PROBIOTICS, PREBIOTICS AND THE GUT MICROBIOTA

ILSI Europe
International Life Sciences Institute

ABOUT ILSI / ILSI EUROPE

PROBIOTICS, PREBIOTICS AND THE GUT MICROBIOTA

by
Nino Binns

CONTENTS

Author: Nino Binns, NB Consulting (UK)

Scientific Editors: Glenn. R. Gibson, University of Reading, (UK);
Mary Ellen Sanders, International Scientific Association for Pro & Prebiotics (US)

Scientific Reviewers: Nathalie Delzenne, Université Catholique de Louvain (BE) and Lorenzo Morelli, Catholic University of Piacenza, (IT)

Concise Monograph Series Editor: John Howlett (UK)

Coordinators: Agnès Méheust, Massimo Ambrosio and Alessandro Chiodini, ILSI Europe (BE)

FOREWORD

Since the introduction of the concept of functional foods in Japan in the 1980s, there has been growing interest in the concept of prebiotics and probiotics, and the synergistic combination thereof, called synbiotics, and their role in human nutrition. Pre- and probiotics are now commonly found in a range of products for infants, young children and adults. What is it that makes the consumer interested in these ingredients? By definition, both pre- and probiotics should convey health benefits. The general population is increasingly interested in maintenance of health and self-care and this may explain the consumers' interest.

Both pre- and probiotics elicit their effects, at least to some extent, through modulation of the intestinal microbiota (formerly called microflora). The results of multidisciplinary research efforts to understand the composition and function of the intestinal microbiota, as well as the role of pre- and probiotics, have recently been published. A summary of these findings is therefore both appropriate, in the context of pre- and probiotics, and timely.

Because of the lack of easily understandable and objective information on the topic for the interested non-specialist life scientist, ILSI Europe Task Forces on both Prebiotics and Probiotics decided to initiate the writing of a Concise Monograph with input from experts in the field.

The purpose of the monograph is to discuss in understandable terms the current abundant scientific knowledge on prebiotics, probiotics and the intestinal microbiota, including the resulting effects on the host. The monograph does not address the detailed regulatory aspects of the topic. The challenge in nutritional sciences is not to tackle disease with a pharmaceutical approach, but rather to maintain and support health and thereby reduce the risk of disease. Instead of testing clinical endpoints of reduction in disease, in nutritional intervention studies it is the markers of health or markers of risk of disease that need to be checked and validated. Influencing the biomarkers of disease risk often requires an in-depth understanding of the underlying mechanisms. This is where future research in pre- and probiotic science will add to existing knowledge and evidence. With their complexity and the complexity of the systems with which they interact (the intestinal microbiota, the immune system, etc.), understanding the mechanisms is scientifically a challenge.

However, the scientific understanding of pre- and probiotic mechanisms has grown substantially in the past decade and efforts in the field are increasing, making us confident that further scientific knowledge will be generated. So far, evidence for the many potential health benefits of different pro- and prebiotics has been documented, the effects very often being strain- and product-specific. Emerging physiological and analytical tools embedded in a multidisciplinary research setting will enable the elucidation of further mechanisms. The latter will be a part of the better understanding of pre-, pro- and synbiotic health effects.

We are convinced that this Concise Monograph, based on sound scientific evidence, will be an important contribution in informing a wide audience about the concepts of pre-, pro- and synbiotic nutrition.

Bernd Stahl
Danone Research, Germany
Arthur Ouwehand
DuPont Nutrition and Health, Finland

INTRODUCTION

Microbes, or micro-organisms, include bacteria, fungi, yeasts and algae. They can be found everywhere on Earth, including hostile environments like volcanoes, the ocean bed and deserts. They are incredibly diverse and have adapted over millions of years to occupy their own particular niches. As far as humans are concerned, microbes are best known for their role in causing disease, but their power has also been harnessed for millennia to the benefit of humankind. They are used in the production of fermented foods including dairy products, breads, vegetables and, of course, wines and beers to name but a few. Owing to their potential for very selective action, microbes are also crucial to the development and production of pharmaceuticals such as antibiotics and to the production of food ingredients such as vitamins and citric acid. Microbes are also involved in the production of many other chemicals and enzymes and are used in waste processing.

Most of the 10^{14} bacteria in the gut are found in the large intestine (colon) and, over the past 30 years or more, interest in the gut microbial population – the microbiota – and its environment has intensified. Numerous research studies have shown that, far from being passive inhabitants of the gastrointestinal (GI) tract, the habitual residents of the gut (commensal micro-organisms) interact with their host in a very intricate manner. They may modulate the effect of potentially harmful bacteria, impact the host's GI tract, digestion, metabolism and immune system, and might even influence functions beyond the gut.

The concept that food-borne bacteria can be beneficial to health emerged at the turn of the twentieth century and is usually attributed to Nobel Prize-winning Russian scientist Ilya Metchnikoff. He hypothesised that consuming large amounts of fermented milk products that contained *Lactobacillus* bacteria ("soured milk") could prolong and improve the quality of life because these bacteria entered the colon and limited the activities of undesirable microbes. Metchnikoff therefore saw the intestinal tract as an organ that could be manipulated to improve health by adding exogenous bacteria to the gut. As a result, commercial yogurts and fermented milks gained some popularity after the First World War, but it was not until the 1980s that the sales of products containing probiotics began to grow rapidly - first in Japan and then extending to Europe during the 1990s.

Probiotic bacteria may be defined as '*live micro-organisms which, when administered in adequate amounts, confer a health benefit on the host*' (FAO/WHO 2001). They can interact with commensal bacteria and can also have a direct impact on the host. Disentangling these interactions is one of the key challenges for future research. Other key challenges are to understand their mechanisms of action, to elucidate more specifically which probiotic strains can offer which health benefits and to define the intake levels needed to achieve those effects.

The prebiotic concept developed more recently. The Japanese were the first to recognise the value of non-digestible oligosaccharides, initially in animal feed where their addition to the feed of piglets helped relieve and prevent scouring (diarrhoea). Japanese researchers also recognised the value of oligosaccharides in human milk and later demonstrated that consumption of fructo-oligosaccharides and galacto-oligosaccharides led to an increase in intestinal bifidobacteria and stimulated their growth in the human gut. However, it was not until 1995 that the scientific concept for human gut microbiota modulation by "prebiotics" was introduced. Since then, a wealth of research information has accumulated. A prebiotic may be defined as "*a selectively fermented*

ingredient that results in specific changes in the composition and/or activity of the gastrointestinal microbiota, thus conferring benefit(s) upon host health" (Gibson – *et al.*, 2011).

Today, over 60% of functional food products are directed towards digestive health, with prebiotics and probiotics probably being the most common, worldwide. Probiotics and prebiotics target the host through the gut by distinct as well as complementary mechanisms of actions.

This Concise Monograph will describe the concepts of probiotics and prebiotics for use in the human diet and will explore the scientific basis for potential human health benefits. In general, research to date indicates that these food ingredients offer possible health benefits and do not pose any risks to health. Indeed, a range of naturally occurring prebiotics and a number of probiotics, primarily from the genera *Lactobacillus*, *Bifidobacterium* and *Saccharomyces*, have long been consumed throughout the world either as part of traditional diets or in the form of modern functional foods.

ROLE OF THE GI TRACT MICROBIOTA IN HEALTH AND DISEASE

Microbiota of the GI tract

Bacteria are normal cohabitants with humans and are associated with many tissues including the skin, the vaginal tract, the respiratory tract and the GI tract. Microbes occur throughout the GI tract (Figure 1), the majority residing in the colon.

FIGURE 1.
The human gastrointestinal tract. CFU, colony-forming units. (Adapted from Sanders, 2007)

Mouth

Stomach
Sparsely colonized
(~10^3 CFU/g of contents)

Small intestine
Comprised (in order of flow through the intestine) of the duodenum, jejunum, and ileum. Microbial colonization is sparse in the duodenum, increasing until it reaches ~10^{6-7} CFU/g of contents in the distal ileum.

Large intestine (colon)
~1.5 m in length, richly colonized with a great diversity of microbes reaching 10^{11} CFU/g of contents

Rectum

Anus

The most common of the numerous bacteria harboured in the oral cavity are streptococci. Bacteria do not colonise the stomach in high numbers because of its low pH and rapid transit; nevertheless, in the healthy adult stomach there may be around 10^3 bacteria in every millilitre (ml) of stomach contents, the main inhabitants being lactobacilli, enterococci, *Helicobacter* and bacilli. The duodenum also tends to be acidic with a rapid transit but additionally receives pancreatic secretions and bile that create a hostile environment for microbes. Here, lactobacilli and streptococci predominate, with total numbers of bacteria at 10^2–10^4 per ml. Along the jejunum and particularly the ileum there is a gradual increase in the numbers and diversity of bacteria present. Finally, the colon contains the majority of GI microbes, with as many as 10^{11} organisms per ml.

Prior to birth, micro-organisms are absent from the GI tract but quickly colonise it during and after birth. Exactly which microbiota develops is dependent on factors such as the method of delivery and the environment in which birth takes place, the mother's microbiota and the manner of feeding. Bifidobacteria dominate the faecal microbiota of healthy breast-fed infants whereas healthy formula-fed infants have a wider range of organisms present, including bifidobacteria, bacteroidetes, clostridia, enterobacteria and streptococci. At weaning, there are changes in the numbers and diversity of the gut microbiota, which gradually begins to resemble those of the adult. Once the adult microbiota is established, by the age of about 2–3 years, it is relatively stable within an individual but nevertheless subject to influence by diet, disease, use of medication (particularly antibiotics) and ageing.

Gut microbes may be commensal (a person's native, colonising microbes) or transient (microbes just passing through). Furthermore, these microbes can be beneficial, potentially harmful or pathogenic. Microbes considered to be beneficial usually ferment carbohydrates, do not

produce toxins and may have a range of potential benefits for the host such as interaction with the immune system and competitive inhibition of pathogens. Such microbes include *Bifidobacterium, Eubacterium* and *Lactobacillus.*

The small intestine is the main target of many exogenous infections such as rotavirus, *Salmonella typhimurium* and some *Escherichia coli* types, which are usually contracted from contaminated water or foods. However, all individuals harbour microbes that have opportunistic, pathogenic potential. Amongst the most important of these is *Clostridium difficile*, which may become prominent and cause serious diarrhoea and inflammation when conditions in the gut are altered by illness or medication. *C. difficile* often becomes a transmittable pathogen through contamination of food or surfaces, especially in hospitals or care homes. Other undesirable colonic microbes such as peptolytic bacteria and sulphate-reducing bacteria do not cause acute disease but can be associated with the production of toxins, pre-carcinogens, carcinogens and toxic gases (such as hydrogen sulphide). In turn, this may result in the host becoming more susceptible to transient pathogens, antibiotic-associated diarrhoea and, possibly, inflammatory bowel disease and irritable bowel syndrome.

Probiotics are transient, although some may belong to species that are also normal commensal organisms. Some, but not all, probiotics are able to replicate and persist in the gut at least temporarily, but they disappear a few days after cessation of their intake.

Although recent research has provided a great deal of information about the overall composition of the gut microbiota, there is little certain knowledge about what constitutes the normal microbial composition (eubiosis) of the gut. In part, this is because it is difficult to study what is happening inside the GI tract of a healthy individual. Hence, there is no definition of the "normal" or "healthy" microbiota, although this is a key objective of current research. Individuals may have a reasonably stable microbiota but there is considerable inter-individual variation.

Deviations in composition or function from the usual microbiota, known as dysbiosis, have been observed in certain disease states (Table 1) but it is not known whether the change in the microbiota causes, or partly causes, the disease state or whether the change in microbes is a result of the disease itself. Changes in the microbiota can certainly result from a GI infection or use of oral antibiotics to treat a disease, but such alterations are usually quite rapidly corrected without intervention and the microbiota returns to "normal" for that individual. However, repeated antibiotic use may result in permanently disrupted microbiota. Whether or not prebiotics and probiotics can hasten or improve the correction of the microbiota following an insult is a subject of research.

TABLE 1. Disease states that have been associated with altered GI microbiota (adapted from Sanders, 2011)
Atopy (allergy) and asthma
Coeliac disease
Colon cancer
Type I diabetes
Type II diabetes
HIV infection
Inflammatory bowel disease (IBD)
Irritable bowel syndrome (IBS)
GI infections
Antibiotic-associated diarrhoea (AAD)
Necrotising enterocolitis
Obesity
Rheumatoid arthritis

Recent research also suggests that the normal microbiota is not simply a collection of micro-organisms, but reflects an inter-relationship between different groups that may work together to the benefit of the host. In addition, the current thinking is that harbouring a wide diversity of organisms in the GI tract is beneficial to the host.

Bacterial fermentation and metabolism

As living organisms, all microbes require a source of energy in order to grow and reproduce. Many microbes ferment carbohydrates (saccharolytic fermentation), an activity that is harnessed by humans in the production of various food products. For example, in wine production, yeast ferments the sugars in grape juice to yield alcohol. In yogurt production, bacteria such as lactobacilli and streptococci ferment milk sugar (lactose) to lactic acid to develop the characteristic tart flavour. In sauerkraut production, the bacteria naturally present in cabbage ferment sugars to lactic acid in the absence of oxygen and the presence of 2-3% salt.

In like manner, microbes in the first part of the colon meet their energy needs by fermenting dietary and endogenous residues that have escaped digestion and absorption in the upper GI tract (Table 2 and Figure 2). Many microbes

TABLE 2. Bacteria, their mode of action on substrates and the products of fermentation (adapted from Salminen, 1998)		
Bacteria	**Mode of action on substrates**	**Fermentation products**
Bacteroides	Saccharolytic, peptolytic, aa-fermenting	Ac, Pr, Su, Am
Eubacteria	Saccharolytic, some aa-fermenting species	Ac, Bu, La, Am, Sul
Bifidobacteria	Saccharolytic	Ac, La, f, EtOH
Ruminococci	Saccharolytic	Ac
Peptostreptococci	Saccharolytic, some aa-fermenting species	Ac, La, Am
Peptococci	aa-fermentation	Ac, Bu, La, Am
Clostridia	Saccharolytic, some aa-fermenting species	Ac, Pr, Bu, La, EtOH, Am, Sul
Lactobacilli	Saccharolytic	La
Propionibacteria	Saccaharolytic, lactate fermentation	Ac, Pr, Am
Actinomyces	Saccharolytic	Ac, Pr
Streptococci	Carbohydrate and aa-fermentation	La, Ac, Am, Sul
Methanobrevibacter	Chemolithotrophic	CH4
Escherichia	Carbohydrate and aa-fermentation	Mixed acids, Am
Desulfovibrio	Various	Ac, Sul
Fusobacteria	aa-fermentation, assimilation of carbohydrates	Bu, Ac, La, Am, Sul

aa, amino acid; Ac, acetate; Am, amines; Bu, butyrate; EtOH, ethanol; f, formate; La, lactate; Pr, propionate; Su, succinate; Sul, sulphides

FIGURE 2.

Diagram showing the principle metabolic activity in the colon
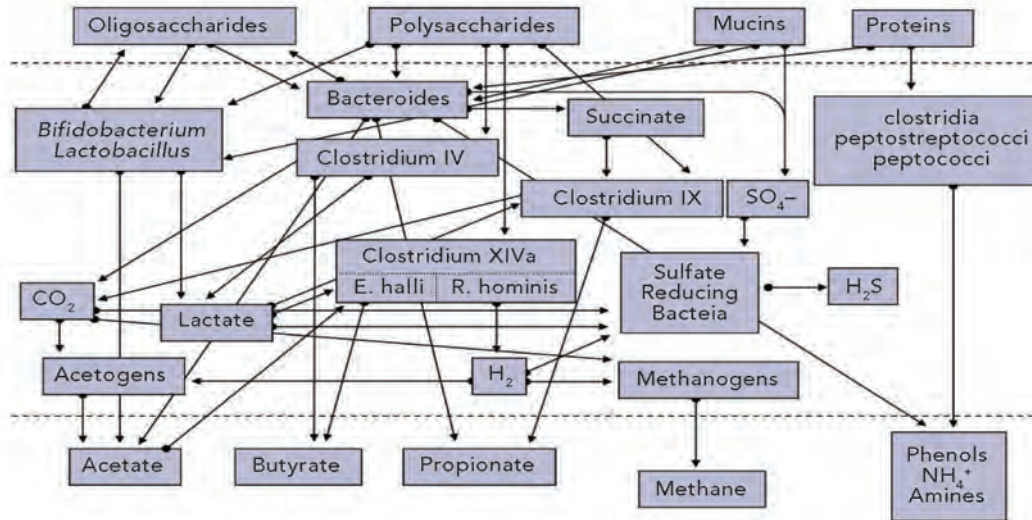
Source: Prof. R. Rastall, University of Reading, UK

metabolise carbohydrates and dietary fibre[1], including polysaccharides (such as pectins, hemicelluloses, gums, inulin and resistant starches), oligosaccharides (such as raffinose, stachyose, fructo-oligosaccharides, galacto-oligosaccharides and resistant dextrins), sugars (lactulose, non-absorbed lactose and non-absorbed fructose) and polyols (such as mannitol, lactitol, maltitol and isomalt). The main species in the colonic microbiota that ferment carbohydrates belong to the genera *Bacteroides, Bifidobacterium, Ruminococcus, Eubacterium* and *Lactobacillus*. This microbial action results in the production of the short chain fatty acids (SCFA) acetic, propionic and butyric acids and of lactic acid (which is mostly converted to acetic and propionic acid by gut microbes). The nature

of the fermentation products depends partly on the substrate fermented and the type of bacteria (Table 2) and also on other individual host factors. SCFA are absorbed, enhancing the uptake of water and salts, and are used as a source of energy by the host. Butyric acid is also the major source of energy of the epithelial cells lining the colon and can impact cell growth and differentiation.

The gases hydrogen, methane and carbon dioxide are also produced and may contribute to the equilibrium of the microbiota. In addition, these gases can cause flatulence and distension, which can lead to intestinal discomfort if the dietary intake of fermentable substrates is suddenly increased.

---

1. Note that the legal definition of dietary fibre differs around the world. The term dietary fibre is used here only in a general sense to refer to the dietary components listed.

Bacteria also metabolise other components found in their environment (Figure 2). In addition to foodstuffs consumed by the host and not fully digested, substrates for bacterial growth include degraded bacterial cells and host-derived mucins, enzymes and sloughed-off intestinal cells. Peptococci and clostridia species metabolise proteins as a source of nitrogen for growth and yield branched chain fatty acids such as isobutyrate and isovalerate as well as a range of nitrogenous and sulphur-containing compounds, some of which may be harmful. For example, ammonia, amines and phenolic compounds can, under certain conditions, lead to the formation of carcinogens, particularly in the left, descending colon where putrefactive conditions can prevail. Phytochemicals such as isoflavones and polyphenols may also be metabolised, yielding smaller components like equol and small phenolic molecules that are more readily absorbed. The impact of this microbial activity on human health is still under investigation.

As bacteria grow in numbers, they contribute to the bulk of the stools that form in the rectum. High stool bulk is related to a shorter gut transit time and also to a lower risk of constipation and bowel cancer. Although non-fermentable dietary fibre sources such as wheat bran fibre are the most important contributors to stool bulk, bacterial mass resulting from the fermentation of more soluble dietary fibres and carbohydrate residues also contributes to the bulk.

The GI epithelial barrier and immune system

The GI tract is sometimes described as the body's largest immune organ. It represents the host's greatest area of mucosal contact with the environment and contains as many as 80% of all antibody-producing cells. The intestinal microbiota is also a vital part of the body's defence system.

At birth, the GI tract is essentially sterile and, in addition, the newborn's immune system is not fully mature. The immune system only becomes functionally mature as a result of exposure to the myriad of foreign substances encountered by the naive intestinal tract. Studies on animals raised in germ-free conditions have shown that the immune system is poorly developed in such animals and that they have lower levels of immunoglobulins and fewer specialised immune cells in their intestinal mucosa. Germ-free animals are thus much more susceptible to disease than are those that are conventionally reared. It is also known from these studies that microbial antigens, derived from the intestinal microbiota as well as the environment, play a crucial role in the maturation of gut-associated lymphoid tissue (GALT) and normal resistance to disease.

The GALT is organised into different compartments such as lymph nodes, lymph follicles and Peyer's patches (Figure 3). The GALT limits the passage of bacteria and food antigens from the GI lumen through the intestinal mucosa. It does, however, allow the passage of antigens (minute samples of viable or dead bacteria and protein and peptide fragments) using specialised cells such as the M cells that cover the Peyer's patches and the dendritic cells that act as sentinels along the mucosa. These so-called antigen-presenting cells (APCs) process and present the antigens to lymphocytes, a type of immune cell. The APCs are thus very important in stimulating a balanced immune response and, as is increasingly documented, having an impact beyond the gut (see the section "Cross-talk with the host"). It has been hypothesised that reduced exposure to exogenous microbes in developed and industrialised countries has led to increased incidence of chronic immune dysfunction, leading to atopic (allergic) and auto-immune disorders or inflammatory bowel disease, because of changes in the way the immune system has matured. This is known as the "hygiene hypothesis".

FIGURE 3.

Schematic overview of the lymphoid elements of the gut-associated lymphatic system
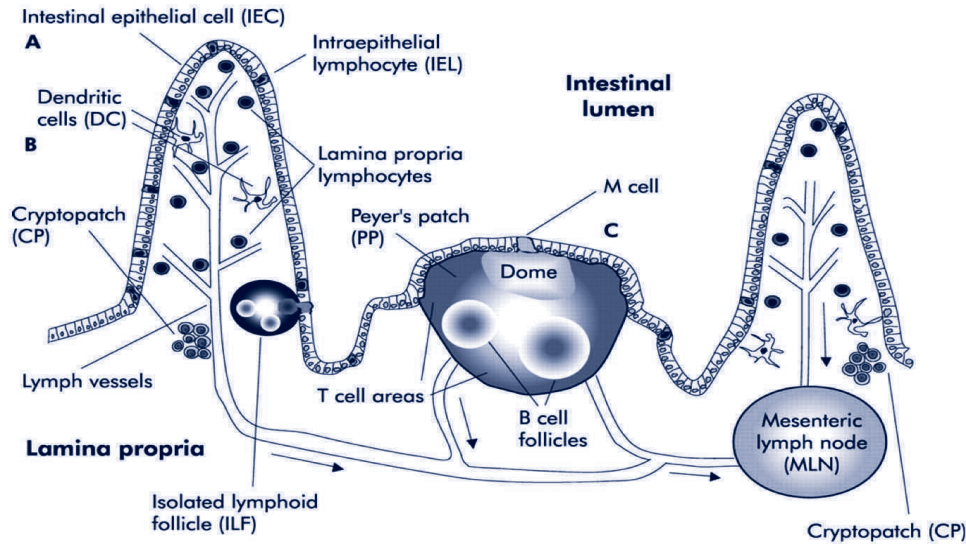
Peyer's patches (PP) and mesenteric lymph nodes (MLN) are organised intestinal lymphoid follicles. (A–C) Pathways of intestinal antigen uptake: luminal antigen can be taken up by (A) intestinal epithelial cells, (B) interdigitating lamina propria dendritic cells, and (C) M cells. The lymphatic drainage of PP and villus lamina propria goes to the MLNs (direction of lymph flow is indicated by arrows). Reproduced from Spahn and Kucharzik (2004) with permission from BMJ Publishing Group Ltd.

The integrity of the epithelial lining of the GI tract is crucial to health and the disruption of this intestinal barrier may increase the risk of certain intestinal disorders or diseases. Epithelial cells have become specialised and adopt a number of strategies for defence against pathogens.

Goblet cells secrete mucins (high molecular weight glycoproteins), which act as a layer that helps protect the underlying epithelial cells from mechanical damage and the direct action of chemical compounds that are ingested or derived endogenously from gut secretions. The amount and composition of mucus produced by the gut varies by site. The small intestine has a thick, quite mobile layer of mucus whereas the colon has two layers: a mobile layer similar to that of the small intestine and a second thinner layer that is much more viscous and impermeable than the mobile mucous layer. Although microbes reside predominantly in the lumen of the GI tract, they are also associated with the mucous layer and may adhere to the cells lining certain areas of the small intestine if the mucous layer is compromised. Here, beneficial microbes may compete with pathogens.

Both the mucous layer and the epithelial cells are designed to allow selected nutrients and other dietary components to penetrate and, in some cases, pass through them.

In addition, some components pass through the inter-cellular spaces. Proteins known as occludins and claudins help police the small intercellular space (tight junction) between cells to control access by foreign molecules and particles.

Specialised Paneth cells, located in the crypts of the small intestine, produce antibacterial peptides known as defensins as well as defensive enzymes (such as lysozyme) and cytokines that help protect the host from pathogenic micro-organisms.

Techniques for exploring the GI microbiota

In the past, microbes taken from their initial source (whether food, blood, tissue or excreta) were characterised after culturing them in a laboratory. The cultured micro-organisms could then be counted and identified by microscopy, biochemical observation and other taxonomical (identification) tests (see section "Characterisation and taxonomy" for information on taxonomy).

Faecal sampling has always been the mainstay of analysis of the human gut microbiota, especially given the limited accessibility of other GI sites. An inherent limitation of this approach is that the micro-organisms expelled in the faeces and cultured in the laboratory do not necessarily accurately reflect what can be found in different segments of the gut, particularly the upper gut. Even colonic biopsy samples may not accurately reflect the *in vivo* situation because, prior to their excision, the colon is cleared with laxatives, which disturbs the endogenous microbiota. Another challenge in understanding the composition of the gut microbiota is that numerous microbes have not yet been successfully cultivated under laboratory conditions.

In the early 1990s, research scientists developed a technique called fluorescence *in situ* hybridisation (FISH). By using fluorescent probes directed to highly variable regions of the 16S ribosomal ribonucleic acid (rRNA) within the bacterial cells, different species and even sub-species of bacteria could be identified and quantified. From the mid-1990s, sequence analysis of 16S ribosomal DNA, often obtained by polymerase chain reaction (PCR), was possible, which enabled microbiologists to detect and identify micro-organisms without the need to culture them. Simultaneously, sequence analysis revealed a far greater diversity than previously detected by culturing. These techniques have allowed more accurate detection and identification of specific species, especially ones that were previously unknown or difficult to culture, from faecal or intestinal samples. Culture-independent analysis of faecal samples has thus led to an increased understanding of the complexity of the intestinal microbiota. Modern techniques also allow very high numbers of samples to be analysed in parallel and thus have increased the knowledge of inter-individual variation and stability of the microbiota within individuals.

The co-development of high-throughput DNA sequencing technology and information technology (bio-informatics) has enabled clustering and analysis of large amounts of data such that researchers have embarked upon major new projects to study the human microbiome – a term that refers to the collective genomes of all micro-organisms present in an ecosystem, in this case, the human body. The Human Microbiome Project (USA-led) and the MetaHIT project (Europe-led) comprise large research consortia that have started to study and characterise the complete microbial population of the human intestine and other parts of the body, with the aim of associating the composition and function of the microbiome with health and disease. A great deal

of current research on probiotics and prebiotics also interfaces with these ongoing research programmes on commensal bacteria. All these projects will help to shed light on the role of microbes, both commensal and ingested, in human health.

Analysis of the intestinal microbiota has made tremendous progress, in particular in the past two decades with the mainstream use of various molecular techniques. These techniques have made it possible to both investigate the unknown members of the microbiota and their functionality as well as follow specific strains. A number of challenges remain, however. Analysis primarily remains restricted to faecal samples that may not be representative of the microbiota higher up the GI tract or the microbiota associated with the mucosal surfaces. On the analytical side, new techniques allow the accurate and quantitative analysis of the microbiota and, although the detection limits may currently still be too high to capture all the minor components of the intestinal microbiota, it is reasonable to assume this will improve in the future. More powerful computers and new statistical algorithms will also be required to deal with the ever-increasing amount of data.

THE PROBIOTIC CONCEPT

Definition and history

The word "probiotic" (origins: Latin *pro* meaning "for" and Greek *bios* meaning "life") was first used in 1954 to indicate substances that were required for a healthy life. Out of a number of definitions, the most widely used and accepted definition is that proposed by a joint FAO/WHO panel (FAO/WHO, 2001): *"Live micro-organisms which, when administered in adequate amounts, confer a health benefit on the host"*.

As mentioned, the original proposal that certain bacteria could benefit human health is usually attributed to Ilya Metchnikoff, who worked at the Pasteur Institute at the beginning of the twentieth century. His insights still have resonance today:

> *"The dependence of the intestinal microbes on the food makes it possible to adopt measures to modify the flora in our bodies and to replace the harmful microbes by useful microbes"* and *"systematic investigations should be made on the relation of gut microbes to precocious old age, and on the influence of diets which prevent intestinal putrefaction in prolonging life and maintaining the forces of the body."*

A French paediatrician, Henry Tissier, also published information at around the same time about his work on young children with diarrhoea. He found that their stools contained fewer unusual Y-shaped ("bifid") bacteria than did stools from their healthy peers and suggested that patients with diarrhoea could be treated with these "bifid" bacteria to help restore a healthy gut microbiota.

Until recently, high quality scientific research supporting the purported benefits of probiotics was limited, partly

because the complexity of the gut ecosystem was largely underestimated. In the last three decades, research has progressed and, with the application of molecular techniques, major advances have been made in the characterisation of specific probiotics as well as in our understanding of their mechanisms of action and health effects.

Selection of probiotic candidates

Beyond safety, the selection of a probiotic strain is driven primarily by its potential to confer a health benefit for humans. It is commonly considered that, for food applications, probiotics need to survive until they reach the part of the GI tract where they exert their intended effect. For example, to be active in the colon, probiotics must resist salivary enzymes, stomach acid, small intestinal secretions of bile and enzymes as well as the pH changes and chemical milieu of other foods and beverages they will encounter during their passage along the GI tract. In addition, they need to compete with the resident microbiota. Finally, a selected strain has to fulfil a number of technological requirements, such as culturability on a large scale, genetic stability and maintaining viability in a food product or supplement. Thus, the identification of suitable probiotic strains worthy of further study is a very complex and detailed process that can take substantial research effort.

The most commonly used probiotics in foods are species from the genera *Lactobacillus* and *Bifidobacterium*, but yeasts such as *Saccharomyces* spp. have also been used. There are a number of important steps required to characterise each strain.

Characterisation and taxonomy

The determination of genus, species and strain is essential for full characterisation of a microbe.

Taxonomy provides a first view of the organism's main physiological and metabolic properties, indicates whether there are any potential safety concerns, and allows discrimination between individual strains. Indeed, full characterisation of probiotics is a requirement for evaluation of a health claim in Europe.

FIGURE 4.

Code of Nomenclature, showing *Lactobacillus acidophilus* as an example

Life	
Domain	Bacteria
Kingdom	Eubacteria
Phylum	Firmicutes
Class	Bacilli
Order	Lactobacillales
Family	Lactobacillaceae
Genus	*Lactobacillus*
Species	*acidophilus*
Strain	Strain xx
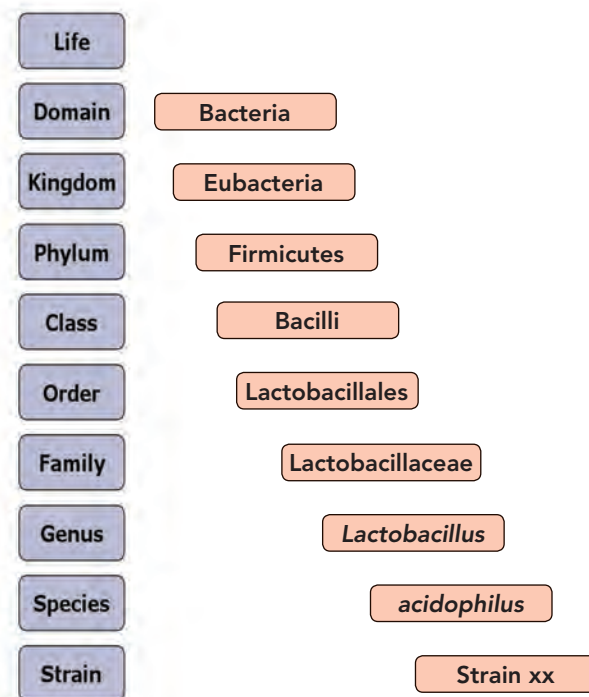
FIGURE 5.

Representation of genomic commonalities and differences for three strains belonging to the same species
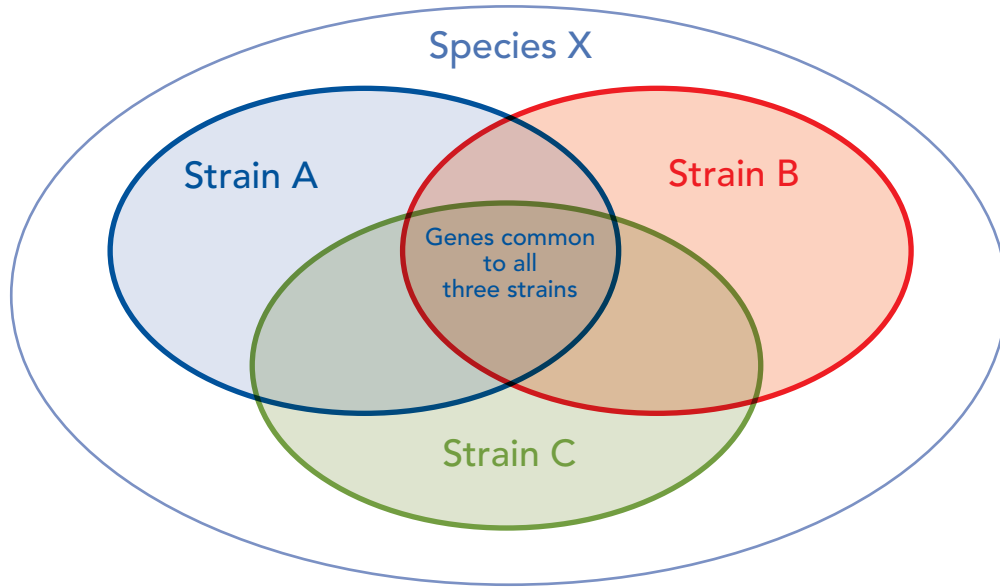
Modern molecular methods should be used for species and strain identification because they are far more reliable than phenotypic methods. Thanks to recent progress in technology, sequencing the full genome of a new strain is no longer very expensive or time-consuming and this opens the way for detailed characterisation of a specific strain and comparison with its close relatives. There is an International Code of Nomenclature that has to be followed in naming all micro-organisms (Figure 4).

Through assessing phenotypic and genotypic properties, microbial taxonomy groups together related species into one genus and, further, related strains into one species. Nevertheless, even when belonging to the same species, different strains can be distinguished by unique genetic and physiological properties (Figure 5).

Safety

Many probiotic organisms belong to genera represented in the functional group of bacteria known as lactic acid bacteria, which have been safely consumed for many years and as such are presumed to be safe ingredients in the food supply. To formalise and underwrite this concept, a system for a pre-market safety assessment was proposed that leads to a 'Qualified Presumption of Safety (QPS)' in the European Community. In summary, a safety assessment of selected groups of micro-organisms from a defined taxonomic group (e.g. genus or group of related species) can be made on the basis of four pillars of information (identity, body of knowledge, possible pathogenicity and end use). If the taxonomic group and characterisation to strain level do not raise safety concerns

or if any safety concerns can be defined and excluded, the organism may be granted QPS status. Thus, for any strain of micro-organism that can be unequivocally demonstrated to be from a qualified QPS group (such as *Lactobacillus* or *Bifidobacterium*), further safety assessment is limited to tests for antibiotic resistance. If a microbe is not covered by QPS, then a comprehensive assessment of safety is likely to be required before it can be used in the food supply.

Application of probiotics in food

Probiotic organisms are used in a variety of foods, the main category being dairy products, but they are also present as food supplements in capsule or tablet form. Since viability is an essential property of a probiotic, the final product must contain an adequate amount of living probiotic(s) until the end of its shelf life. A health claim for the addition of probiotics to foods or food supplements should only be made if there are documented benefits based on good quality human trials conducted with the relevant food product containing the specific strain that is the subject of the claim and using relevant endpoints. These studies should also be able to demonstrate the safe, effective dose of the probiotic organism in food. Like legislation on food safety, regulation of health claims for foods varies by country or region and any claims on commercial products containing probiotics must adhere to requirements, which in some cases include pre-market approval of the claim by the regulatory authorities.

THE PREBIOTIC CONCEPT

Definition and history

As mentioned, the Japanese were the first to recognise the value of fermentable oligosaccharides, initially in feeding piglets and later, during the 1980s, with the identification of human milk oligosaccharides. However, it was not until 1995 that the prebiotic concept for modulation of gut microbiota was introduced. Although a number of definitions have been proposed, there is as yet no full agreement on a single definition of a prebiotic. The most recent was agreed at the 2010 Meeting of the International Scientific Association of Probiotics and Prebiotics (ISAPP) (Gibson *et al.*, 2011):

> *"A dietary prebiotic is a selectively fermented ingredient that results in specific changes, in the composition and/or activity of the gastrointestinal microbiota, thus conferring benefit(s) upon host health."*

Characterisation of prebiotic ingredients

Although not stipulated as a requirement in the definition of a prebiotic, to date only carbohydrate compounds have been studied with regard to prebiotic activity. Most research has been carried out on fructans (i.e. the polysaccharide inulin or fructo-oligosaccharides (FOS) derived from various crops or from sucrose) and galacto-oligosaccharides (GOS). For these ingredients, selective fermentation and a shift in the microbiota have been confirmed in human studies and they have been linked to potential health benefits. Candidate or emerging prebiotics require additional evidence in humans before they can be fully established as prebiotic. Such candidate

prebiotics include the disaccharide lactulose, further oligosaccharides and resistant dextrins, polysaccharides such as polydextrose, arabinoxylans and resistant starches as well as some polyols such as lactitol and isomalt.

Some prebiotics occur naturally in foods such as chicory, cereals, agave and milk. However, most foods contain only trace levels, so the approach of refining the active ingredients from these foods crops or of producing them by synthesis (e.g. enzymatic, chemical or thermal processes) has been undertaken in order to attain levels in foods whereby a prebiotic effect may occur.

Many prebiotics and candidate prebiotics today fall into the nutritional and regulatory definition of dietary fibre and are labelled as nutrients of that category. They share with dietary fibre the properties of resistance to digestion and (for some fibres) fermentability, but established prebiotics are distinguished from dietary fibre by the selectivity of their fermentation. Note that mono- and disaccharides are typically not considered as dietary fibre according to EU and CODEX definitions.

Criteria for prebiotic selection

Prebiotics have an action complementary to, but distinct from, probiotics. Probiotics are exogenous micro-organisms that are ingested to promote a specific health effect. In contrast, the prebiotic concept is based on the selective stimulation of the host's own beneficial microbiota, the prebiotic being the substrate that is (selectively) fermented, stimulating the growth and activity of the particular micro-organism or group of micro-organisms of interest and thus leading to the desired health effect.

It is essential to measure the effect of the candidate prebiotic on bacterial growth; it is not enough simply to know that fermentation has taken place. Although *in vitro* tests can be used to screen potential candidates, the increase in target microbes must be quantified in human trials after a short feeding period at acceptable levels of intake in order to establish prebiotic status. Furthermore, human feeding trials are essential in order to demonstrate a health benefit.

The main site of action for prebiotics is the colon. Thus, a prebiotic should resist the effects of gastric acidity and digestive enzymes in order to reach the colon intact. Once there, prebiotics confer their purported benefits through the stimulation of the growth and/or the metabolic activities of the bacteria that ferment them. The foremost target genera for prebiotic action are bifidobacteria and lactobacilli, although this may change as knowledge of the microbial diversity and functionality expands. It can, however, not be excluded that prebiotics have a direct effect on health e.g. through the immune system or an impact on binding of microbes to receptors.

Prebiotics and probiotics may be combined into "synbiotics". In this case, the effects of the two components should be synergistic. The probiotic may be stimulated to grow in the gut by fermenting the prebiotic and/or the prebiotic may support a more favourable gut environment in which the probiotic may better compete.

Application of prebiotics in food

As noted above some prebiotics or candidate prebiotics are naturally occurring and widely consumed at low levels in the normal diet. The commercial prebiotic ingredients GOS and fructans are used in infant foods when their safety and efficacy has been demonstrated; in some countries this may require premarket approval. In foods for general consumption, the target level of intake of prebiotic ranges from 2 to 20 g per day, depending on the ingredient and the desired effect. These amounts can be readily incorporated into a variety of foods such as cereals, bread, confectionery, biscuits, yoghurts, table spreads, sauces and drinks. Similarly to the case of probiotics, the health benefits of candidate prebiotics need to be demonstrated in clinical trials.

HEALTH EFFECTS OF PREBIOTICS AND PROBIOTICS

Research challenges

In order to demonstrate that probiotics and prebiotics have beneficial effects on human health, evidence should be provided by nutritional intervention studies in human subjects. Supportive evidence may be gathered from animal feeding studies (*in vivo* studies) as well as from laboratory studies that examine blood or tissue samples taken from humans or animals (ex vivo studies), or by examining isolated cells that are grown in culture in the laboratory and subject to various experimental conditions (*in vitro* studies). These non-human studies can provide insights into mechanisms of action, but are not suitable *per se* to substantiate a human health benefit.

One of the factors that has hampered progress in research into the health impact of functional foods, including probiotics and prebiotics, is the lack of generally accepted biomarkers of GI health and immune function. In this context, biomarkers are surrogate markers of health endpoints just as blood cholesterol level is a well-accepted risk factor of disease. Accepted markers of GI function include stool bulk and the transit time through the GI tract, and these can be used to demonstrate the benefit of prebiotics and probiotics. There are numerous markers used in relation to the immune system but knowledge is lacking about the predictive value of single markers of function, such as immune cell function, cytokine levels, or antibody production, in overall immune health. The relevance of these immune markers remains to be established, even when more than one marker is used. The absence of agreed markers means that clinical endpoints such as reduced susceptibility to an infection, enhanced response to a vaccine or reduction in the duration of validated symptoms are still more widely accepted as evidence of benefit than are changes in a biomarker.

Another challenge that is common to all research in humans is inter-individual variation, i.e. the variability in results observed for a specific endpoint in different subjects. Inter-individual variability depends on a wide range of factors including host genetics, diet, microbiota, age, nutritional status and other lifestyle factors. Researchers try to control for these differences but must include sufficient subject numbers to allow for variation. In addition, the effects of an intervention may be more evident in people at high risk of, or diagnosed with, a disease than they are in healthy subjects. This often poses a question as to whether the effect would be observed in healthy people.

In all cases, it is clear that prebiotics and probiotics must be consumed regularly in order to confer a benefit.

Impact on the GI tract of prebiotics and probiotics

Gut microbiota

An increased proportion of bifidobacteria and lactobacilli is thought to represent a "healthier" microbial composition. This is partly based on evidence from infants, which is discussed later in this section as well as in the section on mechanisms. These bacteria are more likely to ferment carbohydrates and produce acids, and they generally lack potential toxicity.

There is ample evidence in human subjects, including infants, as well as in animal and *in vitro* studies that established prebiotics increase the proportion of bifidobacteria and sometimes lactobacilli present in the gut microbiota while having no measurable effects on other groups of bacteria.

In the case of probiotics, the consumption of adequate doses of *Lactobacillus* strains often results in a measurable increase in the lactobacilli in the faeces, and in some cases there may be a decrease in unfavourable organisms such as staphylococci. For pre-term infants, who usually harbour reduced numbers of bifidobacteria, there is good evidence that the ingestion of bifidobacteria not only increases their numbers but may also reduce the numbers of clostridia. In practice, the effect of prebiotics and probiotics on the microbiota is somewhat variable but also difficult to measure because of the factors discussed in the section "Techniques for exploring the GI microbiota".

In addition to considering an increase in the number or proportion of certain microbes, it is also important to consider their functional capacity, which may be changed by prebiotic or probiotic consumption in the absence of an alteration in number or proportion. Recent human data on probiotics using new techniques have enabled measures of components that reflect the genes that are being actively expressed at any given time. The link between gene expression and health outcomes will no doubt be the subject of future research.

Transit time and stool bulking

There is strong evidence that prebiotics and probiotics can influence gut function. This effect for prebiotics is thought to be due to their fermentation in the colon, resulting in increased bacterial mass and osmotic water-binding capacity that contribute to increased stool weight, increased stool frequency and softer stools. There is also some evidence that SCFA, especially butyrate, have a positive effect on the endothelium and on peristalsis, which improves transit. Because there is an inverse link between stool mass and transit time, prebiotics may also decrease transit time. In some studies, prebiotics are reported to reduce symptoms of

intestinal discomfort, such as bloating, abdominal pain and flatulence. Studies on certain strains of probiotic bacteria have demonstrated an impact on gut function, as revealed by normalisation of transit time and reduction of self-reported minor digestive discomfort symptoms. An improved transit time may reduce putrefactive activity in the left colon, as indicated by some studies that have found reduced levels of polyamines and metabolites such as cresol and indoles.

These stool-regulating effects are considered to be beneficial to gut health by decreasing the risk of constipation. An improvement of stool function is likely to be important with respect to the general population since dietary fibre intakes in developed countries are almost universally lower than recommended and the number of people reporting digestive problems is extremely high (more than 80% in some surveys of women). As with all dietary fibre, too-high an intake of prebiotics may need to be avoided by certain individuals because over-consumption could lead to bloating and, in severe cases, to watery stools. However, this subsides if consumption is reduced or stopped.

Chronic inflammatory gut conditions

The inflammatory bowel diseases (IBD) are serious conditions with an as-yet unknown cause. They include Crohn's disease (CD), which can affect the whole gut though mainly affects the small intestine, and ulcerative colitis (UC), which is usually restricted to the large bowel. IBD is associated with a breakdown of the normal barrier function provided by the gut epithelial lining and its associated mucus. Whether the inflammation causes the breakdown of the barrier or if a breakdown of the barrier allows inflammation to develop is not clear. It is known from studies on germ-free animals compared to normal animals that germ-free animals are less susceptible to experimental IBD and that the presence of commensal

bacteria can initiate and/or exacerbate inflammatory bowel conditions. CD and UC may thus result from an inappropriate mucosal immune response to the GI microbiota in genetically susceptible individuals. There is also some evidence from clinical studies that the balance of different groups of commensal bacteria might be altered in IBD patients.

Numerous studies of both probiotics and prebiotics in animal models have shown a positive impact on the prevention or treatment of IBD. Clinical studies in CD subjects have not been effective in prolonging remission of CD, but there are promising data indicating that some probiotics are useful in maintaining remission in UC. In another inflammatory bowel condition known as pouchitis, which can occur after surgery to treat UC, one mixture of probiotic strains appeared to be effective in helping maintain remission. The potential for prebiotics and synbiotics to help the management of IBD has been shown in several small studies with fructans, mainly in the reduction of inflammatory markers, but as yet the data do not allow a final conclusion. Although there are still insufficient data to draw firm conclusions on the effect of pre- or probiotics on IBD, importantly, none of the trials conducted thus far have raised concerns regarding their safety in patients with IBD at the levels of intake tested.

Irritable bowel syndrome

Irritable bowel syndrome (IBS) is a distressing condition that is characterised by an array of symptoms such as abdominal pain, bloating and altered bowel habits that may often alternate between constipation and diarrhoea. As similar symptoms can be observed from time to time in the general population, a specific set of criteria (known as the Rome criteria) was developed to improve consistency of diagnosis of IBS. In industrialised countries, IBS affects between about 5 and 20% of the adult population, with rates higher in women and older people. Recently, there has been interest in the role of inflammatory processes as

a potential cause of IBS. In addition, in a certain subset of subjects, it appears that previous gut infections play a role in onset of IBS (post-infectious IBS). Furthermore, in some studies, lower levels of bifidobacteria have been observed in subjects with IBS compared with healthy subjects.

Because of the lack of good therapy for IBS and the identification of abnormal microbiota in IBS subjects, both probiotics and prebiotics have been investigated for their ability to help subjects manage this condition. A couple of probiotic preparations have been shown to provide reduction in a global symptom score (the sum of a number of different symptom scores) and in reducing abdominal pain; however, no change in diarrhoea, constipation or bloating was confirmed. In other studies, some strains had no effect or resulted in worsening of symptoms. For some prebiotics, studies showed that low doses led to an improvement in the condition, whereas a larger load led to an enhancement of the perceived symptoms. Thus, additional research will be required to determine if consistent benefits can be observed by those experiencing IBS if they use prebiotics and probiotics.

Impact on the GI tract specific to prebiotics

Colon cancer

Colon cancer has been linked to diets low in dietary fibre and thus the potential for prebiotics to reduce colon cancer risk has also been investigated, mainly using *in vitro* techniques and animal models. Results from animal studies with endpoints such as DNA damage, aberrant crypt foci as well as tumours in the colon suggest that prebiotics may reduce the risk of colon cancer. This is supported by ample *in vitro* evidence. Synbiotics were investigated in a few animal studies and were found to be more effective than either prebiotics or probiotics alone. One synbiotic study (SYNCAN

project) in humans found a reduction in DNA damage and a reduction in cell proliferation in colon biopsies. Potential mechanisms for a prebiotic effect on colon cancer risk have been identified in animal studies and include changes in gut bacterial enzyme activities, which modify the fermentation products, and up-regulation of apoptosis (programmed cell death – in this case of the pre-cancerous cells). However, definitive evidence that certain prebiotics might reduce the risk of colon cancer in human subjects is lacking and requires more robust, multi-centre, prospective human trials.

Prebiotics in early life nutrition

Oligosaccharides with fucosyl, galactosyl and sialyl structures are found in human breast milk and are thought to promote healthy microbiota. Intervention studies show that infant formula supplemented with GOS and fructans, alone or in combination, help stimulate the bifidobacteria that are characteristic of breast-fed infants in a dose-dependent manner. Further, infants fed formula with oligosaccharides have microbiota, a stool pH and a SCFA pattern similar to those of breast-fed infants. The stool consistency and stool frequency of prebiotic-fed infants (softer and more frequent) is also closer to that seen for breast-fed infants than for those fed standard formula. The use of specific GOS and fructan prebiotics in infant formula is widespread practice and accepted as safe. The range of the resulting benefits of these prebiotics as well as of other prebiotic candidates is still an active area of research by experts in the field.

Mineral absorption

One specific, well-established effect of prebiotics is on mineral absorption. There is a wealth of data showing that prebiotics increase calcium absorption and increase growth and skeletal mass in rats. In addition, there are some studies showing enhanced absorption of magnesium and iron. Further evidence for an improved mineral absorption is available from pigs, which are considered a better model for the human than are rodents. Numerous human intervention studies consistently show an increase in calcium absorption. So far, there is one long-term human intervention study that assessed the effects of prebiotics on bone health. The study was in adolescents and used a combination of FOS and long-chain inulin (50/50). After one year, bone mineral density and bone mineral content were significantly higher at certain bone sites in the supplemented group. Whether this effect is common to all prebiotics or unique to the particular formulation requires further substance-specific research.

Gut hormones and food intake

Numerous studies in rodents, mainly with fructans, show a consistent effect of feeding prebiotic fibres in reducing food intake and decreasing fat mass, though not necessarily body weight. Additional data from these studies suggests that the mechanism for this is likely to be SCFA-stimulated secretion of gut peptides such as glucagon-like peptide (GLP)-1, peptide YY (PYY) and oxytomodulin and reduced secretion of ghrelin, all of which are secreted by endocrine-type cells in the mucosa. These peptides are known to affect food intake in animals and humans. Overall, evidence from an increasing number of studies in human subjects, mainly with fructans, supports an effect of daily prebiotic consumption in reducing appetite, lowering body weight or fat mass, altering gut peptide levels in blood and improving glucose tolerance. Some, but not all, of these studies examined the composition of the gut microbiota; where examined, shifts in the microbiota were confirmed. The impact of SCFA on glucose and lipid metabolism may also be important.

Impact on the GI tract specific to probiotics

Lactose malabsorption

As discussed in the section on "Bacterial fermentation and metabolism", many micro-organisms ferment lactose, the sugar found in milk and products made from milk. Although infants rely on lactose, which contributes as much as 10% of the energy in breast milk, many populations around the world have a high proportion of adults who are unable to digest this sugar. In humans, and in fact in all mammals, expression of the enzyme lactase is down-regulated in adulthood with the exception of some population groups, particularly those of European origin. Lactose intolerance is a condition in which the colonic fermentation of undigested lactose results in gastrointestinal effects such as abdominal pain, bloating, borborygmi or laxation. There is evidence that the live bacteria of yogurt are able to compensate for the lack of endogenous lactase in the human gut by digesting lactose. The typical measure of improved lactose digestion is a reduction in breath hydrogen excretion (breath hydrogen is usually raised when undigested carbohydrate reaches the colon and is fermented). This improved digestibility reduces the symptoms related to lactose intolerance in some lactose malabsorbers.

Impact on the immune responses

Germ-free animals have, as mentioned, an underdeveloped immune system and GI epithelium, resulting in reduced resistance to infection compared with conventional animals. It is thus accepted that commensal organisms are vital for the maturation of the immune system. The potential for probiotics and prebiotics to impact immune responses and to reduce the risk of infections has been the subject of a number of human studies (discussed below). Such results, combined with evidence from mechanistic studies showing changes in certain immune parameters, support the notion that the effect of probiotics and prebiotics on the immune system can translate into measurable health benefits, but definitive evidence is lacking.

Gastrointestinal infection

The small intestine is the main target of many GI infections such as rotavirus, *S. typhimurium* and some *E. coli* types. As early as 1916, it was reported that *S. typhimurium* was cleared from the GI tract of healthy carriers of the organism when members of the normal gut microbiota were introduced. Probiotics have long been associated with a purported ability to counteract pathogenic bacteria and so recently several potentially beneficial strains have been tested in controlled studies.

The first-line of treatment for the symptoms of diarrhoea is oral rehydration – and no other dietary treatment should be substituted for this, especially in infants. However, in established conditions, some probiotics can be used as an adjunct under medical supervision where appropriate. Certain probiotics seem to be most effective in improving symptoms when the diarrhoea is the result of a viral (rather than bacterial) infection, if they are used early in the course of the infection and are given in sufficient amounts. In terms of reduced susceptibility to infection, some studies have found decreases in the risk of infection in infants (mainly in developing countries) and in institutionalised or hospitalised elderly. Efficacy is clearly strain related, i.e. some strains are effective and others not. In addition, there is some evidence that specific probiotic strains, and some prebiotics, may reduce the risk of traveller's diarrhoea.

Some antibiotics can significantly disrupt commensal bacteria, resulting in side effects such as antibiotic-associated diarrhoea (AAD). The estimated incidence of AAD is as high as 25% for some antibiotics and this can lead to patients failing to complete the course of

treatment. There is evidence that specific probiotics can reduce the risk of AAD and, indeed, several meta-analyses conclude that there may be as much as a halving of the risk of AAD in adults or the elderly, although the effect is less consistent in children. The observed effects relate to a limited number of specific probiotic strains. In the case of prebiotics, it has been shown that FOS administration following an antibiotic treatment reduced the re-occurrence of AAD from more than 30% in the control group to less than 10% in the prebiotic group. As this was not associated with a decrease in subjects testing positive for *C. difficile*, this could suggest that the prebiotic had a stabilising effect on the microbiota, supporting a return of eubiosis.

Clostridium difficile infection is a frequent cause of diarrhoea in institutionalised populations, for example in hospitals and in long-term care homes. It is often associated with antibiotic use but it can occur as a result of other risk factors such as age greater than 65 years or a compromised immune system owing to illness, medication or GI surgery. So far, the results of research to investigate whether probiotics can reduce the risk of *C. difficile* infection or reduce the severity or duration of symptoms in adults are promising but further confirmatory studies are needed.

A bacterium known as *Helicobacter pylori* is present in the stomach of a small proportion of young adults but in as many as 50% of those aged 60 years and over. It colonises the mucous layer next to the gastric epithelium and in some people can cause acute gastritis (i.e. pain, bloating, nausea and vomiting) and can lead to chronic gastritis and peptic ulcers. Treatment involves long-term administration of strong antibiotics and although probiotics do not speed the eradication of *H. pylori,* some have been shown in several studies to reduce the side effects of treatment and may result in less disturbance of the microbiota.

The microbiota in pre-term infants is restricted and differs in composition from those in healthy, full-term infants. In particular, potentially beneficial bifidobacteria are not well established in the pre-term neonatal gut. The microbiota is further challenged by environmental bacteria from the hospital milieu and the common use of antibiotics in pre-term infants, putting this population at increased risk of necrotising enterocolitis (NEC). Although the use of probiotics is not yet established in clinical practice, several trials have shown that specific probiotic strains can reduce the risk of NEC. Additional studies are needed to clarify the preferred strain and dose recommendations. Furthermore, the use of live microbes in such a susceptible population makes confirmation of safety for this use a prime objective.

Other infections

There has been a number of studies on various age groups to investigate the potential for probiotics to impact the susceptibility to upper respiratory tract infection (URTI) and its duration and symptoms. Studies were conducted with a range of different strains; some strains reduced incidence, some reduced duration and most had effects on symptoms. The evidence is promising, but the range of strains and the variation in age groups and study design prevent any firm conclusions. Evidence for an effect of prebiotics is limited to a recent, large, long-term study in which infants consuming formula supplemented with a specific GOS/long-chain FOS combination were less prone to upper URTI and associated fever than were those infants fed formula without a prebiotic.

There has also been interest in the use of probiotics in urogenital medicine. Certain probiotic strains have been shown to improve recovery from bacterial vaginosis during antibiotic treatment. Potential mechanisms for the effect include anti-microbial antagonism, restoration of balanced microbiota or an enhanced immune response.

Vaccinations

Animal studies have convincingly demonstrated that certain probiotic strains can both enhance the immune response to a vaccine and reduce the risk of subsequent infection. Human studies are much fewer, but an increasing number of well-controlled trials are being conducted. Preliminary evidence indicates that the response to vaccines against influenza, cholera or childhood diseases can be enhanced by selected probiotics, as measured by the number of subjects who respond to the vaccine or an increase in the level of serum immunoglobulins. The effects are strain-specific in terms of efficacy of the probiotics and, in the case of influenza, specific to the pathogen strains. In one study, there was limited evidence that subsequent risk of infection with the influenza virus was reduced. In the case of prebiotics, although evidence in animals seemed promising, clinical studies have not yet been supportive of an effect.

Allergic conditions

Allergy can be defined in simple terms as an inappropriate immune reaction or over-reaction to an otherwise harmless foreign antigen (mostly proteins or peptides). In medical terms, it is described as a hypersensitivity reaction mediated by specific antibodies (IgE) or cell-based mechanisms. Common allergies include reactions to certain food proteins (e.g. milk, eggs, peanuts, tree nuts, soy, wheat gluten, fish, shellfish and shrimp) or to environmental allergens such as pollen (hay fever), house dust mites and pet hair. Food allergies are more common in infants and children than in adults. The most serious form of allergy resulting in anaphylaxis (which can be fatal when the throat and respiratory tract swell and restrict breathing) is rare, albeit a lifelong concern. Less severe symptoms of allergies are more common (prevalence is about 2% for food allergies and up to 30% for respiratory allergies) and can substantially reduce the quality of life for allergic subjects.

As noted, the prevalence of allergy has increased in westernised societies. There is growing evidence that the nature of microbiota acquired by the infant in the postnatal period has an important bearing on maturation of the immune system. There is some indication that atopic children tend to have a degree of dysbiosis, with more clostridia and fewer bifidobacteria than non-atopic individuals. In addition, data suggests that breast-fed infants are less prone to allergic conditions. It has thus been suggested that prebiotics may help reduce the risk of developing atopy or reduce the associated symptoms of atopic eczema or allergic rhinitis. There is promising evidence, based on a follow-up of one intervention, that not only can prebiotic-supplemented infant formula reduce susceptibility to atopy but that the benefits persist up to 2 years of age. Furthermore, studies in infants at high risk of allergy who were fed supplemented formulas for 6 months had reduced levels of IgE and some IgG types.

There have been several studies on the impact of probiotics on the development of allergic symptoms in infants at high risk of developing atopic disease. In most of these studies, the mother consumed the probiotic prior to birth and the infant was administered the probiotic after birth. Results showed a decreased risk of eczema at 2 years of age and beyond. Overall, results of the studies point towards strain-specificity and also hint towards two separate windows of opportunity: first, the maternal consumption of probiotics during the perinatal period and, second, the use of probiotics during weaning. Past and ongoing studies have also targeted the management or reduction of allergic symptoms such as those linked to atopic eczema or allergic rhinitis; results are promising but not yet conclusive. This probably reflects the complexity of the allergic diseases spectrum and the fact that a range of different clinical designs was used.

PROBIOTICS AND PREBIOTICS: MECHANISMS OF ACTION

Overall mechanism

Both probiotics and prebiotics are thought to work largely through direct or indirect effects on the gut microbiota and environment and/or on host function. In the case of probiotics, a live micro-organism is consumed, in a range of dosages, spanning from ~10^8 to 10^{12} cells/day, depending on the product. This large number of microbes has the potential for a greater impact in the upper GI tract where lower densities of micro-organisms are found, but is also thought to impact the colon. Prebiotics enhance the growth of the endogenous microbiota or possibly stimulate the growth of probiotics when provided concurrently. Thus, probiotics and prebiotics share many common mechanisms of action mediated through an impact of microbes on the host and these are discussed below. In the case of health effects that relate only to prebiotics or only to probiotics, the mechanisms are less well known and have been alluded to in the section on health effects.

Probiotics and prebiotics (via their stimulation of commensal organisms) act on and interact with the host by two main modes of action, or a combination of actions (Figure 6 – see page 24):

- Impact of micro-organisms or their metabolites/enzymes on the host's GI tract and its microbiota
- Interaction with the host's cells and immune system

GI tract and its microbiota

As noted, bifidobacteria and lactobacilli in the colon preferentially ferment carbohydrates that escape digestion in the upper GI tract, resulting in a reduced pH of the colon. Bifidobacteria can ferment fructans because they have an enzyme, β-fructofuranosidase, that other bacteria either lack or have present at a lower activity, thus giving them a competitive advantage when exposed to fructans in the human gut. Similarly, the presence of β-galactosidase in lactobacilli or streptococci exerts a competitive advantage in GOS fermentation. The metabolism of prebiotic fructans by bifidobacteria yields mainly the acidic compounds acetate and lactate. Cross-feeding of these fermentation products to other species gives rise to butyrate and propionate. Butyrate and propionate are also formed from the direct fermentation of other dietary carbohydrates.

The benefits of a lower pH in the colon are that it encourages the multiplication and survival of commensal organisms that prefer acidic conditions and generally inhibits the ability of some pathogens to adhere, grow, translocate across the epithelium or colonise the GI tract. Furthermore, butyrate has long been known, from *in vitro* studies on fermentable dietary fibres, to enhance mucosal cell differentiation and this may also promote the barrier function of the epithelium.

Saccharolytic fermentation concomitantly reduces the potentially adverse effects of protein fermentation and other processes, which give rise to nitrogen and sulphur-containing compounds such as ammonia, *N*-nitroso- and azo- compounds as well as sulphides.

Many bacteria produce bacteriocins, which are peptides or proteins that are intended to reduce the survival of competing organisms. Bacteriocins produced by probiotic bacteria have been observed in *in vitro* studies to decrease the ability of pathogens such as *E. coli* O157:H7 to adhere to and invade cultured intestinal cells. Bacteriocin

FIGURE 6.

Diagram illustrating potential or known mechanisms of probiotics action
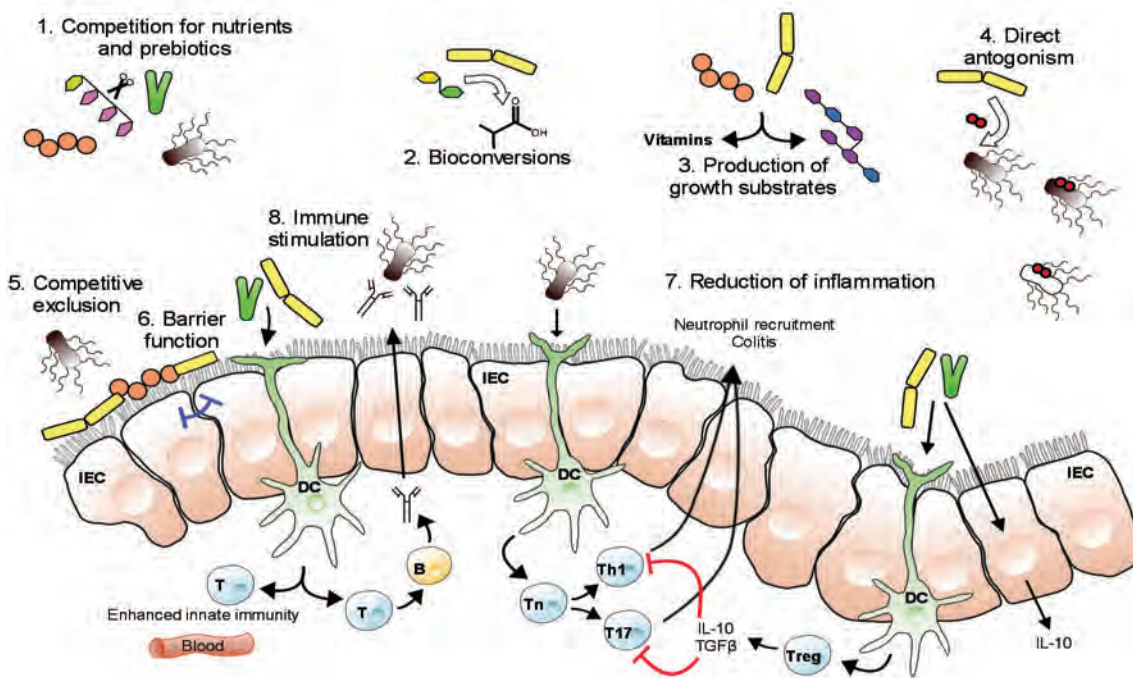
These mechanisms include (1) competition for dietary ingredients as growth substrates, (2) bioconversion of, for example, sugars into fermentation products with inhibitory properties, (3) production of growth substrates, for example, EPS or vitamins, for other bacteria, (4) direct antagonism by bacteriocins, (5) competitive exclusion for binding sites, (6) improved barrier function, (7) reduction of inflammation, thus altering intestinal properties for colonisation and persistence within, and (8) stimulation of innate immune response (by unknown mechanisms). IEC, epithelial cells; DC, dendritic cells; T, T cells. Source: O'Toole and Cooney (2008)

production following prebiotic administration has also been reported. This may be one of the mechanisms by which probiotics and prebiotics decrease the infection rate in humans and animals and increase the survival of mice in studies where a lethal challenge with a pathogen is performed. Additional supporting evidence for this mechanism comes from studies using probiotic bacteria modified in such a way that they can no longer produce bacteriocins. In this case, such organisms lose their ability to prevent adherence and translocation of pathogens during *in vitro* studies and/or to reduce infection rates/survival in infected animals. In addition, probiotics have been shown *in vitro* to alter the gene expression of certain pathogens thereby reducing their virulence.

Some probiotics may improve the barrier function of the mucus layer or epithelial cells. Evidence from cell culture

studies suggests that an increase in the production of mucins may result from an enhancement of gene expression in the mucus-producing Goblet cells that line the GI tract. Increasing the mucous layer helps protect the epithelial cells from potential pathogen translocation and may enhance the clearance of pathogens from the GI tract.

Probiotics may also enhance the ability of specialised Paneth cells in the small intestine to produce the antibacterial peptides known as defensins. This hypothesis is supported by *in vitro* studies, using intestinal epithelial (e.g. Caco-2) cells grown in tissue culture, that have shown that certain probiotics can stimulate human β-defensin mRNA expression and peptide secretion.

In vitro studies suggest that probiotics and prebiotics may affect the barrier function of the epithelium itself by enhancing the resistance of tight junctions, possibly via an effect on tight junction proteins (e.g. occludins and claudins). Increased expression of genes encoding tight junction proteins has recently been shown in a study conducted in human volunteers administered a specific *Lactobacillus* strain.

Animal and *in vitro* studies have found that specific probiotics can compete with pathogens for receptor sites on epithelial cells or in the mucous layer, thereby preventing pathogens from adhering or translocating. In contrast, other probiotics may directly bind to the pathogen, thus reducing its ability to colonise the intestine. There is good evidence from studies on mice that feeding on certain probiotic strains can greatly reduce the ability of pathogens such as *S. typhimurium* and pathogenic *E. coli* to translocate and invade the liver and spleen. *In vitro* studies showed that the same strains compete with the ability of pathogens to adhere to cells. Influence on pathogen translocation in infected animal models has also been shown for some prebiotics.

Cross-talk with the host

The most complex of the postulated mechanisms by which probiotics and stimulated endogenous microbes may act is the interaction with the GI immune cells and lymphoid tissue to modulate the immune and inflammatory responses of the host, which might lead to the potential for an impact beyond the gut (Figure 7 – see page 26).

The mammalian immune system is generally considered to consist of two major arms: the innate (or non-specific immediate) immune response and the acquired (or specific adaptive) immune response. Both parts of the immune system are extremely complex and involve cells (cellular immunity) and other components secreted into the blood (e.g. antibodies and cytokines). The two arms work together to protect the host from pathogens (bacteria, viruses, fungi), other foreign materials (antigens) and also from tumour cells arising in the host. For more information, see the ILSI Europe Concise Monograph on Nutrition and Immunity in Man (ILSI, 2011).

Through so-called bacterial–epithelial cell "cross-talk", it seems that ingested and endogenous microbes can impact both the innate and the adaptive responses of the host immune system. The interaction between microbial cells (commensal, probiotic or pathogen) and host cells is mediated by the interaction with specific receptors such as Toll-like receptors (TLR) that are associated with cells lining the mammalian GI tract. The activation of these receptors initiates a cascade of concerted immune signals leading to different responses. For example, the response can ensure a balanced maturation of T cells (Th1 versus Th2) and T-regulatory cells, which allows an appropriate response to potential pathogens and food antigens. An inappropriate T cell response is thought to be one of the features of allergic conditions, as mentioned previously. Further, activation of the immune pathways can also result in B cell differentiation and production of protective antibodies, such as IgA,

FIGURE 7.

The three levels of action of a probiotic
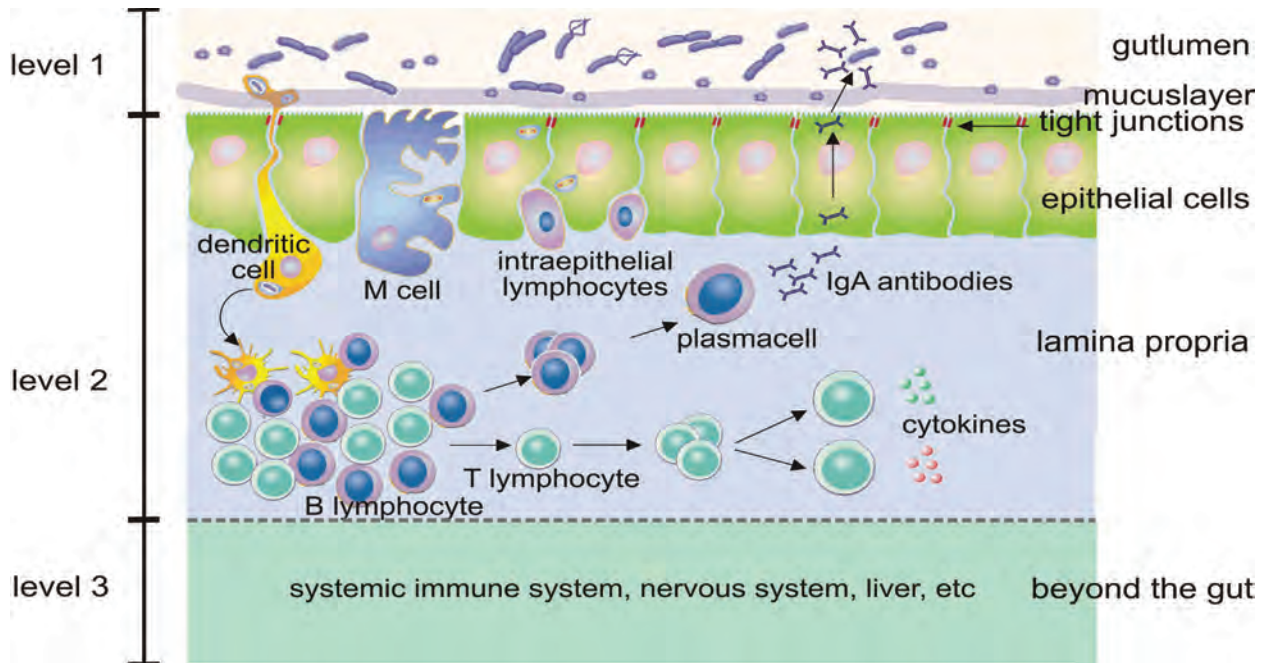
Probiotic bacteria can interfere with the growth or survival of pathogenic micro-organisms in the gut lumen (level 1). Probiotic bacteria can improve the mucosal barrier function and mucosal immune system (level 2) and, beyond the gut, have an effect on the systemic immune system, as well as other cell and organ systems such as liver and brain (level 3). Source: Rijkers (2010)

secreted into the intestinal lumen. Along the same lines, the ingestion of specific probiotic strains or prebiotics in human and animal studies has been found to stimulate an increase in the anti-inflammatory cytokines, such as IL-10 and TGF-β, and a decrease in the expression of pro-inflammatory cytokines, such as TNF-α and IFN-γ. It is proposed that these changes in cytokine balance could be a mechanism by which prebiotics and probiotics may be able to mitigate chronic intestinal inflammation.

The activity of phagocytic cells (neutrophils and macrophages) and natural killer (NK) cells (non-T non-B lymphocytes), which are part of the innate immune response, is also modulated in animals and humans by various probiotics and to some extent by prebiotics or synbiotics. In addition, animal studies have suggested that the so-called G-protein receptors in certain white blood cells may act as receptors for SCFA, increased levels of which result from the ingestion of prebiotics,

thus opening up the possibility of alternative mechanisms impacting the immune system.

Although studies in humans have found changes in biomarkers such as cytokine levels and changes in the number and activity of immune cells, it is nevertheless of prime importance to have studies in human subjects that also measure clinical outcomes. Clinical measures, such as a reduced incidence of infection or enhanced immune response to a vaccine, can then be linked to measures of humoral or cellular immune biomarkers. Even though results from animal studies cannot necessarily be extrapolated to humans, *in vivo* studies in animal models represent a valuable means of understanding the complex signalling cascade underlying a protective immune response.

OVERALL CONCLUSIONS

The science around the concept of probiotics and prebiotics continues to expand. Current global research efforts have greatly contributed to the understanding of the role of GI commensal organisms in their extraordinary symbiotic relationship with humans. Continued research into the microbiota will no doubt help lead to an improved insight into the impact of probiotics and prebiotics on human health.

Probiotics are designed to provide added functions that can compensate for, substitute for, or add to the gut microbiota, and therefore impact the host directly or indirectly through "cross-talk" with the gut microbiota and/or the host. In addition, the effects may be local in the GI tract or systemic. Prebiotics are designed to improve the intrinsic microbiota by selectively stimulating those groups that are thought important for eubiosis.

Research over past decades has demonstrated potential health benefits of dietary probiotics and prebiotics and contributed to our understanding of the mechanisms by which these effects are brought about. The most commonly reported impact of probiotics and prebiotics is on intestinal function, including transit time, AAD and infectious diarrhoea. Evidence continues to emerge that probiotics and prebiotics have an influence on the immune system and thereby may enhance resistance to infections, particularly those of the GI or respiratory tract, and help to mitigate allergies, particularly in infants and young children. Evidence is gradually developing for the potential for probiotics and prebiotics to impact other conditions of the GI tract, such as IBD, IBS and colon cancer. In the case of prebiotics, a well-established role in enhancing calcium absorption remains to be a proven benefit for bone health.

An emerging role for prebiotics and probiotics in appetite control and weight management could be very important. An expanding area of interest for both prebiotics and probiotics is the investigation of their potential for an anti-inflammatory role in conditions beyond the gut such as cardiovascular disease, obesity and metabolic syndrome.

One critically important fact to bear in mind is that reported benefits of probiotics should be considered strain-specific unless otherwise demonstrated. Prebiotics are also likely to have substance-specific effects. Thus, for both probiotics and prebiotics it is vital that future human studies take this into account. Such studies, apart from establishing the effects of each ingredient, should also aim to improve our understanding of the mechanisms of action and, if possible, lead to validated biological markers.

It must be remembered when considering studies on prebiotics that only a few prebiotics are currently established. Similarly, only a limited number of microbes have been documented as probiotic. In all cases, it is clear that prebiotics and probiotics must be consumed regularly in order to confer a benefit.

This monograph attempts to summarise the science and principles valid for prebiotics and probiotics today. It is noteworthy that these ingredients can be readily incorporated into a balanced diet and that there is a growing body of evidence for their potential health benefits.

ABBREVIATIONS

AAD	Antibiotic-associated diarrhoea
CD	Crohn's disease
CFU	Colony-forming units
DP	Degree of polymerisation, i.e. the number of monomers in a molecule
FOS	Fructo-oligosaccharides – typically applied to mixtures of DP3–DP9
GALT	Gut-associated lymphoid tissue
GI	Gastro-intestinal
GOS	Galacto-oligosaccharides – typically applied to mixtures of DP3–DP9
IBS	Irritable bowel syndrome
IBD	Inflammatory bowel disease
IL	Interleukin
NEC	Necrotising enterocolitis
QPS	Qualified presumption of safety
SCFA	Short chain fatty acids
TLR	Toll-like receptors
UC	Ulcerative colitis
URTI	Upper respiratory tract infection

GLOSSARY

Antibody: A specific protein produced in the blood or tissues as part of the immune response to a foreign antigen such as a bacterium, toxin or food protein. The antibody interacts with the antigen, thereby inactivating it and thus forming the basis of immunity.

Antigen: A substance that the body recognises as foreign and that can evoke an immune response. Most often, an antigen is a peptide or protein (e.g. bacterial antigen, food antigen or toxin).

Atopy: A genetic susceptibility to exhibit hypersensitivity reactions (eexaggerated immune responses) to common antigens e.g. atopic eczema in response to a common foodstuff.

Commensal: From the Latin for "common table". It means two organisms living together in a way that is either beneficial to both and or that, at least, is not harmful to either. Hence, commensal bacteria live in the human gut and may be neutral or beneficial.

Cytokines: Low molecular weight proteins (other than antibodies) produced by various cell types and involved in cell-to-cell communication and control of the inflammatory and immune response. Cytokines include interferons, interleukins and lymphokines.

Dysbiosis: The condition of the microbiota of the gut in which one or a few potentially harmful micro-organisms are present in high numbers, thus creating a disease-prone situation or resulting in otherwise noticeable disturbances of the microbiota such as liquid stools, gastrointestinal infections or inflammations.

Eubiosis: Formally referred to as "normobiosis", this characterises the composition of a stable or balanced gut microbiota in a healthy individual. There is incomplete understanding of what constitutes eubiosis and, hence, no general definition in terms of bacterial composition or function.

Fermentation: The anaerobic oxidation of organic compounds to generate metabolic energy in the absence of oxygen as an electron sink. Reduction equivalents are released as hydrogen, ammonia, hydrogen sulphide, methane, organic acids or alcohols. For example, the oxidation of carbohydrates to short chain fatty acids (SCFA), ethanol, lactic acid and/or gases to produce energy in the form of ATP.

Microbe/micro-organism: Small, often single-cell organisms including bacteria, archaea, yeast, mould, fungi, algae and plankton (fungi may also be multicellular). Although definitions vary, we have taken the view that microbes do not include viruses.

Microbiota: All the microbes that are found in a particular region or habitat; hence, gut microbiota describes the whole microbial population found in the gut or gastrointestinal tract. The term "microflora" is no longer used.

Oligosaccharide: A carbohydrate that consists of 3–9 monosaccharide units joined by glycosidic linkages. Some are prebiotics.

Polysaccharide: A carbohydrate comprising ten or more monosaccharide units. Some are prebiotics.

Taxonomy: The science of identifying species and arranging them into a classification.

SOURCE MATERIALS AND FURTHER READING

Blaut, M. and Clavel, T. (2007). Metabolic diversity of the intestinal microbiota: implications for health and disease. *Journal of Nutrition*, 137(3 Suppl 2):751S-755S.

Braegger C., *et al.* (2011). Supplementation of infant formula with probiotics and/or prebiotics: a systematic review and comment by the ESPGHAN committee on nutrition. *Journal of Pediatric Gastroenterology and Nutrition*, 52:238-250.

Cani P.D. and Delzenne N.M. (2011). The gut microbiome as therapeutic target. *Pharmacology & Therapeutics*, 130:202-212.

EFSA (2011). EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA); guidance on the scientific requirements for health claims related to gut and immune function. *EFSA Journal*, 9:1984.

EFSA (2011). EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA); general guidance for stakeholders on the evaluation of Article 13.1, 13.5 and 14 health claims. *EFSA Journal*, 9:2135.

Gibson, G.R., *et al.* (2011). Dietary prebiotics: current status and new definition. *IFIS Functional Foods Bulletin*, 7:1–19.

HMP (2012) Human Microbiome Project. National Institutes of Health, Bethesda, MD, USA. www.hmpdacc.org

ILSI Europe (2011) ILSI Europe Concise Monograph on Nutrition and Immunity in Man, 2nd Edition. ILSI Europe, Brussels. http://www.ilsi.org/Europe/Publications/Nutrition%20and%20Immunity.pdf

ILSI Europe (2010). Guidance for assessing the probiotics beneficial effects: how to fill the GAP. *Journal of Nutrition*, 140 (Suppl 3S-I): 671S-721S. Series of papers presented at a workshop "Guidance for assessing the probiotics beneficial effects: how to fill the GAP" organised by the ILSI Europe Probiotics Task Force in association with the International Dairy Federation, Montreux, Switzerland, 22–24 May 2008.

FAO/WHO (2002) Joint FAO/WHO Working Group report on drafting guidelines for the evaluation of probiotics in food. London, Ontario, Canada, April 30 and May 1, 2002. http://www.who.int/foodsafety/fs_management/en/probiotic_guidelines.pdf

FAO/WHO (2001) Joint FAO/WHO Expert Consultation on evaluation of health and nutritional properties of probiotics in food including powder milk with live lactic acid bacteria. Cordoba, Argentina, October 2001. http://www.who.int/foodsafety/publications/fs_management/en/probiotics.pdf

MetaHIT (2008) Metagenomics of the human intestinal tract. http://www.metahit.eu/

O'Toole, P.W. and Cooney, J.C. (2008). Probiotic bacteria influence the composition and function of the intestinal microbiota. *Interdisciplinary Perspectives on Infectious Diseases*, 2008:175285.

Rijkers, G.T. *et al.* (2010) Guidance for substantiating the evidence for beneficial effects of probiotics: current status and recommendations for future research. *Journal of Nutrition*, 140:6715-6765

Roberfroid, M., *et al.* (2010). Prebiotic effects: metabolic and health benefits. Commissioned by the ILSI Europe Prebiotics Task Force. *British Journal of Nutrition*, 104(Suppl. S2):S1-63

Salminen *et al.*, (1998) Functional food science and gastrointestinal physiology and function. *British Journal of Nutrition* 80(Suppl. 1):S147–S171.

Sanders, M.E., *et al.* (2007). Probiotics: their potential to impact human health. Council for Agricultural Science and Technology (CAST), Issue Paper 36. CAST, Ames, Iowa.

Sanders, M.E. (2009). How do we know when something called "probiotic" is really a probiotic? A guideline for consumers and health care professionals. *Functional Food Reviews*, 1:3–12.

Sanders, M.E. (2011). Impact of probiotics on colonizing microbiota of the gut. *Journal of Clinical Gastroenterology*, 45(Suppl. 3): S115-S119.

Spahn, T.W. and Kucharzik, T. (2004) Modulating the intestinal immune system: the role of lymphotoxin and GALT organs. *Gut*, 53:456-465.

World Gastroenterology Organisation (2011) Practice guideline: probiotics and prebiotics, October 2011. http://www.worldgastroenterology.org/probiotics-prebiotics

ILSI Europe Concise Monographs

- Alcohol – Health Issues Related to Alcohol Consumption
- A Simple Guide to Understanding and Applying the Hazard Analysis Critical Control Point Concept
- Calcium in Nutrition
- Carbohydrates: Nutritional and Health Aspects
- Caries Preventive Strategies
- Concepts of Functional Foods
- Dietary Fibre
- Food Allergy
- Food Biotechnology – An Introduction
- Food, Glycaemic Response and Health
- Functional Foods – From Science to Health and Claims
- Genetic Modification Technology and Food – Consumer Health and Safety
- Healthy Lifestyles – Diet, Physical Activity and Health
- Microwave Ovens
- Nutrition and Genetics – Mapping Individual Health
- Nutrition and Immunity in Man
- Nutritional and Health Aspects of Sugars – Evaluation of New Findings
- Nutritional Epidemiology, Possibilities and Limitations
- Oral and Dental Health - Prevention of Dental Caries, Erosion, Gingivitis and Periodontitis
- Oxidants, Antioxidants, and Disease Prevention
- Principles of Risk Assessment of Food and Drinking Water Related to Human Health
- The Acceptable Daily Intake – A Tool for Ensuring Food Safety
- Threshold of Toxicological Concern (TTC)
- Type 2 Diabetes – Prevention and Management

ILSI Europe Concise Monographs can be downloaded from:
www.ilsi.org/Europe/Pages/ConciseMonographSeries.aspx

ILSI Europe publishes also Reports in its Report Series. ILSI Europe Reports can be downloaded from:
www.ilsi.org/Europe/Pages/ReportSeries.aspx

ILSI Europe publishes articles and proceedings in peer-reviewed journals as well. Some of them can be downloaded from:
www.ilsi.org/Europe/Pages/Proceedings.aspx