *L. plantarum* NCIMB8826 which meant that the teichoic acids present on the surface of the *L. plantarum* contained substantially less D-alanine. This Dlt- mutant was significantly more protective in the murine colitis mouse model when compared to its isogenic parent and the mutant strain exhibited strong pro-inflammatory effects both *in vitro* and *in vivo*, with significant increases in IL-10 secretion (Grangette et al., 2005).

The bacterial-epithelial host cell cross-talk between probiotics, pathogens and the host is extremely complex and although changes in cytokine levels are often observed, this is not necessarily the whole story. One of the major issues with immunological data, in particular cytokine profiles obtained from *in vitro* studies, is that they are mainly performed using immortalized cell lines which

exhibit aberrant gene expression and often have highly variable phenotypes. These cell lines are cultured under highly artificial conditions and do not accurately replicate the complex interplay between the multiple cell types within a 3D matrix. Observed changes in cytokine profiles ideally should be supported by data from clinical trials to obtain greater biological significance (Delcenserie et al., 2008; Grangette et al., 2005).

30.6.12 Conclusion

Probiotics are being used consistently in veterinary medicine to modify animal nutrition, enhance immunity, improve feed conversion ratios and to competitively exclude pathogens (Fuller, 1992; Krehbiel et al., 2003). It is clear from the CE work of Nurmi and Rantala in broilers that the microbiota of an adult host can protect newly hatched chicks from environmental stresses and from the colonization of pathogens. The pressures of modern farming often mean that new born animals often do not have contact with the mother and therefore require assistance in order to rapidly acquire a protective normal microbiota. It is well documented that if animals do not have a fully functional normal microbiota they are more susceptible to bacterial infections such as *E. coli* and *Clostridium* species. Diseases such as necrotic enteritis (*Clostridium perfringens*) and colisepticemia (*E. coli*) can lead to high levels of morbidity and mortality and therefore the use of effective probiotics can significantly increase animal welfare.

Currently, available probiotic supplements need to be further characterized with regards to the carriage of transferrable antimicrobial resistance genes and accurate identification of probiotic candidates to the species level. Selection criteria must also be agreed which can be used *in vitro* to select defined "probiotic traits," which show clear efficacy *in vivo* (Timmerman et al., 2004). Currently, the use of single strain probiotics has been shown to be efficacious in poultry (La Ragione and Woodward, 2003; La Ragione et al., 2001, 2004), whereas studies in pig clearly advocate multi-species probiotics (Casey et al., 2007). Both types of probiotic have been shown to reduce the colonization of pathogens, for example, *S.* Typhimurium *in vivo* (Bernet et al., 1994; Bernet-Camard et al., 1997; Corr et al., 2007). However, the mechanisms behind probiotic efficacy *in vivo* still elude us. There is substantial evidence showing *in vitro* efficacy, including the production of antimicrobial compounds (Alakomi et al., 2000; Bernet-Camard et al.,1997; Fayol-Messaoudi et al., 2005; Makras et al., 2006; Mikolajczyk and Radkowski, 2002; Rodriguez et al., 2003), the inhibition of quorum sensing (Medellin-Pena et al., 2007; Valdez et al., 2005), the antagonism of receptor

sites (Servin, 2004; Servin and Coconnier, 2003), the immunomodulation (Delcenserie et al., 2008) and the enhancement of barrier function (Johnson-Henry et al., 2008). To date, very few studies have shown *in vitro* efficacy and have then proved the mechanism of action *in vivo*. Two such studies both used *Lactobacillus* strains which produced bacteriocins or uncharacterized antimicrobial molecules (Bernet-Camard et al., 1997; Corr et al., 2007). Corr et al. showed that the bacteriocin producing *L. salivarus* UCC118 conferred significant protection against *Listeria monocytogenes*. They identified the bacteriocin as the primary mediator of the probiotic effect in this CE experiment in two ways. They made the *Listeria* immune to the bacteriocin by adding the immunity peptide from *L. salivarus* and they also knocked-out the genes responsible for the bacteriocin production. In both cases, the *Listeria* were still able to successfully infect the mice (Corr et al., 2007). Prior to this work, Bernert-Camard et al. demonstrated the *in vitro* efficacy of *L. acidophilus* LA1 against *S.* Typhimurium in tissue culture assays and their subsequent *in vivo* mouse model showed that *L. acidophilus* conferred protection against *S.* Typhimurium although the antimicrobial compound was never identified (Bernet et al., 1994; Bernet-Camard et al., 1997).

30.7 Prebiotics

30.7.1 The Use of Prebiotics in Livestock

Prebiotics have been utilized in experimental *in vivo* studies in a range of animals such as mice, poultry, cattle and pigs to investigate the effects on:

- The microbiome.
- The immuno-modulation of the host.
- The suppressive effects on enteric and systemic infections by pathogens.
- The efficacy in weight again and in the general welfare of animals.

30.7.2 The Effects of Prebiotics on the Microbiome

The principle aim of prebiotics is to stimulate the resident microbiota of host to proliferate, to suppress deleterious bacteria, and to confer health benefits to the host. Diets enriched with prebiotic oligosaccharides, such as GOS, increase

the growth of certain gastrointestinal bacteria, specifically the LAB bifidobacteria and/or to a lesser extent lactobacilli and/or their fermentation products in the colon (Macfarlane et al., 2008).

It is widely accepted that members of the microbiota, such as bifidobacteria, are able to utilize oligosaccharides as substrates for fermentation and that they cannot be utilized by detrimental bacteria such as the coliform *Salmonella*. Specifically, it has been illustrated that IMOS can be utilized by cecal isolates of bifidobacteria, thus increasing their numbers, whilst not being utilized by *Salmonella* which lack the enzymes required to ferment the oligosaccharides (Chung and Day, 2004). Oligosaccharides are enzymically synthesized from enzymes such as fructofuranosidase (for FOS) and xylanase (for XOS) and galactosidase (for GOS) derived from fungi and bacteria, such as *Aspergillus* spp., *Trichoderma* spp., and bifidobacteria. Many of these or similar microorganisms harboring the enzymes are naturally present in the colon and therefore will be able to selectively ferment the oligosaccharides.

Studies have been conducted in poultry and swine to examine the effects of prebiotics on the bacterial microbiome (i.e., the total bacterial flora of the GI tract). Techniques such as cultivation fail as it is recognized that there are many hundreds of non-cultivable organisms in the GI tract, whereas 16S sequencing, denaturing gradient gel electrophoresis (DGGE), fluorescence in situ hybridization (FISH) and more recently massively parallel high throughput sequencing have been utilized to assay for changes in bacterial populations. A disadvantage of the traditional cultivation method is that the technique relies on cultivating viable bacteria and due to difficulties in culturing obligate anaerobes such as bifidobacteria, and inappropriate choices of selective media the numbers seen in a sample may not be a true representation of the numbers seen in the GI tract. Consequently, molecular techniques, such as FISH, are favored as they do not rely on the presence of viable bacteria. The principle of this technique is to enumerate the levels of specific bacteria within samples due to the detection of hybridized Cy3 labeled oligonucleotide probes which are specific for the 16S rRNA sequence of bacteria.

The supplementation of poultry and pig diets with oligosaccharides is generally associated with subsequent stimulation of members of the microbiota to proliferate. Specifically, in pigs, GOS has been associated with increasing the numbers of bifidobacteria both *in vivo* and *in vitro* (Tzortzis et al., 2005) (› *Table 30.8*). In this study, supplementing the diet of pigs with 4% (w:w) GOS mixture significantly increased the population of bifidobacteria ($p < 0.001$) as detected by FISH (Tzortzis et al., 2005). Additionally, the concentration of the

Table 30.8

Commercially available prebiotic products, including production details and experimental evidence of their use (*Cont'd* p. 1166)

Commercial product	Active prebiotic component	Supplier	Details of production	Experimental aims	Experimental results	Statistical significance	Reference
Bio-Mos®	MOS	Alltech Inc., Nicholasville KY 40356	Yeast cell wall fragments derived from *Saccharomyces cerevisae*	Investigate the effect of MOS on cecal fermentation parameters, microbiota and enteric pathogens in chicks	No effect on microflora, pH, volatile fatty acid	–	Spring et al. (2000)
					Reduced cecal levels of *Salmonella* Typhimurium and *Salmonella* Dublin	$p < 0.05$	
				Investigate the effect of MOS and FOS on immune function, microbial populations and nutrient digestibility in dogs	Reduced fecal total aerobes	$p = 0.05$	Swanson et al. (2002)
					Increased lactobacilli and serum IgA	$p = 0.13$	
					Increased lymphocyte populations	$p < 0.05$	

Raftilose P95®	Oligofructose	Orafti, Tienen, Belgium	From partial hydrolysis of inulin. Consists of 75% fructose only chains, 2–7 d.p.	Investigate the efficacy of oligofructose and inulin on enteric and systemic protection from pathogens and protection from carcinogens in mice	Reduced aberrant crypt foci	$p < 0.05$	Buddington et al. (2002)
					Reduced *Candida albicans* incidence in small intestine	$p < 0.05$	
					Reduced mortality caused by *Listeria monocytogenes*	$p < 0.05$	
Raftline HP®	Inulin/long chain FOS	Orafti, Tienen, Belgium	From hydrolysis of chichory root. Additionally contains glucose, fructose and sucrose. Average d.p. >23	Investigate the efficacy of GOS/FOS; ratio 9:1 in an influenza vaccination model in mice	Enhanced delayed-type hypersensitivity response	$p < 0.05$	Vos et al. (2006)
				Investigate the efficacy of oligofructose and inulin on enteric and systemic protection from pathogens and protection from carcinogens in mice	Reduced aberrant crypt foci	$p < 0.05$	Buddington et al. (2002)
					Reduced *Candida albicans* incidence in small intestine	$p < 0.05$	
					Reduced mortality caused by *Listeria monocytogenes* and *Salmonella* Typhimurium	$p < 0.05$	

Table 30.8 (*Cont'd* p. 1168)

Commercial product	Active prebiotic component	Supplier	Details of production	Experimental aims	Experimental results	Statistical significance	Reference
Oligomate 55®	GOS	Yakult, Acton, London, UK	Produced by the action of galactosidases. Consists of 55% GOS, 45% glucose galactose and lactose	Investigate the selectivity of a novel GOS and oligomate 55 at a species level when fermented by colonic microorganisms	Increased the growth of bifidobacteria, lactobacilli and bacteroides in a batch culture of mixed fecal bacteria	–	Rabiu et al. (2001)
				Investigate the ability of prebiotic oligosaccharides to inhibit the adherence of EPEC to HEp-2 and Caco-2 cell lines	Reduced the adherence of EPEC to HEp-2 and Caco-2 cells by 65 and 70%, respectively	$p < 0.05$	Shoaf et al. (2006)
Nutraflora®	FOS	Golden Technologies Company, Westminster, CO	Produced by the action of β-fructofuranosidase from *Aspergillus niger* in the presence of sucrose. It consists of 1-kestose (GF_2), nystose (GF_3), and 1-β-fructofuranosylnystose (GF_4)	Evaluate the *in vivo* effects of selected oligosaccharides on cecal and fecal SCFA, pH and concentrations of the rat intestinal microbiota	Increased cecal butyrate, SCFA pools, bifidobacteria and anaerobes	$p < 0.05$	Campbell et al. (1997)
					Reduced total aerobes	$p < 0.05$	

Vivinal® GOS syrup	GOS	Borculo Domo, Zwolle, The Netherlands	Produced from the β-galactosidases of *Bacillus circulans* in the presence of lactose. It consists of 45% GOS, 15% lactose, 14% glucose and 1% galactose, 3–9 d.p.	Investigate the safety of Vivinal GOS syrup at 2,500 and 5,000 mg/kg bw/day	No toxicological effects of GOS	–	Anthony et al. (2006)
					Mean feed consumption was lower	$p < 0.05$	
					Feed efficacy was increased	$p < 0.05$	
Raftline® ST	FOS	Orafti, Tienen, Belgium	From hydrolysis of chichory root. It consists of oligosaccharides and polysaccharides (900–940 g/kg), sucrose (40–80 g/kg), glucose and fructose (0–40 g/kg). 10–12 d.p.	Investigate the effects of oligosaccharides in weaning diet of piglets on fecal physio-chemical characteristics and fecal microbial populations	No effect on the pH, organic acid concentration, microbial populations and growth performance of piglets	–	Mikkelsen et al. (2003)
Elix'or®	TOS	Borculo Whey Products, Holland	Produced by β-galactosidases. Consists of 600 g/kg TOS, 200 g/kg lactose, 200g/kg glucose and galactose. TOS fractions consists of DP2 (330g/kg), DP3 (390g/kg), DP4 (180g/kg), DP5 (70g/kg), and DP6-8 (30g/kg)	Investigate the effects of oligosaccharides in weaning diet of piglets on faecal physio-chemical characteristics and feacal microbial populations	No effect on the pH, microbial populations and growth performance of piglets	–	Mikkelsen et al. (2003)
					Increased number of yeast and concentration of butyrate	$p < 0.01$	

Table 30.8

Commercial product	Active prebiotic component	Supplier	Details of production	Experimental aims	Experimental results	Statistical significance	Reference
Bimuno®	GOS	Clasado Inc., Milton Keynes, UK	Produced from the galactosyltransferases from *Bifidobacterium bifidum* NCIMB 41171 in the presence of lactose. Consists of 50% GOS (disaccharides, trisaccharides, tetrasaccharides and pentasaccharides) mainly in the β1–3, β1–4, and β1–6 linkages and a dissaccharide fraction of α1–6 galactobiose	Investigate the microbial changes in fecal and colonic microbiota, pH and SCFA in pigs fed GOS, and the anti-adhesive effect against *Salmonella* Typhimurium and EPEC	Increased bifiobacteria in three-stage continuous culture system	$p < 0.05$	Tzortzis et al. (2005)
					Increased bifidobacteria, acetate concentration and decreased pH in pigs *in vivo*	$p < 0.001$	
					Reduced the attachment of EPEC and *Salmonella* Typhimurium to HT29 cells	$p < 0.01$	

				Investigate the efficacy of Bimuno® in inhibiting adherence and invasion of *Salmonella* Typhimurium using *in vitro* and *in vivo* models	Reduced the invasion of *Salmonella* Typhimurium into HT29 cells	$p < 0.05$	Searle et al. (2009)
					Reduced the colonisation of *Salmonella* Typhimurium in all organs sampled from mice	$p < 0.05$	
					Reduced the pathology associated with murine salmonellosis *in vitro* and *in vivo*	–	

MOS Mannan-oligosaccharides, *FOS* fructooligosaccharide, *GOS* galactooligosaccharide, *TOS* transgalactooligosaccharides, *EPEC* enteropathogenic *E. coli*, *d.p.* degree of polymerization

SCFA acetate was significantly increased and the pH of the colonic contents was significantly decreased ($p < 0.001$) (Tzortzis et al., 2005). Furthermore, a three stage continuous culture system, fed with pig fecal slurry and 1% (w/v) GOS mixture, was utilized *in vitro* to sample for alterations in bacterial populations in response to GOS. This carefully controlled system represented the proximal, traverse and distal regions of the colon. Significant increases in bifidobacteria were detected in the proximal and traverse regions of the model ($p < 0.05$) (Tzortzis et al., 2005) (*Table 30.8*). Taken collectively, the studies by Tzortzis et al. (2005) suggest that prebiotics such as GOS selectively increase the number of LAB in the GI tract, specifically those bacteria harboring the specific enzymes required for their production and hydrolysis. This accounts for why prebiotics stimulate specific bacteria to proliferate. Care must be taken when interpreting *in vitro* data, such as that produced from a three stage continuous culture system, as there is an unnatural accumulation of compounds such as organic acids within the vessels due to the lack of a selectively permeable membrane. As with all *in vitro* studies they do not truly represent the complex conditions found within the host. However, new developments in technologies are being made to better mimic the host, for example the production of the Toegepast-Natuurwetenschappelijk onderzoek (TNO) *in vitro* model (TIM). The TIM, unlike the three stage continuous culture system, has a dialysis membrane that functions to remove nutrients that are digested (Meunier et al., 2008). This more complex model offers a more realistic alternative for assaying for changes in the microbial populations or physiology of the gut in response to prebiotics or probiotics. The model has been used in the study of digestion of both humans and animals, for example it has been utilized to study the impact of carbohydrates on the digestion of pigs (Marteau et al., 1997; Meunier et al., 2008). Furthermore, the model has been used to model the survival of probiotics and prebiotics in the gut (Marteau et al., 1997; van Nuenen et al., 2000).

Significant increases in populations of bifidobacteria ($p < 0.05$) and an increase in SCFA production ($p < 0.05$) were associated with pigs fed a diet supplemented with 3.5% GOS and 3% inulin (Loh et al., 2006; Smiricky-Tjardes et al., 2003). Additionally, in the GOS fed piglets, populations of lactobacilli were significantly increased (Smiricky-Tjardes et al., 2003). The two types of oligosaccharide, GOS and inulin, had different effects on SCFA production, with butyrate and propionate significantly increased in the GOS fed piglets and only butyrate significantly increased in the inulin fed pigs (Loh et al., 2006; Smiricky-Tjardes et al., 2003). Clearly, there are differences in responses *in vivo* relating to

the type of oligosaccharide used. This probably reflects the metabolic capability of the microbiota and may reflect selective enrichment by the specific oligosaccharide. In other studies where, perhaps it might be anticipated there would be a selective enrichment, certain discrepancies have arisen. For example, Mikkelsen et al. (2003) were unable to show using a metagenomic approach any effects of 40g/kg GOS on the total number of organisms, nor on the numbers of LAB detected in piglets (› *Table 30.8*). Furthermore, supplementing the diet of pigs-with sucrose thermal oligosaccharide caramel (STOC) did not alter the host microbiota (Orban et al., 1996), whereas, in poultry, STOC significantly increased cecal levels of bifidobacteria ($p < 0.03$) and significantly reduced total aerobes and coliforms ($p < 0.05$) (Orban et al., 1997). The differences observed may be due to deviations in the type and/or dose of prebiotic used, differences in the basal diet, differences in the type and/or age of animal used and the choice of sampling methods. It is perhaps naïve to think that one prebiotic will have the same effect in different species especially as each species (and indeed each animal in that species) is likely to have a specific GI-flora pertinent to its health requirements.

30.7.3 Fermentation in the GI Tract: Short Chain Fatty Acids (SCFAs)

Fermentation products, predominantly SCFA's, increase after prebiotic supplementation as a consequence of the resident microbiota fermenting the available oligosaccharides. SCFA production is one of the most important physiological processes of colonic microorganisms (Macfarlane et al., 2008) and approximately 95% of SCFA's produced through their fermentation are absorbed by colonocytes in the GI tract and therefore the host is able to obtain energy from food that is not digested in the upper GI tract (Macfarlane et al., 2008). In the review by Macfarlane et al. (2008) the potential benefits of prebiotics are discussed, such as that SCFA's serve as an energy source for the colonic epithelium and are involved in colonic cell health, in the differentiation and proliferation of colonocytes, and in calcium, magnesium and water absorption. Additionally, they have downstream consequences in reducing the luminal pH, suppressing putrefying bacteria, inhibiting inflammation and suppressing tumor growth. In this review the role of butyrate in stimulating apoptosis and immunomodulation is discussed, implicating that it may have a protective role for neoplasia (Macfarlane et al., 2008).

The wide range of implications of SCFA on the host emphasizes that prebiotics may be valuable in the day to day husbandry of livestock.

In poultry, prebiotics, such as MOS, have been utilized to modulate the resident gut flora of the host and subsequently reduce the incidence of pathogen colonization (Fernandez et al., 2002). It is feasible that increasing the abundance of bifidobacteria present in the colon reduces the availability of host receptors that are required for pathogens to adhere to. Alternatively, the observed reduction in pathogen colonization may be due to receptor mimicry or depletion of nutrients that are available to the pathogen as a consequence of a stimulated microbiota. The evidence indicates that prebiotics may aid the competitive exclusion of pathogens from the GI tract, a concept that will be discussed at length in subsequent sections.

Stimulating the microbiota of the host to proliferate can have a range of down stream effects on the host, such as effecting the health and immune status of the host, short chain fatty acid (SCFA) production, the pH of the colon and the out-competition of pathogens. Specifically, bifidobacteria are associated with increased resistance to infection and diarrhea diseases, possibly through allowing the out-competition of pathogens or producing compounds such as organic acids, hydrogen peroxide and bacteriocins that have antagonistic effects on other microorganisms (Alakomi et al., 2000; Bernet-Camard et al., 1997; Fayol-Messaoudi et al., 2005; Makras et al., 2006; Mikolajczyk and Radowski, 2002; Skrivanova et al., 2004, 2007). Furthermore, they are associated with stimulating the host's, immune response and protect from cancer. Thus, prebiotics can be associated with improving the welfare of the host, indirectly, through having an effect on the indigenous microbiota of the host.

30.7.4 Prebiotics Use for Reducing Pathogens in Livestock

In 2006 the EU banned the use of antibiotics as growth promoters (Castanon, 2007) and whilst this has reduced the total usage of antibiotics, alternative strategies are sought to replace some of the prophylactic and medicinal uses of antibiotics as discussed earlier. The application of probiotics and prebiotics may provide such an alternative as there is a growing body of evidence that indicate probiotics and prebiotics are capable of reducing the colonization of gastrointestinal pathogens *in vivo* and in some instances alleviate the severity of the clinical

symptoms and pathology that are associated with infections (Searle et al., 2009) (Table 30.8).

In livestock, the majority of studies investigating the use of prebiotics as an intervention strategy have been performed in poultry. The application of MOS (Bio-MOS®) in broiler chickens has been associated with significant reductions in the ability of *S.* Typhimurium to colonize the caeca ($p < 0.05$) (Spring et al., 2000) (Table 30.8). Furthermore, it has been demonstrated that MOS suppresses the proliferation of *C. perfringens*, the causative agent of necrotic enteritis in poultry and an increasing concern for commercial broiler farmers (Agunos et al., 2007). Additionally, β1–4 mannobiose has been shown to reduce the colonization of *S.* Enteritidis in the liver of broiler chickens at 7, 14 and 23 days post infection ($p < 0.05$) and the fecal shedding of the enteric pathogen ($p < 0.05$) (Agunos et al., 2007). Furthermore, β1–4 mannobiose reduced the severity of the pathology associated with *S.* Enteritidis infection (Agunos et al., 2007), such as inflammation, edema and mucosal hyperplasia. This is of biological significance as *S.* Enteritidis is detected in a range of poultry flocks in both live birds, contaminated meat and eggs (Agunos et al., 2007) which poses a threat for both animals and people.

30.7.5 The Mode of Action of Prebiotics

The application of prebiotics may be valuable in reducing the incidence of enteric pathogens, however, from the available published evidence, the exact mechanism by which prebiotics reduce pathogenic infections is unclear. It is feasible that through modulating the microbiota to proliferate it allows the out-competition of pathogens for receptors and/or nutrients. Alternatively, members of the microbiota, such as lactobacilli and bifidobacteria, may produce antagonistic compounds such as bacteriocins or antibiotics that have a detrimental effect on the pathogen. Additionally, the microbes may be having an immuno-modulatory effect on the host, priming the host to overcome challenges by pathogens. Contrary to these microbiota-dependent theories, it may be that prebiotics are able to directly affect the pathogen or host in a microbiota-independent manner. Mechanisms such as receptor mimicry inhibiting host–pathogen interactions, or immuno-modulation have been proposed (Vos et al., 2007).

In regards to receptor mimicry, glycoconjugates containing oligosaccharides have been implicated in the interactions between bacterial and epithelial cells (Schnaar, 1991). Intestinal pathogens utilize short chain oligosaccharides as

receptors, implying that oligosaccharides may have the ability to directly inhibit the attachment or invasion of pathogens by acting as receptor mimics, thus reducing the likelihood of pathogens interacting with host receptors. Typical host receptors include membrane glycolipids, mucin like glycocalyx and transmembrane stalked glycoproteins. It has been suggested that the adhesion of *S.* Typhimurium to the immortalized human intestinal epithelial cell line Caco-2 is dependant upon the Galβ(1–3)GalNAc epitope, recognized by the peanut agglutinin (PNA) lectin, and that these host cell receptors are crucial in not only bacterial adhesion but may also play a role in host specificity (Giannasca et al., 1996). The archetypal example of a monosaccharide blocking the adhesion of a pathogen to both intestinal explants and epithelial cells *in vitro* is mannose. Mannose has shown great efficacy in blocking the binding site for Type 1 pili present in most *Enterobacteriacae* and can greatly reduce type 1 pili mediated adhesion (Lin et al., 2002). It is therefore highly likely that oligosaccharides can block other receptor sites that pathogens utilize, especially when these glycoconjugates are often highly glycosylated, making receptor mimicry a likely occurrence (Lin et al., 2002; Pan et al., 1997). An excellent example of this is the use of tyrosinamide-linked oligosaccharides, specifically Man9(GlcNAc)2 to inhibit the binding of *E. cloacae* pili to HT-29 cells (Pan et al., 1997). Furthermore, oligosaccharides, such as GOS, have been shown to reduce the adherence of enteric pathogens to host cells *in vitro*. Specifically, GOS significantly inhibited the adherence of enteropathogenic *E. coli* (EPEC) to HEp-2 and Caco-2 cells ($p < 0.05$) (Shoaf et al., 2006) and inhibited the adherence of EPEC and *S.* Typhimurium to HT29 cells ($p < 0.01$) (Tzortzis et al., 2005) (*Table 30.8*). These organisms are causative agents of gastroenteritis, specifically EPEC, in a wide host range and therefore prebiotics may be useful *in vivo* to reduce the severity and the burden of such infections. Interestingly, *in vitro* organ culture (IVOC) experiments utilizing Danish Landrace pigs fed either commercial GOS or FOS demonstrated that FOS reduced the association of *E. coli* O9:K36:H19 to porcine jejunal tissue, but neither prebiotic showed a statistically significant reduction in *S.* Typhimurium association (Naughton et al., 2001).

With regard to the immuno-modulatory properties of prebiotics for clearing pathogenic infections, correlations between bacterial clearance and secretory IgA (sIgA) levels have been observed in chickens (Agunos et al., 2007). sIgA is an important component of the mucosal immune defense against pathogens, thus, prebiotics may be useful in not only stimulating the normal flora to proliferate and outcompete pathogens but also in priming the immune response to overcome subsequent infections. Furthermore, prebiotics have been used as adjuvants

for vaccines (Agunos et al., 2007; Silva et al., 2004) in livestock, highlighting the awareness that oligosaccharides may be capable of evoking an immune response on the host themselves or when in conjunction with an appropriate antigen. An adjuvant is defined as an immunological agent that increases antigenic responses; for example, they have been used in conjunction with vaccines to potentiate the immune response of the host. The potential role of prebiotics as adjuvants will be discussed in subsequent sections of this chapter.

In addition to increasing the resistance of poultry to pathogens such as *S.* Enteritidis and *C. perfringens* (Agunos et al., 2007; Spring et al., 2000) that have been previously discussed, MOS has been associated with increasing the degradation of Aflatoxin B1 in poultry (Zaghini et al., 2005). Aflatoxin B1 is a potent mycotoxin produced by *Aspergillus flavus* and *A. parasiticus* and has negative effects on egg production, quality and the susceptibility of poultry to infections (Zaghini et al., 2005).

Prebiotics have been utilized *in vivo* to reduce the incidence of enteric pathogens in poultry, such as infections by *S.* Enteritidis and *C. perfringens* (Agunos et al., 2007; Lowry et al., 2005; Spring et al., 2000) and in swine to reduce the incidence, colonization and pathology associated with swine influenza virus (Jung et al., 2004). Through stimulating the normal flora to proliferate, improving the well-being of the host and reducing the incidence of infections, prebiotics appear to be attractive alternatives to the prophylactic application of antibiotics.

30.7.6 The Immuno-Modulatory Effects of Prebiotics

Macfarlane et al. (2008), reviewed the importance of the colonic microbiota for the development and maturation of the hosts immune system, specifically, documenting that bifidobacteria and lactobacilli have been associated with increasing sIgA levels and phagocyte number. Furthermore, prebiotics such as β1–4 mannobiose are associated with increasing *S.* Enteritidis specific secretory IgA (sIgA) concentrations in the ceca and bile of poultry (Agunos et al., 2007). sIgA is an important component of the mucosal immune response against pathogens and viruses, thus the ability of prebiotics to increase sIgA levels may have implications on the resistance of the host to pathogenic infections. The interaction between prebiotics and the immune system has been highlighted in *in vitro* and *in vivo* studies (Agunos et al., 2007; Lee et al., 2001), to collectively indicate that prebiotics have not only an immuno-stimulatory effect on the host but also act as adjuvants to boost vaccine-induced immune responses.

β-glucans, a polysaccharide that is derived from cereals, yeasts or bacteria, have been used to improve the immune function of both pigs and chickens and thus provide a level of protection against infections. In chick, which are extremely susceptible to infection as a consequence of having an immature immune system and microbiota supplementing the diet with β-glucan significantly reduced the invasion of *S.* Enteritidis into the liver/spleen ($p < 0.05$) (Lowry et al., 2005). Furthermore, in this study the immune response of the chickens appeared to be enhanced, as evidenced by significant increases in bactericidal killing, oxidative bursts, and phagocytosis of heterophils ($p < 0.05$) (Lowry et al., 2005). This indicates that prebiotics may prime the heterophils of the host to phagocytose and kill invasive *Salmonella.* In further support of this, β-glucan, when given in the diet of pigs, was associated with the stimulation of the immune response and the reduction of viral replication and of the pathology associated with swine influenza virus (Jung et al., 2004; Vos et al., 2007). Feeding β-glucan to pigs correlated with significant reductions in swine influenza virus nucleic acid and its associated microscopic lesions in the lungs of pigs ($p < 0.05$) and significant increases in IFN-γ and nitric oxide (NO) levels ($p < 0.05$) (Jung et al., 2004). It can be speculated that the latter two, which are associated with improved viral clearance and immuno-modulation, are increased due to β-glucan interacting with specific receptors on mononuclear phagocytes (Jung et al., 2004).

A functional immune system is essential for enteric health and prevention of infections. Calves and other young offspring are highly susceptible to infections due to the functional immaturity of the immune system and are reliant on the passive transfer of sIgA from the mother in colostrum. As a consequence, prebiotics such as FOS have been included in calf milk replacers, to reduce the incidence of enteric disease and to promote weight gain (Quigley et al., 2002). Incidentally, FOS is used in this formulation as a replacement for the traditional antibiotics. As anticipated, a FOS/Serum Ig mix used to supplement the calf milk replacers was associated with improved enteric health (Quigley et al., 2002). Furthermore, supplementation of cow diets with MOS was shown to increase the passive transfer of rotavirus neutralization titres to calves ($p = 0.08$) (Franklin et al., 2005). Whilst these findings indicate that prebiotics may help the immune function of the host, further studies are required to investigate the efficacy of prebiotics in immune-stimulation. Specifically, the efficacy of FOS supplemented milk must be further investigated as it is unclear whether the protective effect was conferred by the serum Ig or the prebiotic component.

Studies in rodents have demonstrated the positive effects of prebiotics on Peyer's patches and lymphocytes; sIgA secretion, cytokine levels and lymphocyte

numbers have been shown to be elevated. Specifically, increases in Th1, Th2 cytokine responses and sIgA levels were observed in the Peyer's patches of BalbC mice when supplemented with FOS and raffinose (Hosono et al., 2003; Nagura et al., 2002; Vos et al., 2007). Furthermore, FOS has been associated with increasing the number of B cells within the Peyer's patches of mice (Manhart et al., 2003), and 1–10% GOS/FOS significantly increased delayed type hypersensitivity responses post vaccination in mice ($p < 0.05$) (Vos et al., 2006) (*Table 30.8*). Similarly, probiotics have been associated with reducing delayed type hypersensitivity responses in mice and therefore it may be that the immunomodulatory effects of prebiotics are microbiota dependant.

As discussed previously, due to the proposed immuno-stimulatory effects of prebiotics, investigations into their role in adjuvanticity has been explored. Adjuvant therapies such as Freunds complete adjuvant, are delivered in combination with a vaccine to promote a Th1 immune response in the host and thus enhance the immune response to vaccines. As a consequence of their use, however, they produce some local and systemic toxicity and therefore alternative adjuvants are being explored. The application of prebiotics as adjuvants may serve as a safe, non-toxic, cost-effective alternative to traditional adjuvants as they modulate the immune response of the host through a Th1, Th2, cell mediated and humoral, immune response. MOS has been shown to be an effective adjuvant in both poultry and cattle, by increasing vaccine responses to Newcastle disease virus (Agunos et al., 2007) and rotavirus (Franklin et al., 2005). In the latter case, supplementing cow diets with MOS resulted in increased rotavirus neutralization titres ($p = 0.04$) when compared to controls and in a trend of passive transfer of immunity to calves (Franklin et al., 2005). Thus, prebiotics may be useful in reducing the incidence of rotavirus in calves, a virus which is responsible for circa 62% of mortality associated with diarrhea and gastrointestinal problems (Franklin et al., 2005). Additionally, inulin derived adjuvants are being explored (Silva et al., 2004), as γ-inulin has been shown to activate, complement and thus influence the immune response through a Th1, Th2 response (Silva et al., 2004; Vos et al., 2007). Furthermore, γ-inulin has enhanced the function of murine antigen presenting cells *in vitro* (Vos et al., 2007) and has acted as an adjuvant in influenza and hepatitis B vaccinations in mice (Silva et al., 2004).

There are many theories concerning the working mechanisms of prebiotics and their interaction with the immune system. These theories involve both microbiota dependant and independent stimulation of the immune system. It is possible that, through stimulating their proliferation, the bacteria and/or their metabolites interact with receptors or immune cells to evoke an immune response

(Vos et al., 2007). Alternatively, prebiotic oligosaccharides may directly interact with immune cells or their receptors (Vos et al., 2007). Dendritic cells may play a pivotal role in stimulating an immune response to prebiotics. These immune cells sample the intestinal lumen, take up antigen (such as bacteria, bacterial antigens or oligosaccharides) and subsequently migrate to mesenteric lymph nodes to induce an immune response. Alternatively, M cells in the Peyer's patches of the gut associated lymphoid tissue (GALT) may take up bacteria or antigens and deliver them to underlying macrophages or dendritic cells. Little evidence, to date, is available for the direct effects of oligosaccharides on immune cells, however, a review by Vos et al. (2007) documents that RAW264.7 cells, a murine macrophage-like cell line, are activated by inulin and β-glucan. Specifically, β-glucan has been shown *in vitro* to increase phagocytosis and TNF-α cytokine production by RAW264.7 cells (Lee et al., 2001). As discussed previously, there are limitations to the interpretation of *in vitro* results; in this case, the direct application of prebiotics to immortalized macrophage cell lines is very artificial and thus extrapolations between the cytokine responses seen *in vitro* and the *in vivo* situation cannot be made. Future studies are required to study the cytokine responses of macrophages derived from *in vivo* studies in order to determine whether prebiotics are able to interact with macrophages to stimulate an immune response in the host.

The implication of prebiotics in boosting the immune response of the host, accentuating vaccine responses and reducing pathogenic infections by bacteria such as *S.* Enteritidis and viruses such as swine influenza virus indicates that prebiotics may serve as a safe, cost-effective method to reduce the morbidity and mortality seen in livestock. However, further studies are required to investigate the adjuvant effects of prebiotics on a wider range of vaccination therapies and to determine the mechanisms by which oligosaccharides effect the immune response of the host, be it by microbiota dependant or independent mechanisms.

30.7.7 Implications of Prebiotics Use in Weight Gain and the Incidence of Diarrhea

The evidence supporting the effect of prebiotics on growth promotion is equivocal. Specifically, supplementing STOC into the diets of broilers was associated with significant increases in feed intake ($p < 0.001$) and thus improved feed conversion and weight gain compared to controls (Orban et al., 1997). Furthermore, STOC was associated with significant decreases in aerobes and significant

increases in bifidobacteria ($p < 0.05$), which have been shown to be a good indicator of health in animals (Orban et al., 1997). Conversely, STOC did not affect weight gain, feed conversion or microbial populations in pigs (Orban et al., 1996). Similarly, GOS and FOS have not been associated with improving weight gain in pigs (Mikkelsen et al., 2003; Tzortzis et al., 2005) whereas the pig diets supplemented with MOS was associated with significantly increased growth of pigs ($p < 0.03$) (Rozeboom et al., 2005). In this latter study a 7% increase in growth was observed, but the effectiveness of the oligosaccharide was variable amongst individual farms. This is possibly due to the differences in the susceptibilities of the farms to disease; for example, one out of three of the farms had high disease rates (Rozenboom et al., 2005).

Unlike in cattle (Franklin et al., 2005), the efficacy of oligosaccharides in weight gain in chickens appears to be more unified. It has been demonstrated that FOS increases weight gain and feed utilization in chickens (Orban et al., 1997). Furthermore, 2g/kg MOS increased feed efficacy in the starter phase of chickens (Yang et al., 2008). This indicates that feed conversion can increase close to post-hatching, possibly by stimulating the development of the microbiota and thus improving enteric health and reducing the susceptibility to infections. Furthermore, as with probiotics, it could be speculated that prebiotics may be more effective when animals are kept under sub-optimal conditions, however, carefully controlled studies are required to prove or disprove this.

From the current literature, it is unclear as to why growth rate is influenced by prebiotic supplementation, but it is possible that weight gain increases due to prebiotics improving the overall intestinal health of the host. Specifically, lactobacilli and bifidobacteria, which are markers of enteric health, increase after prebiotic supplementation and are associated with augmenting the immune response of the host and reducing the incidence of pathogenic infection. Other factors, such as increased energy levels in colonocytes through the provision of butyrate may contribute to more efficient nutrient uptake. Discrepancies in the efficaciousness of prebiotics in weight gain are observed, which may be due to differences in the host's response to prebiotics, differences in the doses of the prebiotic supplied, or differences in the susceptibilities of the animals to infection and diarrhea. Thus, these discrepancies make it difficult to ascertain whether prebiotics benefit feed conversion and furthermore, extrapolations from one animal to another are not possible.

There is increasing awareness of the importance of the microbiota in gut health and improving GI functions. Prebiotics may be useful in improving GI health and thus reducing the occurrence of diarrhea, possibly through modulating

the microbiota and the production of SCFA's. Diarrhea is a major cause of mortality in pigs, calves and poultry if they are not dehydrated. During diarrhea, in addition to the massive loss of electrolytes and water, the normal flora is disturbed often showing reductions in bifidobacteria and lactobacilli and, therefore, the application of oligosaccharides along with oral electrolyte solutions may help to restore the host's electrolyte levels and microbiota. The addition of FOS to oral electrolyte solutions replenished salt and water loss and aided the recovery of the normal flora by increasing lactobacilli populations in pigs that were administered cholera toxin to induce diarrhea (Oli et al., 1998). Furthermore, inulin has been demonstrated to attenuate diarrhea in piglets (Kien et al., 2004).

The application of prebiotics to increase weight gain and improve gastrointestinal health has interesting implications for livestock as farm workers will want their animals to recover from infections rapidly, restoring the microbiota to normal levels and thus promoting host well-being. A minor caution is that prebiotics can be associated with osmotic diarrhea if too higher dose is used; specifically, lactulose is not widely used due to its diarrhea-inducing properties. Further studies are required to correlate these effects on the overall microbiome and host well-being, as currently the experimental evidence is limited.

30.7.8 Anti-Inflammatory and Anti-Tumor Effects of Prebiotics

Prebiotics have been implicated in having anti-inflammatory and anti-tumor effects, which could hold great promise in veterinary medicine. Potentially, prebiotics could be used to reduce the incidence and/or severity of inflammatory conditions such as mastitis, where inflammation of the parenchyma of the mammary gland is observed, in mammals, and other conditions such as inflammation of the bowel that is seen in pigs and sheep. Furthermore, prebiotics could be used potentially to reduce the incidence of cancer such as tumors of the liver and lungs in sheep, cattle and chickens and ovarian tumors in chickens.

The majority of studies focusing on the anti-tumor and anti-inflammatory effects of prebiotics have been conducted in rodents. Nevertheless, prebiotics have been implicated in preventing tumors; for example, intra-tumor injection of γ-inulin has been shown to induce the regression of squamous cell carcinomas in sheep, equine sarcoids and malignancies in dogs (Silva et al., 2004). Additionally, prebiotics such as scFOS have been demonstrated to significantly reduce colonic carcinomas in mice (Pierre et al., 1997) and act as adjuvants in cancer therapy

(Taper and Roberfroid, 2005). It may not be cost effective or practical to give anti-cancer therapy to livestock, however, if through the application of prebiotics you can reduce the incidence of cancers along with conferring health benefits to the host, this could be of great benefit in veterinary medicine. Additionally, oligofructose and inulin have been associated with reducing significantly the incidence of aberrant crypt foci in the distal colon of mice when 1,2-dimethylhydrazine was used as a promoter of colorectal cancer (Buddington et al., 2002) (*Table 30.8*). Furthermore, they were associated with significantly reduced mortality associated with *L. monocytogenes* and *S.* Typhimurium infections ($p < 0.05$) (Buddington et al., 2002).

30.7.9 Effects of Prebiotics in Lipid Metabolism and Mineral Absorption

Oligosaccharides have been implicated in lipid metabolism and mineral absorption (Macfarlane et al., 2008). Oligosaccharides have been implicated in lipemia and triglyceridemia in animals. Specifically, inulin reduces triacylglycerol and phospholipid concentrations in serum, possibly by inhibiting the lipogenic enzymes such as fatty acid synthase that are involved in fatty acid synthesis (Delzenne and Kok, 2001). Furthermore, inulin has been demonstrated to reduce significantly plasma and hepatic cholesterol levels in mice ($p < 0.001$) (Rault-Nania et al., 2006). It is, therefore, feasible that prebiotics and/or their metabolites inhibit the enzymes involved in fatty acid synthesis.

Increased mineral absorption may be accounted for by SCFA concentrations increasing after prebiotic supplementation, which acidifies the intestinal lumen and increases the solubility of minerals. Alternatively, it may be that improving the health of enterocytes may aid them in more efficient mineral absorption. Improving mineral absorption may have benefits in veterinary medicine by reducing the anemia that is often observed in sheep and pigs as a consequence of iron deficiency and also to reduce rickets, fractures and osteoporosis that is observed in pigs as a consequence of calcium deficiency.

30.7.10 Conclusion

Prebiotics have been utilized in rodents and farm animals to stimulate the microbiota to proliferate and thus confer health benefits to the host, such as reducing the incidence of diarrhea, the general welfare of the animals, and in

some cases improving weight gain. Furthermore, prebiotics have been implicated in reducing the incidence and severity of both gastrointestinal and systemic infections, possibly through inducing an immuno-modulatory effect on the host. The mechanisms by which prebiotics work are still to be fully elucidated, however, be it by microbiota dependent or independent mechanisms, prebiotics appear to be valid alternatives to the prophylactic use of antibiotics and in improving the welfare of the target host.

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31 Safety Assessment of Probiotics

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31.1 Introduction

Viable microbes have been a natural part of human diet throughout the history of mankind. Today, different fermented foods and other foods containing live microbes are consumed around the world, including industrialized countries, where the diet has become increasingly sterile during the last decades. By definition, probiotics are viable microbes with documented beneficial effects on host health. Probiotics have an excellent safety record, both in humans and in animals. Despite the wide and continuously increasing consumption of probiotics, adverse events related to probiotic use are extremely rare. Many popular probiotic strains such as lactobacilli and bifidobacteria can be considered as components of normal healthy intestinal microbiota, and thus are not thought to pose a risk for the host health – in contrast, beneficial effects on health are commonly reported. Nevertheless, the safety of probiotics is an important issue, in particular in the case of new potential probiotics which do not have a long history of safe use, and of probiotics belonging to species for which general assumption of safety cannot be made. Furthermore, safety of probiotics in high-risk populations such as critically ill patients and immunocompromized subjects deserves particular attention, as virtually all reported cases of bacteremia and fungemia associated with probiotic use, involve subjects with underlying diseases, compromised immune system or compromised intestinal integrity.

Several approaches to the evaluation of the safety of probiotics have been applied. Assessment of the safety of a probiotic begins with the correct identification of the strain. Laboratory tests applied in the safety assessment of probiotics include *in vitro* assays assessing different intrinsic properties of the strains such as resistance to antibiotics or production of toxic metabolites, and different animal models, which can be used to evaluate the potential of

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probiotics to translocate from the host's gut into the host's bloodstream and tissues, or assess the infectivity of the probiotics in different disease models. In addition, the safety of probiotics may be evaluated in clinical trials. In this review, the different approaches for the safety evaluation of probiotics are reviewed. In addition, the adverse events associated with probiotic use to date are outlined, and the factors affecting the likelihood of adverse events are discussed.

31.2 Taxonomy and Identification as the Basis of Safety Evaluation

The study of taxonomy comprises of different sub-disciplines including classification, identification and nomenclature. Classification assigns microorganisms to a known taxonomic group (taxa) according to the similarity between the microorganism and other members of the taxa, allowing the prediction of the properties of the microorganism based on what is already know on the taxa. Reliable identification confirms the identity of a microorganism, for example a strain isolated from fermented milk. Nomenclature, which includes assigning names to taxonomic groups and specific microorganisms, allows not only scientific communication but also proper labeling of products containing probiotic microorganisms (Felis and Dellaglio, 2007). Reliable labeling of probiotic products requires both correct identification of the bacterial species and strain used and use of up-to-date nomenclature. Establishing the identity of microorganisms constitutes the first step for the assessment of their safety. In fact, the Qualified Presumption of Safety (QPS) approach, recently established by the European Food Safety Agency (EFSA), considers identification the first pillar of the safety assessment of microorganisms (EFSA, 2007). In this respect, a FAO-WHO expert group recommended that phenotypic tests should be conducted first, followed by genetic identification, using methods such as DNA/DNA hybridization, 16S RNA sequencing or other well-established methods (FAO/WHO, 2006). The availability of such methods makes the improper identification and labeling of probiotics unacceptable. Failure to properly identify the strains may lead to the inclusion of potentially harmful microorganism in the food chain.

Many different microorganisms are being used as probiotics, including both gram-positive and gram-negative bacteria. Most of the currently used probiotics belong to the genera *Lactobacillus* and *Bifidobacterium,* which are two genera of gram-positive, non-sporeforming microorganisms. Lactobacilli are generally

aerotolerant whilst bifidobacteria are anaerobes. The genus *Bifidobacterium* shares phenotypic features and habitat with many lactobacilli and other lactic acid bacteria (LAB), and for practical reasons some authors have considered this genus to be part of the LAB. However, they are phylogenetically distinct, with bifidobacteria having DNA with high guanine and cytosine (G + C) content (55–67%) and belonging to the phylum *Actinobacteria*, whilst LAB form part of the so-called *Clostridium* branch of the phylum *Firmicutes*, and are characterized by a low G + C content. In addition, bifidobacteria possess a particular metabolic pathway for hexose fermentation, characterized by the fructose-6-phosphate phosphoketolase (F6PPK) enzyme activity. Determination of F6PPK constitutes a reliable test for the identification of the family *Bifidobacteriaceae* (Felis and Dellaglio, 2007). The F6PPK pathway leads to the production of acetic and lactic acid in a ratio of 3:2, whilst in LAB the major end product of sugar fermentation is lactic acid (Felis and Dellaglio, 2007).

Proper strain identification constitutes a critical starting point for probiotic studies. Special attention should be paid to the strain identification, as a number of studies have reported that the identity of microorganisms isolated from probiotic products often does not correspond to the information stated on the product label (Gueimonde et al., 2004; Hamilton-Miller et al., 1999). In fact, a recent EU-funded project showed that 28% of the commercial probiotic cultures were misidentified already by their manufacturers or distributors, which may partly explain the disagreements observed between the label information and the true identity of the isolated microorganisms in many products (Huys et al., 2006). Accurate and reliable identification of probiotic strains is thus necessary to evaluate both the documented health benefits and the safety of probiotic products, and to avoid the inclusion of potentially pathogenic microorganisms in commercial products. Pathogenic microorganisms can be found all around the domain Bacteria, indicating the lack of common "pathogenicity" determinants and making the identification of all the potentially pathogenic microorganisms difficult. It is therefore important to clearly identify the pathogenicity traits associated with a specific microorganism. In some studies similar properties have been found between clinical isolates and commercial probiotic strains (Ouwehand et al., 2004a, b), indicating that not only bacterial factors, but also factors associated with the host play a role in pathogenicity. In this context, it is necessary to clearly identify the possible risks associated with each probiotic strain, as different strains can possess different characteristics. The first step in identifying the possible risks is the proper identification of the strain, which allows the preliminary establishment of the potential risks of the strain based

on the previous knowledge on the corresponding taxonomical unit. An example of misidentification and possible deliberate mislabeling of a probiotic is that of *Bacillus coagulans*, which in some products may be labeled according to old and long outdated nomenclature as *Lactobacillus sporogenes*. It is widely known that the correct identification of this strain is *Bacillus coagulans*, but despite this, the old and incorrect nomenclature of *L. sporogenes* is continuously used in many products sold as "probiotics." Given the long and good safety record of *Lactobacillus* and the lack of safety assessments of *Bacillus coagulans*, it is possible that the incorrect nomenclature is sometimes used on purpose to benefit from the safety and efficacy status of members of the genus *Lactobacillus* (De Vecchi and Drago, 2006). This example highlights the importance of proper identification and labeling of probiotic products.

Traditional phenotypic identification of probiotic bacteria can be tedious and not always reliable, since certain species cannot be distinguished by these methods. Molecular techniques have emerged in recent years to replace or complement the traditional phenotypic tests for the identification and comparison of strains of probiotic bacteria. Two strains are considered to belong to the same species if their DNA-DNA relatedness is 70% or higher. The DNA-DNA hybridization method has become the gold standard for the determination of bacterial identity. However, this method is laborious and difficult to perform and hence expensive, and therefore not suitable for large scale typing. Phylogenetic approaches such as comparison of DNA sequences have therefore become commonly used frequently techniques in bacterial identification. Amongst the sequence-based methodologies, sequence analysis of the 16S rRNA gene and the 16–23S internally transcribed spacer regions have proven to be useful tools for bacterial identification. In general, if two microorganisms share a 16S rRNA gene homology higher than 97%, they are considered to belong to the same species. Nevertheless, it is important to underline that in some cases the 16S rDNA sequencing has limited resolution and is not enough for discrimination of closely related species, some of which are frequently used in probiotic preparations (Felis and Dellaglio, 2007; Vankerckhoven et al., 2008b). Among lactobacilli, the most complex groups to identify are the *Lactobacillus delbrueckii*, the *L. casei*, and the *L. plantarum* groups. Within the genus *Bifidobacterium* the most challenging groups are *B. animalis* and *B. longum*. The 16S rDNA sequences do not allow proper identification within these groups, and therefore complementary information may be required by using other molecular methods. For example, the sequencing of certain protein-encoding genes may be of help in the development of standardized methods for identification.

In the future, the increasing availability of genome sequences will allow genome-wide and/or multilocus phylogenetic analysis. It is important to point out that when comparing a gene sequence with sequences found in the databases, the quality (number, accuracy and proper identification of the microorganisms) of the sequences deposited in the database has a great impact on the accuracy of the identification. To this regard, the EU-funded project PROSAFE concluded that biochemical tests should not be used as a stand-alone approach for identification of probiotic cultures. The use of 16S rRNA gene sequence analysis was considered the best tool for routine determinations but it was also underlined that public sequence databases contain unreliable, poorly documented or incomplete sequence entries, and the need for a list of validated complete 16S rRNA gene sequences for the purposes of identification was recognized. Moreover, the use of sequence-based methods was encouraged given the high reproducibility and data exchangeability of these techniques (Vankerckhoven et al., 2008b).

Correct identification of the probiotic species used is of critical importance but it is very important to keep on mind that the safety aspects of probiotics are often strain-specific. Highly discriminatory molecular methods, such as randomly amplified polymorphic DNA (RAPD), amplified rDNA restriction analysis (ARDRA), repetitive DNA element-PCR (rep-PCR), or pulsed field gel electrophoresis of macrorestriction fragments (PFGE) among others, are also available for strain characterization (genetic typing) (Huys et al., 2006). DNA macrorestriction followed by PFGE is considered to be the gold standard (FAO/WHO, 2006) and has been used for differentiating commercial probiotic strains (Gueimonde et al., 2004). Moreover, it is widely recognized that the comparison of the results obtained by using different molecular methodologies (polyphasic approach) is the best way to establish strain identity.

It is clear that strains used by the food industry and scientists should be identified using molecular methods and up-to-date taxonomical nomenclature. Importantly, the manufacturers of probiotic products have the responsibility on the product composition. It is also important to make all relevant strains easily available in international culture collections to all research groups participating in the assessment of the health effects, the safety and the mechanisms of probiotics. The FAO-WHO working group on probiotics strongly urged for the deposit of probiotic strains in internationally recognized culture collections (FAO/WHO, 2006). Even today, many scientific articles are published with no access data for the tested strains or sometimes even without mentioning the strain designation, which hampers the progress of scientific development in this area.

31.3 *In Vitro* Safety Assessments of Probiotics

In vitro assessments offer means to investigate the safety of probiotics based on the intrinsic properties of the strains. *In vitro* safety assessments should always precede the use of potential probiotic strains in animals and in humans. Assessment of the antibiotic resistance determinants of potential and established probiotic strains has received much interest, but also other *in vitro* assessments targeting the safety aspects of probiotics have been proposed. It should be noted however that classical risk assessments commonly used for pathogens may not always be directly applicable for probiotic strains such as lactobacilli and bifidobacteria, which become members of the normal healthy intestinal microbiota soon after birth and are also components of normal human diet (Borriello et al., 2003). In pathogens, pathogenicity is normally a consequence of several properties of the strain. The presence of such a property in a strain of low infective potential and low clinical significance does not necessarily imply that the strain has pathogenic potential or poses a risk to health under certain conditions (Borriello et al., 2003). An example of this is the ability to adhere to human mucosa, which is a virulence factor in the case of true pathogens, but is also an essential feature of many commensal microbes with very low pathogenic potential. To date, no clear virulence factors similar to those associated with pathogenic microorganisms have been identified for lactobacilli (Vesterlund et al., 2007) or bifidobacteria (Ouwehand et al., 2004a). Screening for the presence of such virulence factors is more applicable for genus such as *Enterococcus* and *Bacillus*, which include known pathogenic organisms but also some strains which have been proposed as probiotics (Eaton and Gasson, 2001). Here, the *in vitro* assessments used in the safety assessments of probiotics are reviewed. Examples of such assessments are listed in *Table 31.1*.

31.3.1 Antibiotic Resistance of Probiotics

One of the main targets of the *in vitro* safety assessments of existing and potential probiotic strains is the determination of antibiotic resistance properties. Resistance of a probiotic strain to a certain antibiotic is clinically relevant only in the case of infections, and infections related to probiotics are extremely rare. The presence of antibiotic resistance genes in the probiotic genomic content is not a safety concern in itself, as long as the genes are not mobilized and transferred to other bacteria. Theoretically, probiotics possessing antibiotic resistance genes

Table 31.1

Proposed *in vitro* safety assessments of probiotics

Assessment	Notes
Presence of antibiotic resistance genes	Commonly used safety assessment
Mobility of antibiotic resistance genes	Commonly used assessment; particularly relevant for *Enterococcus*
Adhesion to host tissues	Not recommended as part of safety assessment (Vankerckhoven et al., 2008b)
Resistance to host defense mechanisms	Commonly used safety assessment (Vesterlund et al., 2007)
Presence of virulence genes and toxic metabolites	Particularly relevant for *Bacillus* and *Enterococcus* (Tompkins et al., 2008)
Hemolysis	Very rare among probiotics (Vesterlund et al., 2007)
Bile salt deconjugation	Irrelevant as safety measurement (Vankerckhoven et al., 2008b)
Presence of macrocapsules	Rarely used safety assessment (Baumgartner et al., 1998)

could serve as a reservoir of resistance for potential pathogens. Therefore, microorganisms intended for use as probiotics have to be systematically screened for antibiotic resistance susceptibility in order to avoid the transfer of antibiotic resistance genes, since the ability of these determinants to transfer in the food and gut environment has been demonstrated. However, the current methodologies may not always unequivocally demonstrate the absence of transfer, and it should be noted that the transfer rates can be completely different under *in vitro* and *in vivo* conditions. Thus, it is of great interest to investigate whether probiotics can act as reservoirs for antibiotic resistance genes, from which they could be spread to opportunistic or pathogenic bacteria.

The EFSA considers that the nature of any antibiotic resistance determinant present in a candidate microorganism for QPS status evaluation needs to be determined. However, antibiotic resistance per se is not a safety issue; it only becomes a safety issue when horizontal transfer is concerned (EFSA, 2008). Currently, it is generally accepted that the possibility of transfer is related to the genetic basis of the resistance mechanism, i.e., whether the resistance is intrinsic, acquired as a result of a chromosomal mutation(s), or acquired by horizontal gene transfer. Intrinsic (or natural) resistance is inherent to a bacterial species or genus. Such is the case of the vancomycin resistant phenotype of some lactobacilli, the best characterized intrinsic resistance among LAB. In certain

Lactobacillus species, such as *L. casei, L. rhamnosus* and *L. plantarum,* the terminal D-alanine residue of the muramyl pentapeptide in the cell wall is replaced by D-lactate, thus preventing vancomycin binding (Delcour et al., 1999). For probiotic use, intrinsic resistance might be clinically relevant in some cases of *Lactobacillus*-related bacteremia (Cannon et al., 2005). In addition, chromosomal mutations leading to antibiotic resistance phenotypes have been described in lactobacilli. A single A-to-G transition mutation in the 23S rRNA gene reduces drastically the affinity of erythromycin for the ribosome. Such mutation has been suggested as the most plausible cause of macrolide resistance in a strain of *Lactobacillus rhamnosus* (Florez et al., 2007). In this respect, the transfer risk is considered to be very low for intrinsic resistance or acquired resistance due to chromosomal mutation(s).

Horizontally transferred antibiotic resistance genes, particularly those carried within mobile genetic elements, are the most likely to be transmitted between different microbes and thus deserve particular attention. A major step in the differentiation between the intrinsic and the acquired antibiotic resistance in probiotic bacteria is the determination and the comparison of antibiotic susceptibility patterns of representative numbers of different strains from each species. Unfortunately there is still a lack of agreement on the resistance susceptibility breakpoints for most antibiotics in lactobacilli and bifidobacteria. This is mainly due to the multiplicity of methods used, which include antimicrobial gradient strips, agar dilutions, disc diffusions, microbroth cultures, and others, and to the lack of standardized guidelines. However, major advances in this field have been achieved during recent years in order to harmonize methods for antimicrobial susceptibility testing in probiotics, and new susceptibility breakpoints for some species of *Lactobacillus* and *Bifidobacterium* have been proposed (Florez et al., 2008b; Klare et al., 2007; Mättö et al., 2007). Also, with the help of new molecular biology methods, such as microarray analysis and various PCR techniques, the genetic basis responsible for the acquired resistance phenotypes is beginning to be elucidated. The essay reviews the current evidence on antibiotic resistance determinants of probiotics, and their potential importance in the safety assessment of probiotic bacteria used in human and animal feed.

31.3.1.1 Antibiotic Resistance in *Lactobacillus*

In regard to antibiotics acting on cell wall, lactobacilli are usually sensitive to penicillin and β-lactamase inhibitors, but more resistant to cephalosporins. Many

Lactobacillus species show a high level of resistance to vancomycin, as previously mentioned. Also, most inhibitors of nucleic acid synthesis seem to have a low inhibitory effect among the majority of *Lactobacillus* species. On the other hand, lactobacilli are generally susceptible to low concentrations of many inhibitors of protein synthesis, such as chloramphenicol, macrolides, lincosamides, and tetracycline, but their resistance to aminoglycosides is often higher. Resistance to other antibiotics varies greatly among lactobacilli.

Several genes responsible for atypical antibiotic resistance properties among lactobacilli have been identified. Chloramphenicol resistance genes (*cat*; chloramphenicol acetyltransferases) have been identified in *L. acidophilus, L. delbrueckii* subsp. *bulgaricus* (Hummel et al., 2007) as well as in *L. reuteri* (Lin et al., 1996) and *L. plantarum* (Ahn et al., 1992). In addition, erythromycin resistance genes, responsible for the macrolides, lincosamides, and streptrogramins (MLS) resistance phenotype, have been identified in several *Lactobacillus* species (› *Table 31.2*), with the *erm*(B) gene, which encodes a rRNA methylase acting on the 23S ribosomal subunit, being the most frequent of such genes. The presence of genes coding for macrolide efflux pumps, such as *mef*(A), has also been reported (Cauwerts et al., 2006), as well as genes for lincosamide transferase (*lnu*(A)) (Cauwerts et al., 2006) and streptogramin A acetyltransferases (*vat*(E)) (Gfeller et al., 2003). However, the most common resistance determinants found in lactobacilli are the tetracycline resistance genes, and to date at least 11 different tetracycline resistance genes have been detected among lactobacilli, including genes coding for ribosomal protection proteins (*tet*(W), *tet*(M), *tet*(S), *tet*(O), *tet*(Q), *tet*(36), *tet*(Z), *tet*(O/W/32/O/W/O), *tet*(W/O)) and tetracycline efflux pumps (*tet*(K) and *tet*(L)) (› *Table 31.2*). Some strains were even found to harbor various tetracycline resistance determinants (Ammor et al., 2008b). On the other hand, aminoglycoside resistance genes, such as *aac*(6′)-*aph*(2″), *ant*(6), and *aph*(3′)-IIIa, *aph*(E) or *sat*(3), and β-lactam resistance genes (*blaZ*) were found much less frequently in lactobacilli (Aquilanti et al., 2007; Rojo-Bezares et al., 2006). It is important to point out that many of the genetic determinants mentioned above are sometimes found in potentially mobile elements, such as transposons and plasmids, which may spread the antibiotic resistance genes mainly by conjugation mechanisms. The localization of these genes within the genome, the nucleotide content, and the analysis of the flanking regions surrounding the antibiotic resistance genes may yield important clues to the acquisition process of these determinants, and their source or origin (Aquilanti et al., 2007; Florez et al., 2006; van Hoek et al., 2008a). Remarkably, some of these genes have been found to be transferred *in vitro* between strains of *Lactobacillus* but also

Table 31.2

Examples of the main antibiotic resistance determinants identified and characterized in lactobacilli and bifidobacteria

Gene(s)	Resistance phenotype	Mechanism of action	Location (when studied)	References
Lactobacillus				
erm(B)/*erm*(C)/*erm* (T) *erm*(LF)/*erm* (GT)	MLS	Ribosomal methylation	Plasmid transposon chromosome	Ammor et al. (2008a), Aquilanti et al. (2007), Cauwerts et al. (2006), Gfeller et al. (2003), Hummel et al. (2007), Klare et al. (2007), and Tannock et al. (1994)
mef(A)	Macrolide	Efflux	–	Cauwerts et al. (2006)
Cat	Chloramphenicol	Antibiotic acetylation	Plasmid	Ahn et al. (1992), Hummel et al. (2007), and Lin et al. (1996)
tet(W)/*tet*(M)/*tet*(S) *tet*(O)/*tet*(Q)/*tet* (36) *tet*(Z)/*tet* (W/O) *tet*(O/W/32/O/W/O)	Tetracycline	Ribosomal protection	Plasmid transposon chromosome	Ammor et al. (2008a), Ammor et al. (2008b), Aquilanti et al. (2007), Klare et al. (2007), and van Hoek et al. (2008b)
tet(K)/*tet* (L)	Tetracycline	Efflux	Plasmid	Ammor et al. (2008b), Aquilanti et al. (2007)
Bifidobacterium				
tet(W)/*tet*(M)/*tet* (O) *tet*(W/32/O)/*tet* (O/W)	Tetracycline	Ribosomal protection	Chromosome	Ammor et al. (2008a), Florez et al. (2006), Kazimierczak et al. (2006), van Hoek et al. (2008b)
tet(L)	Tetracycline	Efflux	Chromosome	van Hoek et al. (2008b)
erm(X)	MLS	Ribosomal methylation	Transposon	van Hoek et al. (2008a)

from lactobacilli to different Gram-positive bacteria, including food pathogens, such as *Staphylococcus* (Tannock et al., 1994). On the other hand, lactobacilli may be able to acquire antibiotic determinants from other Gram-positive bacteria (Vescovo et al., 1983). In addition to *in vitro* studies, the potential risks associated with lactobacilli carrying transferable antibiotic have also been demonstrated in experimental animal modes (Mater et al., 2008). The transfer of these

determinants may be enhanced in the presence of antibiotic selective pressure (Feld et al., 2008). Taken together, these results support the hypothesis of the resistance gene reservoir within intestinal bacteria, and their role as traffickers in antibiotic resistance genes.

31.3.1.2 Antibiotic Resistance in *Bifidobacterium*

Most *Bifidobacterium* species are resistant to aminoglycosides, metronidazole and Gram-negative spectrum antibiotics. They are also intrinsically resistant to mupirocin, an antibiotic that is being used in the selective isolation of this genus. In contrast, bifidobacteria are very susceptible to macrolides/lincosamides, vancomycin, rifampicin, spectinomycin, chloramphenicol, and β-lactams. The susceptibility to tetracyclines and cephalosporins varies widely among strains (Zhou et al., 2005a). Compared to lactobacilli, the data on antibiotic resistance determinants in bifidobacteria are much scarcer. In the case of the macrolide resistance determinants, the presence of the gene *erm*(X) has been described in *B. animalis* subsp. *lactis* and in *B. thermophilum*. This resistance determinant was part of transposon Tn*5432* that has been detected in several opportunistic pathogens (van Hoek et al., 2008a). Also, multidrug resistance transporters able to confer erythromycin resistance have been described in *B. longum* and *B. breve*, although their contribution to a macrolide resistance phenotype is supposed to be very limited (Margolles et al., 2005). Tetracycline resistance in this genus deserves a separate mention. Current knowledge suggests that a potential concern for the safe use of *Bifidobacterium* probiotic strains is the presence of tetracycline resistance genes, especially *tet*(W), although other genes, such as *tet*(M), *tet*(O), *tet*(L), *tet*(W/32/O), and *tet*(O/W) have been detected, albeit much less frequently (Ammor et al., 2008a; Florez et al., 2006; Kazimierczak et al., 2006; van Hoek et al., 2008b). Several studies have shown a high frequency of positive isolates of these determinants in human isolates, with some strains containing up to three different *tet* genes (Florez et al., 2006; van Hoek et al., 2008b). In bifidobacteria *tet* genes seem to be integrated in the chromosome, and thus far they have not been found to be associated with transposons or plasmids, but they are very often flanked by putative transposase genes (Florez et al., 2006; Kazimierczak et al., 2006; van Hoek et al., 2008b). Transposases are enzymes that catalyze the movement of DNA segments among different locations by recognizing insertion sequences in the DNA, and they are thought to be involved in the mobilization of *tet*(W) genes in bifidobacteria. In fact, chromosomically encoded *tet*(W) have

been shown to transfer at low frequency from *B. longum* to *B. adolescentis in vitro*, and the site of chromosomal insertion in the *B. adolescentis* transconjugant was shown to be identical to that of the donor strain, consistent with a transposase-mediated site-site specific insertion event (Kazimierczak et al., 2006). However, transfer within the genus *Bifidobacterium* is not a safety concern; more concerning would be transfer to other genera or even worse to pathogens, but currently there are no indications that such transfer is likely to occur.

31.3.1.3 Antibiotic Resistance in Other Probiotic Species

Currently, different species of bifidobacteria and lactobacilli are the most commonly used probiotics, but other bacteria as well as the probiotic yeast *Saccharomyces boulardii* (*Saccharomyces cerevisiae*) are also used as probiotics. *Saccharomyces* is a member of the domain Eukaryota, and hence naturally resistant to all antibiotics. In the case of *Saccharomyces*, resistance to fungicides is more relevant. *Lactococcus* and *Pediococcus* are LAB present in the commensal intestinal flora of humans and animals. Strains of these genera are frequently used as large-scale starter cultures in the food industry (Klare et al., 2007), and have also been proposed as potential probiotics. Genes conferring resistance for chloramphenicol, tetracycline, erythromycin and streptomycin have been found in different *Pediococcus* species (Danielsen et al., 2007; Hummel et al., 2007; O'Connor et al., 2007; Rojo-Bezares et al., 2006). Remarkably, a plasmid from *P. acidilactici* encoding resistance to clindamycin, erythromycin [*erm*(B)] and streptomycin (*aadE*) has been shown to be able to replicate in *Lactococcus* and *Lactobacillus* species. Moreover, the gene *aadE* was 100% identical to an *aadE* gene found in a *Campylobacter jejuni* plasmid, suggesting a recent horizontal gene transfer event between Gram-positive and Gram-negative intestinal bacteria (O'Connor et al., 2007). Relating to *Lactococcus* strains, in Perreten et al. (1997) described a *Lactococcus lactis* strain resistant to streptomycin, tetracycline and chloramphenicol isolated from a raw-milk cheese. The three resistances were encoded by three different genes, located in a multi-antibiotic resistance plasmid, and these genes were almost identical to others previously found in *Staphylococcus aureus* and *Listeria monocytogenes*. This was the first strong evidence that antibiotic resistance can be spread in a food environment (Perreten et al., 1997). Since then, many genes coding for proteins conferring resistance to several antibiotics, mainly tetracycline and erythromycin, have been described in *Lactococcus lactis* (Ammor et al., 2008a;

Aquilanti et al., 2007), and transfer from *Lactococcus* to other bacteria, including Gram-positive pathogens, as well as the acquisition of resistance genes, has been demonstrated (Florez et al., 2008a). Probiotic strains of different *Bacillus* species have been proposed (Duc et al., 2004), and antibiotic susceptibility patterns of potential probiotic *Bacillus* strains have been determined (Sorokulova et al., 2008; Tompkins et al., 2008). Antibiotic resistance of *Bacillus clausii* to certain antibiotics has been shown to be chromosome-encoded and not linked to transferable genetic elements (Girlich et al., 2007), thus suggesting a low transfer possibility. Antibiotic resistance assessment has also been applied to study the safety of a probiotic strain of *Streptococcus salivarius*, intended for an application in the oral cavity (Burton et al., 2006).

A major issue of concern is the safety of cultures containing enterococci. These LAB constitute a significant percentage of probiotics in the worldwide market. However, in recent years, the genus *Enterococcus* has become increasingly relevant clinically, due to its increasing incidence as a cause of diseases, mainly in nosocomial infections. Further concerns on the safety of *Enterococcus* have been raised because of the widespread distribution of transferable virulence factors among the genus, and because antibiotic therapies are being compromised by evolving antibiotic resistance, and therefore the antibiotic susceptibility profiles in enterococci have been extensively studied and numerous resistance determinants have been identified (Eaton and Gasson, 2001; SCAN, 2003; Vankerckhoven et al., 2008b). Enterococci are able to acquire high-level drug resistance through horizontal gene transfer. Examples of acquired resistance genes by enterococci include those that confer resistance to tetracycline, aminoglycoside, macrolide, streptogramin and chloramphenicol, with resistance to vancomycin being the most clinically relevant (Florez et al., 2008a; SCAN, 2003). Often, these resistance genes are mobilized via transposons or plasmids. Furthermore, transfer among *Enterococcus* strains, and from *Enterococcus* to other Gram-positive pathogens has been reported (Lester et al., 2006), stressing the need for a careful and rigorous examination of the antibiotic resistance of *Enterococcus* strains intended to be used as probiotics.

31.3.1.4 Summary of Antibiotic Resistance of Probiotics

In summary, the potential ability of probiotic strains to transfer antibiotic resistances to pathogenic bacteria in the food and gut environment should be taken into account in the safety assessment of probiotics. Bacterial products

intended for use as food and feed additives must be examined to determine the susceptibility of the strain(s) to a relevant range of antimicrobials, starting from appropriate *in vitro* tests. The detection of minimal inhibitory concentrations above the breakpoint requires further investigations to make the distinction between acquired and intrinsic resistance. When a strain of a typically susceptible species is resistant to given antibiotic, the presence of acquired resistance determinants is indicated, and clearly the presence of these genes in mobile genetic elements presents the highest risk for lateral spread (EFSA, 2008; SCAN, 2003). In this respect, the scientific community must provide clear and convincing evidence to establish a risk assessment of antibiotic resistant probiotics. Currently, many open questions regarding the antibiotic resistance of probiotics remain. Numerous discrepancies have been encountered between the available phenotypic data and the genetic basis of the resistance. For example, in susceptible strains, antibiotic resistance determinants are sometimes detected. On the other hand, in atypically resistant strains, such determinants are not always detected. This suggests the existence of novel resistance genes which have escaped the detection, or the presence of silent genes that may be activated under specific conditions. In fact, recent studies have shown that gastrointestinal conditions may induce the appearance of antibiotic resistance (Noriega et al., 2005), and a higher proportion of tetracycline-resistant bifidobacteria has been detected during antibiotic/probiotic intervention in humans (Saarela et al., 2007). Also, it appears that the gastrointestinal tract may comprise a more favorable environment for antibiotic resistance transfer than conditions provided *in vitro* (Feld et al., 2008). Thus, future *in vivo* experiments should shed some light on the transfer events occurring from, via, or to probiotics. However, to put the risks associated with antibiotic resistance of probiotics into context, it should be noted that antibiotic resistance is not a property of probiotic strains alone and for example wild-type strains of lactobacilli and bifidobacteria also carry antibiotic resistance genes similar to those of the probiotic isolates. Therefore, probiotic strains do not pose any more risk in this respect than the lactobacilli and bifidobacteria occurring naturally in the human intestine.

31.3.2 Virulence Genes and Toxic Metabolite Production

The potential presence of virulence genes may raise concerns on the safety of certain microorganisms used as probiotics. For example, species belonging to the genus *Enterococcus* often harbor such genes (Vankerckhoven et al., 2008b).

Generally, *Enterococcus faecium* strains found in foods and used as probiotics are free from virulence determinants (Eaton and Gasson, 2001; Vankerckhoven et al., 2008a), while strains of *E. faecalis* typically possess multiple determinants (Eaton and Gasson, 2001). Tompkins et al. (2008) detected no virulence genes in a strain of *E. faecium* marketed as a probiotic. They did however detect a PCR product for the adhesion factor $efaA_{fm}$, but the role of this factor in virulence has not been clearly demonstrated. The potential presence of virulence determinants in *Enterococcus* strains proposed as probiotics is a potential risk factor and the safety of these strains requires critical evaluation. Certain strains of *Bacillus* are also being marketed as probiotics. Duc et al. (2004) demonstrated that three strains of *Bacillus cereus*, marketed as probiotics, produced enterotoxins, making them unsafe for human use. Toxin producers can be also found among strains of *Bacillus subtilis* (From et al., 2005), although not all *Bacillus subtilis* carry toxin genes (Tompkins et al., 2008). The presence of virulence factors has also been used to demonstrate the safety of other probiotic species. For example, Ouwehand et al. (2004a) detected no virulence factors in strains of *Bifidobacterium*, and Burton et al. (2006) detected no streptococcal virulence genes in a probiotic strain of *Streptococcus salivarius*.

Since many *Lactobacillus* species produce both L-lactic acid and D-lactic acid as their metabolic products, and since excessive D-lactic acid may cause D-lactic acidosis in certain high risk populations such as children with short-bowel syndrome, the safety of D-lactic acid producing probiotics in infant formulas has raised concerns. However, current evidence suggests that the D-lactic acid producing probiotics are safe to use also in infant formulas (Connolly and Lönnerdal, 2004). D-lactic acid is being effectively metabolized by humans, but in fact only little of the D-lactic acid produced in the gastrointestinal tract is absorbed by the host, as other bacteria in the gut quickly consume lactic acid to produce e.g., butyrate. Many of the naturally occurring microbes in the gut produce both D- and L-lactic acid, also in infants. The risk of D-lactic acidosis is limited to children with short-bowel syndrome, and no data suggest that the ingestion of DL-producing lactobacilli by healthy infants is by any account harmful (Connolly and Lönnerdal, 2004).

31.3.3 Adhesion of Probiotics to Host Tissues

Adhesion is considered an important mechanism for probiotic action, as it contributes to the ability of the beneficial strains to interact with the host,

remain temporarily colonized, and displace potential pathogens (Collado et al., 2007, 2008). However, in true pathogenic bacteria, adhesion is a negative trait which may be associated with the ability of the bacteria to translocate and to cause infection. Strains of *Lactobacillus* and *Bifidobacterium* are also known to adhere to human tissues, and adhesion to different host tissues has been proposed to be included in the *in vitro* safety assessment of these microorganisms (Harty et al., 1994). Many probiotic strains have good adherence to host mucus and intestinal epithelial cell lines as well as adhesion to extracellular matrix proteins such as fibronectin, fibrinogen and collagen (Schillinger et al., 2005). No difference was observed in the adhesion properties to host extracellular matrix proteins between fecal, blood and probiotic isolates of *Lactobacillus* (Vesterlund et al., 2007). However, blood isolates were more adherent to mucus compared to probiotic isolates. Blood culture isolates of *Lactobacillus* spp. have been reported to adhere to intestinal mucus in greater numbers than isolates from human feces or dairy products (Apostolou et al., 2001). However, adherence of bacteremia-associated *Lactobacillus* strains varies significantly between the isolates, suggesting that adhesion to mucus is not a prerequisite to *Lactobacillus* bacteremia and does not serve as a good marker of potential of *Lactobacillus* strain to cause bacteremia (Kirjavainen et al., 1999). Moreover, many widely used probiotic strains such as *B. animalis* subsp. *lactis* and *L. acidophilus* show good adhesion to host mucus *in vitro*, but have not been associated with cases of probiotic sepsis.

Vankerkhoven et al. (2007) found no differences in adhesion to fibrinogen, fibronectin, collagen and laminin between endocarditis and probiotic *L. rhamnosus* and *L. paracasei* isolates. Apart from one fecal isolate of *L. paracasei*, all tested lactobacilli adhered only weakly to immobilized host matrix proteins. Apart from strains of *Lactobacillus*, adhesion properties have also been included in the *in vitro* safety assessments of other strains such as bifidobacteria (Ouwehand et al., 2004a) as well as strains of *Enterococcus* and *Bacillus* (Tompkins et al., 2008). The ability of translocated bacteria to bind to fibrinogen may be more relevant in relation to the risk of endocarditis than binding to fibronectin (Vankerckhoven et al., 2007), but current evidence does not suggest that the adhesion to any of the host extracellular matrix proteins provides a good marker for the potential of *Lactobacillus* strains to cause bacteremia. The recent EU-PROSAFE project (Vankerckhoven et al., 2008b) concluded that currently, adhesion assays are not recommended as part of safety assessment of probiotics.

31.3.4 Platelet Aggregation

The ability to aggregate human platelets is considered a pathogenic trait among true pathogens. Platelet aggregation may be a relatively common trait among genus *Lactobacillus* (Harty et al., 1994). Some bacteremia-associated strains of *Lactobacillus* are able to aggregate platelets, while others are not, suggesting that platelet aggregation is neither a prerequisite of *Lactobacillus* bacteremia nor a good marker for the ability of these strains to cause bacteremia (Kirjavainen et al., 1999). The inability to induce human platelet aggregation has been used to demonstrate the safety of certain specific probiotic strains, including *L. rhamnosus* HN001 and *B. lactis* HN019 (Zhou et al., 2005b).

31.3.5 Hemolysis

Hemolysis is a known virulence factor among pathogenic microorganisms. Assessment of hemolytic activity has also been used in the *in vitro* evaluation of probiotic safety (Baumgartner et al., 1998). No evidence of hemolytic activity was found in fecal, blood and probiotic *Lactobacillus* strains (Vesterlund et al., 2007). Similarly, no hemolytic activity could be detected among strains of *Bifidobacterium* (Ouwehand et al., 2004a) or *L. rhamnosus* (Ouwehand et al., 2004b). However, some strains of lactobacilli express α-hemolysin (Baumgartner et al., 1998).

31.3.6 Resistance to Host Defense Mechanisms

Resistance to host defense mechanisms may enhance the survival of translocated microbes and increase the risk of infections, and *in vitro* assessments of host defense resistance have been applied in the safety assessment of probiotics. Probiotic lactobacilli have been found to be less resistant to intracellular killing by macrophages in cell culture than the clinical *Lactobacillus* isolates (Asahara et al., 2003). Moreover, similar differences were observed in the sensitivity of the strains to nitric oxide, a compound which plays a role in the killing of bacteria by macrophages. Notably, the study also suggested that differences in the sensitivity to host defense mechanisms exist between the different probiotic strains (Asahara et al., 2003). Vesterlund et al. (2007) assessed the ability to avoid the induction

of respiratory burst in peripheral blood mononucleocytes and the resistance to human serum of fecal, blood and probiotic isolates of lactobacilli. Probiotic *Lactobacillus* strains induced a lower respiratory burst in comparison to clinical *Lactobacillus* isolates, and tended to survive better in human serum in comparison to fecal isolates. Similar results were reported for strains of *L. rhamnosus* by Ouwehand et al. (2004b). Vankerckhoven et al. (2007) did not find differences between endocarditis and probiotic isolates in susceptibility to platelet microbicidal proteins. The above-mentioned factors and their relevance to the safety of probiotics may require further investigation. Resistance to serum by *L. rhamnosus* strains was earlier demonstrated by Baumgartner et al. (1998). Ouwehand et al. (2004a) investigated the resistance to the bactericidal effect of human serum and the induction of respiratory burst of strains of bifidobacteria, and concluded that these are unlikely risk factors for the genus *Bifidobacterium*.

31.3.7 Bile Salt Deconjugation

The role of bile salt deconjugation ability in the safety assessment of probiotics is controversial. While there are some implications that free bile acids may affect tumor promotion, there is insufficient evidence for the suggested harmful effects of free bile acids in general and no evidence suggesting that bile salt deconjugation by probiotics is harmful in humans (Vankerckhoven et al., 2008b). In fact, it has been suggested that bile salt deconjugation activity of probiotics may have beneficial effects on human health by lowering serum cholesterol. The EU-PROSAFE project concluded that bile salt deconjugation activity is irrelevant for safety assessment of probiotics (Vankerckhoven et al., 2008b).

31.3.8 Summary of *In Vitro* Assessment of Probiotic Safety

Several different *in vitro* approaches have been used in the safety assessment of probiotics (*Table 31.1*). *In vitro* tests assessing the resistance to antibiotics and the presence of mobile antibiotic resistance genes are common. Several studies have attempted to identify relevant virulence determinants for bacterial species used commonly as probiotics. However, to date such determinants have not been identified. Certain properties which are considered to be virulence

factors for true pathogens may be present in probiotic bacteria, but the presence of such factors (e.g., adhesion to host tissues) does not correlate with the infective potential of the probiotics. This is likely to result from the minimal infectivity of the probiotics in general. Nevertheless, certain *in vitro* measurements may be relevant in probiotic research, even in the case of organisms which are generally considered as safe. Certain microorganisms belonging to bacterial groups for which the general assumption of safety cannot be applied have also been suggested as potential probiotics. For such microorganisms, extensive *in vitro* safety evaluation is required, e.g., to determine the presence of virulent genes or transferable antibiotic resistance.

31.4 Animal Models in the Safety Assessment of Probiotics

31.4.1 Animal Models in Probiotic Research

Preclinical laboratory testing of the safety and efficacy of probiotics can be carried out using *in vivo* animal models. In contrast to *in vitro* assays, the *in vivo* models are dynamic systems in which the complex interactions between the administered probiotics and the host can be assessed in physiological environment. For scientific, regulatory and ethical reasons, studies using animal models should only be carried out following prior *in vitro* tests have been completed. *In vivo* testing of a probiotic strain is essential for scientific and regulatory purposes before the strain can be accepted for widespread use in humans or animals. *In vivo* models are important for studies in which different interactions between probiotics and the host, such as effects on host metabolism and immune system as well as distribution of probiotics following administration are investigated. Moreover, *in vivo* models are essential for safety studies, which may include studies on toxicity, bacterial translocation, and effects of probiotics in seriously ill and immunocompromized hosts. It is important to emphasize that experiments using animals are rigorously regulated by legislation, and they must be conducted humanely and only when similar results cannot be obtained by alternative methods. Similarly, *in vivo* testing should be conducted in animals with lowest degree of neurophysiologic sensitivity, and the lowest number of animals should be employed. Animal welfare and experimental procedures should be improved as much as possible to minimize animal distress and suffering and also to achieve good scientific practices.

Various animal models have been shown to contribute to our knowledge on the function of probiotics, and they have proved particularly useful in the investigation of mechanisms of action, effects on health, and safety of probiotics. The *in vivo* models used in probiotic research typically involve vertebrate laboratory animals, most commonly mice and rats. Also other animals such as pets, livestock or fish have been used in probiotic research, particularly in the field of veterinary science.

The choice and suitability of the animal models is influenced by numerous factors (and their interactions), including the animal species and strain used, the animal genotype and phenotype, the possible use of specific disease model, the number of animals required, the target outcomes, the choice of probiotic strains, and the quantity and quality of biological samples collected. When choosing animal models for probiotic research, the biological, physiological and genetic similarity between the model animal and the ultimately targeted host (e.g., human) should be evaluated. However, phylogenetic closeness is not always a guarantee for a valid model. The use of animal models to assess the safety of probiotics focuses on the microbe-host interactions. Microbiological factors, such as the microbiological quality of the animal facilities and feed are one of the most important factors to be considered by the scientists working with probiotics, because of these factors have the potential to confound and invalidate results and conclusions drawn from the animal experiments (Nicklas et al., 1999, 2002). Therefore, the use of *in vivo* models in probiotic research requires the selection of animals with high standards of microbiological quality. In order to achieve reliable, valid and reproducible experimental results, it is crucial that the microbiological status of the laboratory animal model is defined and free from unwanted microbial agents, such as viruses, mycoplasmas, bacteria, fungi and parasites specific for the animal species (i.e., SPF or specific pathogen free). Likewise, it is important to remark that most rodent infections are latent and do not to cause overt clinical symptoms, but are nevertheless capable of causing various degrees of abnormalities in the experimental results and increase biological variation. This may lead to the need to increase the number of animals used in order to counter the increased variation, which in turn has effects on the project cost as well as on the animal welfare.

The relevance and the extrapolation of the results obtained from animal studies to the ultimate target of probiotic use (e.g., human) depends on the choice of the animal model as well as other exogenous factors including the quality of the work, the targeted outcomes and the microbiological factors. At times, the data generated from animal models are not directly applicable

to the target host and *vice versa*. This is particularly true in microbiological models, where factors such as the high variability in species-specific responses and the differences between the compositions of the commensal microbiota of different species may not always allow the direct extrapolation of the results. Nonetheless, there are many published examples on probiotic research where health data generated from an animal model has been associated with similar outcomes in the ultimate target of probiotic use. However, careful assessment of animal experiment data and the relevancy to the targeted host is needed before the results may be extrapolated.

31.4.2 Examples of Probiotic Safety Assessments Using Animal Models

Various animal models have been used to assess the safety of probiotics (› *Table 31.3*). For the most commonly used probiotics, in particular lactobacilli and bifidobacteria, no clear virulence determinants have been identified, indicating general lack of pathogenicity. This makes the selection of *in vivo* models for safety assessment of probiotics challenging. Probiotic bacteria have a good safety record, but in rare cases these microorganisms have been isolated from infections in subjects with severe underlying diseases (Boyle et al., 2006). For this reason, most of the models used in probiotic safety assessment correspond to different

Table 31.3

Examples of animal models used in the safety assessment of probiotics

Animal model	Example
Healthy	Zhou et al. (2000b)
Neonatal	Lee et al. (2000)
Colitis	Daniel et al. (2006)
Infective endocarditis	Asahara et al. (2003)
Immunodeficient (congenital)	Wagner et al. (1997)
Immunodeficient (induced)	Zhou and Gill (2005)
Liver injury	Osman et al. (2005)
Acute pancreatitis	van Minnen et al. (2007)
Helminthic infections	Dea-Ayuela et al. (2008)
Intestinal resection	Mogilner et al. (2007)

disease models, both induced and spontaneous, and microbial translocation and/or organ colonization have been frequently used as the outcomes of these studies.

The risk of translocation associated with gut barrier disturbance, for example in the case of intestinal inflammation, has been studied extensively by using different models of induced colitis (Daniel et al., 2006; Pavan et al., 2003). In addition, healthy animals have been used in this respect, for example in the safety evaluation of the probiotic strains *L. rhamnosus* HN001, *L. acidophilus* HN017 and *B. lactis* HN019 (Zhou et al., 2000a, b). Also acute oral toxicity tests, using very high doses of probiotics, have been carried out (Kabeir et al., 2008; Tompkins et al., 2008; Zhou et al., 2000b). Immunocompromized animal models, of both adult and young animals, have also been used to evaluate the safety of probiotics. Congenitally immunodeficient animals (Wagner et al., 1997, 1998), and induced immunocompromized animal models (Dandekar et al., 2003; Zhou and Gill, 2005) are available. Many of the animal models applied in the safety assessment of probiotics were originally developed to study pathogenic microorganisms in which virulence traits are present and the infections caused by these pathogens, and therefore they may not be optimal for studying translocation of non-virulent microorganisms such as probiotics. Moreover, some of these models have been found to be resistant to translocation of ingested probiotics (Vankerckhoven et al., 2008b). The translocation ability of probiotics has also been assessed using neonatal animal models (Lee et al., 2000; McVay et al., 2008). Neonate animals may be considered to be immunocompromized due to the lack of properly established gut barrier function. Therefore, they may offer a good model for the determination of the safety of early probiotic intervention. In the light of the certain reports of probiotic sepsis in humans, it should be noted that the potential for probiotics to cause sepsis has also been observed in animal models. Wagner et al. (1997) colonized athymic mice with probiotic strains *L. reuteri, L. acidophilus* NCFM, *B. lactis* Bi-07 or *L. rhamnosus* GG (LGG). While athymic adult mice were not adversely affected by the probiotics, colonization with the probiotics *L. reuteri* and LGG did lead to death in some athymic neonatal mice, suggesting that the neonates with immune deficiency may be at elevated risk of probiotic sepsis.

Some cases of bacterial endocarditis due to lactobacilli have been reported in the literature (Salminen et al., 2004). This has drawn the attention of researchers to the identification of traits related with the ability to colonize heart valves. Animal models of induced experimental endocarditis are currently available (Gibson et al., 2007). Using one of these models it has been shown that lactobacilli are 100- to 10,000-fold less infective than the most common endocarditis pathogens;

Staphylococcus or *Streptococcus* strains. Most strains were even less infective than the non-pathogenic control *Lactococcus lactis* (Vankerckhoven et al., 2008b). Asahara et al. (2003) used rabbit experimental infective endocarditis model to demonstrate the safety of two probiotic strains *L. casei* Shirota and LGG, which were compared to endocarditis clinical isolates of *Staphylococcus*, *Streptococcus* and *Lactobacillus*. LGG was found to be more infective in the mouse model than *L. casei* Shirota, and this correlated with the *in vitro* ability of these strains to resist inactivation by host innate defense mechanisms. The colitis, immunocompromized and endocarditis models are the most commonly used disease models used in probiotic research, but many other models including induced acute liver injury models (Osman et al., 2005), a model for susceptibility to helminthic infections (Dea-Ayuela et al., 2008), models of interleukin deficient animals (Pena et al., 2005), or models of intestinal resection (Mogilner et al., 2007) have also been used. Animal models have also been used to investigate the possible transfer of antibiotic resistance genes between lactobacilli and other bacteria *in vivo* (Mater et al., 2008). Good results in animal models do not always correlate with good results in human clinical trials. Van Minnen et al. (2007) demonstrated the safety and the efficacy of a probiotic mix in a rat model of acute pancreatitis. The same probiotic mix was subsequently used in a human clinical trial assessing the efficacy of probiotics on severe acute pancreatitis. In the human study, compared to the placebo group, the rate of mortality was found to be higher in the group administered with probiotics (Besselink et al., 2008). This demonstrates that caution must be used when extrapolating the results obtained from animal disease models to humans. Moreover, it is important that the animal models used in the safety assessment reflect the real-life situations. In the case of the severe acute pancreatitis study, the animal model involved rats which were administered intragastrically with probiotics before the onset of pancreatitis (van Minnen et al., 2007), while in the human trial, the probiotic mixture was administered through nasojejunal tube to already gravely ill patients with severe complications (Besselink et al., 2008) (e.g., the organ failure rate was already high prior to the treatment in the probiotic group).

31.4.3 Concluding Remarks on Animal Models in the Safety Assessment of Probiotics

Animal models provide the opportunity to investigate many different aspects safety of probiotics (*Table 31.3*). Animal models are used to investigate

scientific questions which can not be answered by using *in vitro* or human trials. Examples of this include the studies focusing on the translocation of bacteria into host tissues, and acute toxicity tests. It is known that the extremely rare but clinically significant adverse effects related to probiotic use in humans are practically always associated with severe underlying diseases and compromised immune system of the host (Salminen et al., 2006). Animal models offer a way to study the safety of probiotics in severely ill hosts. When using the currently available animal models it should be keep on mind that most of them were developed to study virulence traits of pathogenic microorganisms, and caution is required when drawing conclusions from the studies with non-pathogenic microorganism, such as probiotics, in which the potential mechanisms of both beneficial and possible adverse effects but also the potential risks are different from pathogens. To date, the use of animal models has not revealed any specific virulent or pathogenic determinants among the different probiotic microorganisms studied, demonstrating the general safety of probiotics. Animal models designed specifically to assess the safety of probiotics should further be developed, to allow improved safety assessment of new and existing probiotic organisms.

31.5 Human Interventions in the Safety Assessment of Probiotics

Clinical trials assessing the safety of probiotics, along with the widespread and safe use of probiotics worldwide, constitute the most compelling evidence of the safety of probiotics. Clinical safety trials enable the *in vivo* evaluation of the effects of probiotics in humans in a controlled manner, with a special focus on attributes relevant to the safety of the administered probiotic and the factors contributing to possible adverse events. Clinical trials assessing the safety of new probiotics should be carried out following appropriate *in vitro* and animal model safety assessments, but preferably prior to introducing products containing the probiotic to market. However, many organisms belonging to *Lactobacillus* and *Bifidobacterium* are generally regarded as safe (EFSA, 2007), and therefore extensive studies on the safety of these strains are not always carried out. Outcome measures of clinical probiotic safety studies often include stool consistency, defecation frequency and gastrointestinal complaints (Mäkeläinen et al., 2003), as well as serum and immune markers and the frequency of adverse events. Apart from trials specifically designed for assessing the safety of the probiotic

administration, the clinical trials assessing the efficacy of probiotic treatments also contribute to the clinical evidence of probiotic safety. Although the main target outcomes in these probiotic intervention trials focus on the health benefits of probiotics, the safety of the probiotic administration and the potential adverse events are often reported as a secondary outcome (Kajander et al., 2008; Peng and Hsu, 2005; Rautava et al., 2002). In particular, safety aspects are of interest in trials involving diseased patients and other potential risk groups.

Clinical investigations of the safety of probiotics are commonly carried out with healthy volunteers. No adverse effects on gastrointestinal health were seen during probiotic administration of *Bifidobacterium longum* 46 and *B. longum* 2C to healthy volunteers (Mäkeläinen et al., 2003). Safety of the administration of high dose of *L. reuteri* ATCC 55730 (1×10^{11} cells/day) to healthy volunteers has been demonstrated (Wolf et al., 1995). Clinical trials have been conducted to demonstrate the safety of the strain *Streptococcus salivarius* K12 strain used as a probiotic targeted at oral health (Burton et al., 2006), and the lack of tetracycline resistance gene transfer during concomitant ingestion of *L. acidophilus* LA-CH5, *B. animalis* subsp. *lactis* Bb-12 and antibiotics (Saarela et al., 2007). Long term safety studies of probiotics are rare. Laitinen et al. (2005) assessed the effects of perinatal administration of LGG on the subsequent growth of children, and found the early probiotic administration to be safe.

Safety of the probiotics may be of particular interest in specific age groups, such as neonates, who have compromised immune system. In neonates and low-birth-weight infants, successful clinical interventions have been carried out (Agarwal et al., 2003; Hoyos, 1999) but serious adverse events have not been reported. Clinical trials suggest that probiotics are safe to use in follow-up formulas and growing-up milks (Haschke et al., 1998). Clinical evaluation of probiotics in elderly populations is of special interest, since elderly subjects commonly have health related problems including infections and gastrointestinal problems, and may also have altered dietary habits and gut microbiota composition compared to healthy adults. For the very same reasons, the elderly subjects in particular may benefit from the use of probiotics. The safety and the lack of adverse events following the consumption of *B. longum* 46 and 2C (Pitkälä et al., 2007) as well as other strains including *B. lactis* HN0019 and *L. rhamnosus* HN001 (Gill et al., 2001) by elderly subjects has been demonstrated.

As expected, the commonly used probiotics such as strains belonging to *Lactobacillus* and *Bifidobacterium* perform well in studies involving the safety assessment of probiotics in healthy volunteers. Clinical intervention studies and the widespread and long-term consumption of fermented foods and probiotic

products clearly demonstrate the safety of probiotic administration in general population. However, the possible risks associated with probiotics may be elevated in certain high risk populations, and the demonstration of the safety of a certain probiotic strain in general population does not necessarily imply that the administration of the same strain is equally safe in high risk populations. A limited number of clinical safety assessments of probiotics have been conducted in high risk populations. In a clinical trial to assess the safety of enteral administration of *L. casei* Shirota to critically ill children, no evidence was found of bacteremia or colonization of the probiotic in surface swabs from various sites, endotracheal aspirates, sputum, blood, urine, cerebrospinal fluid, sterile body fluid samples or tip cultures of arterial and long venous line catheters (Srinivasan et al., 2006). Probiotics have also been used successfully in patients with necrotizing enterocolitis (Bin-Nun et al., 2005). Immunocompromized patients have an increased risk for translocation and infections, and are therefore a group of special interest for clinical safety assessment of probiotics. In a small placebo-controlled trial in patients infected with the Human Immunodeficiency Virus (HIV) designed to assess the safety of probiotics in this patient group, no changes were found in safety parameters such as serum chemistry, hematology, immune profile, urinalysis, gastrointestinal tolerance, fecal microbiota and physical examination parameters (Wolf et al., 1998), suggesting that the administration of *L. reuteri* ATCC 55730 was safe in this population. Several other probiotic intervention studies have also been conducted in this patient group. Clinical trials involving severely ill patients are associated with an elevated risk of adverse events. Careful preclinical safety assessment is required before such intervention trials are conducted. Even then, the potential for adverse events may remain high, as demonstrated by the recent study carried out in severely ill patients with acute pancreatitis (Besselink et al., 2008). In this study, adverse events were observed in the probiotic group, despite that earlier data from an animal model (van Minnen et al., 2007) and from clinical intervention trials (Olah et al., 2002) suggested the safety of the probiotic intervention.

Taken together, clinical safety trials of probiotics provide valuable information on the effects of these organisms *in vivo*. The importance of such trials is underlined by the fact that the results of *in vitro* safety assessments and animal models cannot be directly extrapolated to humans. The current evidence strongly suggests that probiotics are extremely safe for general population. However, for certain high-risk populations, more thorough safety evaluation may be required to confirm the safety of probiotic use.

31.6 Adverse Events and Potential Risks of Probiotics

Probiotics overall have an excellent safety record in humans. In clinical studies, probiotics have also been fed to in particular high risk populations without significant adverse effects, including subjects infected with HIV (Heiser et al., 2004) and premature infants suffering from necrotizing enterocolitis (Bin-Nun et al., 2005). In Finland there has been a marked increase in the use of the probiotic LGG since its introduction into the country in 1990, but during this period no significant increase in *Lactobacillus* bacteremia or bacteremia attributable to probiotic strains was observed by Salminen et al. (2002). When 47 *Lactobacillus* bacteremia isolates from Finland were species-characterized, 53% of the isolates were identified as *L. rhamnosus* and furthermore, in 23% of the cases the isolate was indistinguishable by PFGE from LGG (Salminen et al., 2004). In a survey from Sweden, lactobacilli were found to represent less than 1% of the total number of bacteremia cases each year, and commonly used probiotic strains were not identified among the clinical isolates (Sullivan and Nord, 2006). Although commercially available probiotic strains are widely regarded as safe, there are concerns with respect to safety in particular high-risk populations.

31.6.1 Sepsis Related to Probiotic Use

The most commonly reported serious adverse event from probiotic treatment is sepsis. In the absence of probiotic supplementation, *Lactobacillus* species are a known, albeit rare, cause of endocarditis in adults and other forms of sepsis in children. Certain reports have directly linked cases of sepsis to the ingestion of probiotic supplements (*Tables 31.4* and *31.5*). A case of a 74 year old diabetic woman who developed LGG liver abscess (isolate indistinguishable from the commercial strain using PFGE of chromosomal DNA restriction fragments) and pneumonia 4 months after commencing daily LGG supplements has been reported (Rautio et al., 1999). *L. rhamnosus* endocarditis after a dental extraction in a 67 year old man with mitral regurgitation who was taking daily probiotic capsules has been reported (Mackay et al., 1999). No differences between the probiotic and the infective *L. rhamnosus* were found using standard API 50 CH biochemical analysis and pyrolysis mass spectrometry. Although highly suggestive of probiotic supplement related sepsis, the aforementioned reports do not conclusively prove that the infectious agents were indeed originating from the

probiotic products, as bacterial strains seemingly indistinguishable from probiotic strains may sometimes be found in the intestinal microbiota of healthy humans (Presterl et al., 2001).

Table 31.4

Cases of bacterial sepsis in humans temporally related to probiotic use (*Cont'd* p. 1221)

Study	Age	Risk factors	Probiotic	Method of identification	Form of sepsis
Rautio et al. (1999)	74	Diabetes mellitus	LGG	API 50 CH; PFGE of DNA restriction fragments	Liver abscess
Mackay et al. (1999)	67	Mitral regurgitation Dental extraction	*L. rhamnosus* 3×10^9 cfu/day	API 50 CH; Pyrolysis mass spectrometry	Endocarditis
Kunz et al. (2004)	3 months	Prematurity Short gut syndrome	LGG	No confirmatory typing	Bacteremia
	10 weeks	Prematurity; Inflamed intestine; Short gut syndrome;	LGG	PFGE of DNA restriction fragments	Bacteremia
De Groote et al. (2005)	11 months	Prematurity; Gastrostomy; Short gut syndrome; CVC; Parenteral nutrition; Rotavirus diarrhea	LGG ¼ capsule/day	rRNA sequencing	Bacteremia
Land et al. (2005)	4 months	Cardiac surgery; Antibiotic diarrhea	LGG 10^{10} cfu/day	Repetitive element sequence-based PCR DNA fingerprinting	Endocarditis
		Cerebral palsy; Jejunostomy feeding; CVC; Antibiotic diarrhea	LGG 10^{10} cfu/day	Repetitive element sequence-based PCR DNA fingerprinting	Bacteremia

Table 31.4

Study	Age	Risk factors	Probiotic	Method of identification	Form of sepsis
Richard et al. (1988)	47	Not stated	*B. subtilis* 8×10^9 spores/day	Antibiotic susceptibility	Bacteremia
	25	Not stated	*B. subtilis* 8×10^9 spores/day	Antibiotic susceptibility	Bacteremia
	63	Neoplastic disease	*B. subtilis* 8×10^9 spores/day	Antibiotic susceptibility	Bacteremia
	79	Not stated	*B. subtilis* 8×10^9 spores/day	Antibiotic susceptibility	Bacteremia
Oggioni et al. (1998) and Spinosa et al. (2000)	73	Chronic lymphocytic leukemia	*B. subtilis* 10^9 spores / day	16S rRNA sequencing	Bacteremia

CVC, Central venous catheter. Adapted from Boyle et al. (2006)

In children, cases of bacterial sepsis related to probiotic use and short gut syndrome have been reported. Two premature infants with short gut syndrome who were fed via gastrostomy or jejunostomy developed *Lactobacillus* bacteremia while taking LGG supplements (Kunz et al., 2004). Similarly, catheter-related LGG bacteremia has been reported in an 11-month-old patient (De Groote et al., 2005). In both cases, the bacteremic strain and probiotic strain were found to be indistinguishable. Cases of probiotic sepsis have been seen in two severely ill children with antibiotic-related diarrhea, related to cardiac surgery or cerebral palsy, due to enteral administration of LGG (Land et al., 2005). Cases of bacteremia associated to other probiotic strains have also been reported. *Bacillus subtilis* bacteremia and cholangitis have been described in three reports (Oggioni et al., 1998; Richard et al., 1988; Spinosa et al., 2000), of which one included confirmation of the strain homology between the probiotic and pathogenic bacteria by molecular typing. Several cases of *Saccharomyces boulardii* fungemia in subjects taking *S. boulardii* supplements have also been described (*Table 31.5*). Molecular typing was used to demonstrate the homology between the probiotic and infective organisms in many cases. Significant sepsis due to *S. boulardii* administered to a neighboring patient, but not the patient developing

sepsis, has been reported in two cases (Cassone et al., 2003; Perapoch et al., 2000). Such cases may have been due to contaminated vascular catheters (Hennequin et al., 2000). Despite of the widespread use of the genus *Bifidobacterium* and in particular the species *B. animalis* subsp. *lactis* in commercial probiotic products,

Table 31.5

Cases of fungal sepsis in humans temporally related to probiotic use (*Cont'd* p. 1223)

Study	Age	Risk factors	Probiotic	Method of identification	Sepsis
Hennequin et al. (2000)	30 months	Cystic fibrosis; CVC; Poor nutritional state; Intestinal surgery; HIV infection; CVC; Diarrhea	*S. boulardii* 750 mg/day	PFGE of mitochondrial DNA restriction fragments	Fungemia
	36	Antibiotic diarrhea; Upper gastrointestinal surgery for malignancy	*S. boulardii* 1.5 g/day	PFGE of mitochondrial DNA restriction fragments	Fungemia
	47	Peptic ulcer; Chronic Renal Failure;	*S. boulardii* 2 g/day	PFGE of mitochondrial DNA restriction fragments	Septic shock
	78	Pneumonia/COPD	*S. boulardii* 1.5 g/day	PFGE of mitochondrial DNA restriction fragments	Fungemia
Cassone et al. (2003)	34	CVC; Intensive care unit	No direct treatment	PFGE of undigested chromosomal DNA	Fungemia
	48	CVC; Intensive care unit	No direct treatment	PFGE of undigested chromosomal DNA	Fungemia
	75	CVC; Intensive care unit	No direct treatment	PFGE of undigested chromosomal DNA	CVC colonization
	35	Intensive care unit	Unclear	PFGE of undigested chromosomal DNA	Fungemia

Table 31.5 (*Cont'd* p. 1124)

Study	Age	Risk factors	Probiotic	Method of identification	Sepsis
Perapoch et al. (2000)	3 months	CVC; Diarrhea; Parenteral nutrition	*S. boulardii* 100 mg/day	PFGE of mitochondrial DNA restriction fragments PFGE of undigested chromosomal DNA	Fungemia
		Short bowel syndrome; CVC	Not received directly (in cot next to first patient)	PFGE of mitochondrial DNA restriction fragments	Fungemia
	Infant	Parenteral nutrition		PFGE of undigested chromosomal DNA	
Lherm et al. (2002)	50–82	Acutely unwell on intensive care unit with respiratory failure; CVC	*S. boulardii* 1.5–3 g/day	PFGE of nuclear and mitochondrial DNA restriction fragments	Fungemia
Bassetti et al. (1998)	51	Immunosuppression; *C. difficile* diarrhea; CVC	*S. boulardii* 1 g/day	PFGE of DNA restriction fragments	Fungemia
Riquelme et al. (2003)	42	Kidney/Pancreas transplant; Immunosuppression; *C. difficile* diarrhea	*S. boulardii* 1 g/day	PFGE of DNA restriction fragments	Fungemia
	41	HIV; Diarrhea	*S. boulardii* 750 mg/day	PFGE of DNA restriction fragments	Fungemia
Fredenucci et al. (1998)	49	Antibiotic diarrhea; Immunosuppressed	*S. boulardii* 200 mg/day	API 32C PFGE of undigested chromosomal DNA	Fungemia
Cesaro et al. (2000)	8 months	Acute myeloid leukemia; CVC; Neutropenia	*S. boulardii*	API 32C	Fungemia
Cherifi et al. (2004)	89	*C. difficile* colitis; Gastrostomy	*S. boulardii* 300 mg/day	No formal identification described	Fungemia

Table 31.5

Study	Age	Risk factors	Probiotic	Method of identification	Sepsis
Henry et al. (2004)	65	Malignancy; Immune compromise; Mucositis; Diarrhea; Parenteral Nutrition	*S. boulardii*	No formal identification described	Fungemia
Niault et al. (1999)	78	Antibiotic diarrhea; Intensive Care unit; Intragastric feeding	*S. boulardii* 1.5 g/day	No formal identification described	Fungemia
Viggiano et al. (1995)	14 months	Burns; Diarrhea; Gastrostomy	*S. boulardii* 200 mg/day	No formal identification described	Fungemic shock
Zunic et al. (1991)	33	Inflammatory bowel disease; Intensive care unit; Parenteral nutrition	*S. boulardii* 1.5 g/day	No formal identification described	Fungemia
Pletincx et al. (1995)	1	Parenteral nutrition; Antibiotic diarrhea; CVC	*S. boulardii* 600 mg/day	No formal identification described	Septicemia
Rijnders et al. (2000)	74	Colitis; Nasogastric feeding	*S. boulardii* 600 mg/day	No formal identification described	Fungemia
Lestin et al. (2003)	48	Diabetes; *C. difficile* diarrhea	*S. boulardii* 150 mg/day	API 32C	Fatal fungemia

CVC, Central venous catheter; *COPD*, Chronic obstructive pulmonary disease. Adapted from Boyle et al. (2006)

these probiotics have never been related to sepsis associated with probiotic use, demonstrating the extremely low pathogenic potential of bifidobacteria.

31.6.2 Gastrointestinal Symptoms Related to Probiotic Use

It is clear from dose ranging studies that high dose probiotic treatment can lead to increased frequency and softening of feces (Larsen et al., 2006). Gastrointestinal adverse events such as vomiting and diarrhea are also rarely seen in probiotic treatment trials. In particular, one study would suggest that there is an increased risk of such adverse events associated with the use of heat-inactivated rather than viable probiotics. Kirjavainen et al. (2003) were forced to terminate their study of

the probiotic LGG in allergic infants due to adverse gastrointestinal symptoms and diarrhea in those treated with a heat-killed form of the probiotic, something which they did not find in those infants treated with viable LGG. However, bacteriological data of the study indicated that high numbers of *Clostridium* and *Bacteroides* may have been a predisposing factor for the observed side effects. The group treated with heat-killed LGG had higher numbers of both *Clostridium* (11%) and *Bacteroides* (12%) already before the treatment than did the group treated with live LGG (4 and 5%, respectively), which may also explain why the adverse gastrointestinal were more common in the group receiving heat-inactivated probiotics.

31.6.3 Other Adverse Events Related to Probiotic Use

A recent clinical trial identified the most severe potential risk of probiotic treatment reported to date – that of fatal bowel ischemia. Besselink and colleagues (Besselink et al., 2008) investigated the effects of a probiotic mix (*L. acidophilus, L. casei, L. salivarius, Lactococcus lactis, B. bifidum* and *B. infantis*) at a total dose of 10^{10} CFU/day in a randomized placebo-controlled trial of 296 adults with a first episode of high risk acute pancreatitis. The probiotic mix was administered by nasojejunal feeding tube, and had been specifically designed to inhibit the growth of pathogens important in pancreatic necrosis. Preliminary data in rats (van Minnen et al., 2007) and humans with less severe illnesses (Besselink et al., 2008) suggested that this probiotic mix would be safe and efficacious in the prevention of infectious complications of pancreatitis. The authors found a 2.53-fold increase in mortality risk in probiotic treated participants, and nine cases of bowel ischemia (eight fatal) in the probiotic group, with no bowel ischemia in the placebo group (Besselink et al., 2008). The bowel ischemia occurred after a median of 3 days probiotic treatment (range 2–11 days). It is known that small bowel ischemia, increased intestinal permeability and increased bacterial translocation are all associated with acute pancreatitis. In these cases the direct application of probiotic bacteria to an already damaged small intestinal mucosa may have precipitated a local inflammatory response leading to increased risk of small bowel ischemia. However, it is not known why exactly mortality was higher in the treatment group, and although the mortality was associated with randomization, this does not necessary implicate that the probiotic itself was the causative factor. Indeed, the observed results may have (at least partly) been due to unsuccessful randomization, because the rate of organ failure, a consequence of hemodynamic

disturbance parallel to bowel ischemia, was significantly higher in the probiotic group (n = 20) compared to the placebo group (n = 7) already at the day of randomization (Reid et al., 2008).

Probiotics have been proposed to have a role in the management of childhood allergies. In general, prevention of the occurrence of allergies appears to be more effective than the treatment of allergies by probiotics. Despite the promising results in this area, there are also some indications of adverse effects of probiotics in this respect. A recent systematic review demonstrated that the probiotic LGG leads to a minor worsening of disease severity when used to treat eczema in young children, whereas subgroup analysis of probiotics other than LGG suggested significant improvement in eczema severity (Boyle et al., 2008). In a 7-year follow-up of a study assessing the efficacy of administration of LGG during infancy in the prevention of atopic eczema, it was observed that children who received LGG during infancy had lower rate of eczema at 7 years of age compared to children who received placebo, but the rate of respiratory allergies and asthma tended to be higher in the probiotic group (Kalliomäki et al., 2007). Kopp et al. (2008) reported no reduction in the rate of atopic dermatitis in children following administration of LGG during late pregnancy and early infancy. Instead, increased frequency of recurrent episodes of wheezing bronchitis was observed in the probiotic group. Finally, Taylor et al. (2007) reported that postnatal administration of *L. acidophilus* LAVRI-A1 to infants was associated with increased allergen sensitization at 12 months of age. Despite these reports, the role of probiotics in the management of allergies appears to be beneficial in general, but the indications of the possible adverse effects following early probiotic administration deserve further attention in the future.

31.6.4 Factors Affecting the Adverse Effects Associated with Probiotics

31.6.4.1 Underlying Diseases and Treatments

To date, there have been no reports of sepsis related to probiotic use in otherwise healthy individuals. All reported cases of probiotic bacteremia or fungemia have occurred in patients with underlying chronic disease, or immune compromised or debilitated state. In most cases, probiotic sepsis has resolved with antimicrobial therapy, but in some cases patients have developed septic shock (Hennequin et al., 2000). In some cases the outcome has been fatal, but apart from a case of

a 48 year old diabetic patient with diarrhea attributable to *Clostridium difficile* who died from multiple organ failure and septic shock in association with a toxic megacolon and probiotic fungemia (Lestin et al., 2003), these fatalities have been related to underlying disease rather than being directly attributable to probiotic sepsis (Boyle et al., 2006). In the report by Lestin et al. (2003), the only case suggestive of fatal probiotic sepsis, molecular methods were not used to confirm homology between the probiotic and pathogenic fungi. Treatment of diarrhea and short bowel syndrome are common targets of probiotic therapies, but these pre-existing intestinal pathologies may also potentially increase the risk of probiotic translocation through the intestinal mucosa. Ongoing antibiotic treatment may increase the risk of *Lactobacillus* bacteremia, in particular in the case of *L. rhamnosus*. Salminen et al. (2006) reported that in approximately half of the 85 cases, the patient had received antimicrobial treatment prior to *Lactobacillus* bacteremia. Administration of probiotics via jejunostomy tube, bypassing the effect of gastric acid and the dilutional capacity of both stomach and duodenum, may increase the numbers of viable probiotic bacteria that reach the intestine. Some cases of adverse events in patients administered with probiotics via jejunostomy tube have been reported. Central venous catheter, a common finding in cases of probiotic sepsis, may serve as a possible source of sepsis (Hennequin et al., 2000). Premature infants and patients who are debilitated or have compromised immune function are overrepresented in the cases of sepsis associated with probiotics (› *Tables 31.4* and › *31.5*). In the case of lactobacilli, cardiac valvular disease may be a risk factor, as certain species of *Lactobacillus* may in rare cases colonize heart valve and cause endocarditis. In the light of the recent results reported by Besselink et al. (2008), enteral administration of probiotics to patients with severe acute pancreatitis or patients in high risk of developing bowel ischemia may be particularly risky.

31.6.4.2 Probiotic Strain Selection and Characteristics

The beneficial health effects of one probiotic cannot be assumed for another probiotic species, or even for different strains of the same species. The same applies for the rare adverse events associated with probiotics. The case reports of probiotic sepsis published to date suggest that *Saccharomyces boulardii,* LGG and *Bacillus subtilis* may be probiotics that carry a higher risk of sepsis than other strains (› *Tables 31.4* and › *31.5*). When 85 blood isolates from cases of *Lactobacillus* bacteremia in Finland were examined, 46 isolates were identified as

L. rhamnosus, 12 isolates of both *L. casei* and *L. fermentum*, and three isolates each of *L. jensenii*, *L. salivarius* and *L. gasseri* were identified (Salminen et al., 2006). Of the *L. rhamnosus* isolates, 22 (48%) were judged to be indistinguishable from LGG by pulsed-field gel electrophoresis, but it is important to note that it is not known whether the patients in this study had actually consumed LGG. In addition, phenotypic differences have later been demonstrated between some of the above-mentioned *L. rhamnosus* isolates and the probiotic strain LGG (Ouwehand et al., 2004b). Certain probiotic strains such as LGG and *S. boulardii* may be more frequently administered to subjects with underlying diseases, including antibiotic associated diarrhea, than other strains. This may partly explain the association of these strains with the reported cases of probiotic sepsis, but the differences in the intrinsic properties of probiotic strains, such as the properties increasing the potential of bacterial translocation, clearly also affect the probability of certain probiotic strains to cause sepsis. Results from animal model experiments also suggest that certain *Lactobacillus* strains have higher infectivity than others (Vesterlund et al., 2007). Other groups such as probiotics in the genus *Bifidobacterium* appear to have less pathogenic features and are underrepresented in case reports of probiotic sepsis (Boyle et al., 2006).

For probiotic sepsis, bacterial translocation appears to be a key event. Good adherence to host mucus and epithelium is thought to play a role in many of the beneficial effects of probiotics. However, theoretically, strong adherence to epithelial layer may also increase the likelihood of bacterial translocation, in particular in subjects with disturbed gut permeability, immune deficiency and intestinal immaturity (Boyle et al., 2006). Adherence alone should not be considered a risk factor for strains of commencal microbes and strains with low infectivity. Properties other than adherence are also required for increased likelihood of translocation and sepsis, as demonstrated by the fact that certain probiotic species such as *B. animalis* subsp. *lactis* and *L. acidophilus* are characterized with good adhering properties, but are not associated with the cases of probiotic sepsis, despite their widespread use as probiotics. Moreover, some clinical isolates of *Lactobacillus* exhibit only low level of adhesion (Apostolou et al., 2001).

The intestinal microbiota is important in stimulating normal immune development, particularly the development of the gut associated lymphoid tissue. The crucial role of the intestinal microbiota in normal immune development and function suggests that manipulations designed to alter this microbiota may have significant immunomodulatory effects. Immune modulation is thought to be one of the key mechanisms of the beneficial effects of probiotics. Although currently there is no evidence linking immune modulation of probiotics to adverse events, such effects remain a possibility (Boyle et al., 2006). For example,

administration of the probiotic LGG to infants with atopic eczema has been shown to induce systemically detectable low grade inflammatory responses characterized by increased levels of c-reactive protein and interleukin-6 (Viljanen et al., 2005). While these effects may serve as a mechanism for the beneficial effects of probiotics on atopic eczema, the potential for adverse events linked with systemic proinflammatory effects cannot be ruled out. At present there is little support for the hypothesis that probiotics lead to adverse immune development from empirical studies, but this is an area that warrants further investigation.

Potential pathogenic determinants to be taken into account in the safety assessment of probiotics, including transferable antibiotic resistance, hemolysis, platelet aggregation, production of deleterious metabolites and resistance to host defense mechanisms, are reviewed elsewhere in this chapter. In addition to the characteristics of administered probiotic strain, the administration dose should be taken into account when evaluating the safety of probiotic regimens. Since high dose regimens of probiotics may be associated with looser stools (Larsen et al., 2006), the dosing of probiotics may also have effect on certain potential side-effects, although it should be noted that in general, even very high doses of probiotics are well-tolerated.

31.7 Conclusion

The current evidence strongly suggests that probiotics are safe to use in general population. Several approaches for the safety evaluation of current and potential new probiotics are available, ranging from *in vitro* assessments to randomized, controlled clinical trials. Most commonly used probiotics are considered to be generally safe, and in Europe such microorganisms have been granted a QPS status (EFSA, 2007). Safety of probiotics, similar to the beneficial effects of probiotics, is a strain-dependent feature, and differences exist between different probiotics. For example, in the case of transferable antibiotic resistance elements, strains of *Enterococcus* are particularly problematic. The problems associated with the potential production of toxic compounds are especially evident among strains belonging to genus *Bacillus*. In regard to the potential of probiotics to cause sepsis, the currently reported cases suggest that strains of *Saccharomyces*, *L. rhamnosus* and *Bacillus* may posses higher risk of adverse events than other probiotics. Notably, among *L. rhamnosus*, clear differences exist between the infective potential of the strains belonging to this species (Vankerckhoven et al., 2007).

Despite the excellent overall safety record of probiotics, they should be used with caution in certain specific patient groups – particularly critically patients, those with immune deficiency and patient groups with increased risk for bacterial translocation due to disturbed intestinal mucosal barrier function. The risk of adverse events is likely to differ with each probiotic strain, and published literature has highlighted some strains which may carry higher risks than others. The dose and mode of administration are also important, with higher dose regimens being associated with gastrointestinal symptoms. Careful consideration should be given to these issues before using probiotic supplementation in high risk populations.

Taken together, the beneficial effects of probiotics clearly outweigh the possible risks of probiotic use. Probiotics provide a variety of health benefits for humans, from healthy subjects to patients with many different diseases.

31.8 Summary

- Probiotics have an excellent overall safety record.
- Several *in vitro* assessments of probiotics are available, but clear markers for potential infectivity have not been identified.
- Animal models allow the safety assessments focusing on bacterial translocation and underlying diseases.
- Numerous clinical trials assessing the safety of probiotic contribute to the safety of probiotics.
- In certain high-risk populations such as critically ill patients, the use of probiotics should be carefully considered.

List of Abbreviations

ARDRA	amplified rDNA restriction analysis
COPD	chronic obstructive pulmonary disease
CVC	central venous catheter
EFSA	European Food Safety Authority
FAO	Food and Agriculture Organization
HIV	human immunodeficiency virus

LAB	lactic acid bacteria
LGG	*Lactobacillus rhamnosus* strain GG
MLS	macrolide, lincosamide and streptrogramin
PCR	polymerase chain reaction
PFGE	pulsed field gel electrophoresis
QPS	qualified presumption of safety
RAPD	randomly amplified polymorphic dNA
Rep-PCR	repetitive DNA element-PCR
SPF	specific pathogen free
WHO	World Health Organization

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