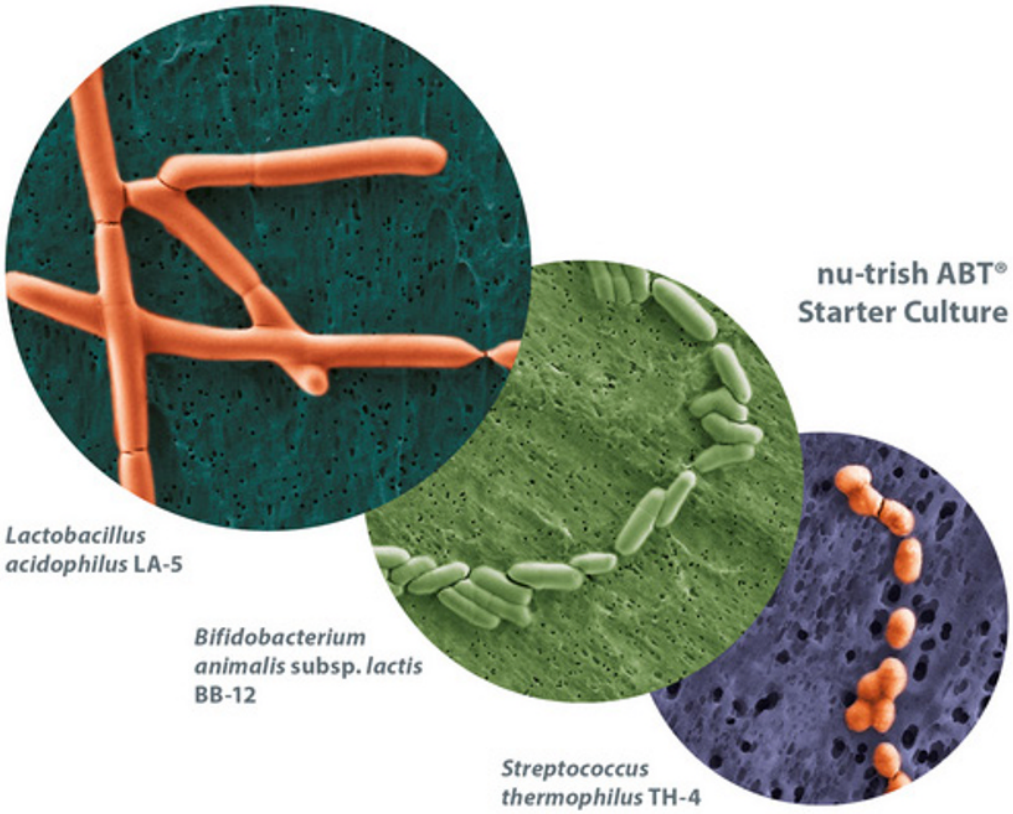
Probiotic Dairy Products

SECOND EDITION

Edited by Adnan Y. Tamime and Linda V. Thomas



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Probiotic Dairy Products

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Probiotic Dairy Products

Second Edition

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Contents

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Preface to the Technical Series, Second Edition

For more than 70 years, the Society of Dairy Technology (SDT) has sought to provide education and training in the dairy field, disseminating knowledge and fostering personal development through symposia, conferences, residential courses, publications, and its journal, the *International Journal of Dairy Technology* (previously known as *Journal of the Society of Dairy Technology*).

Through this time, there have been major advances in our understanding of milk systems, probably the most complex natural food available to man. Improvements in process technology have been accompanied by massive changes in the scale and efficiency of many milk and dairy processing operations, accompanied by an ever widening range of sophisticated dairy and other related products.

In 2005, the Society embarked on a project to produce a Technical Series of dairy-related books, to provide an invaluable source of information for practicing dairy scientists and technologists, covering the range from traditional to modern large-scale operations. The 2nd edition of 'Probiotic Dairy Products', under the editorship of Drs Adnan Tamime and Linda Thomas, provides a timely update on the advances that have been made in the understanding of the human gut microbiota, the characterisation, enumeration and production of probiotics together with their relationship with prebiotics and the commercial implications for dairy and other products within the legislative constraints.

Andrew Wilbey
Chairman of the Publications Committee, SDT
October 2016

Preface to the Technical Series, First Edition

For more than 60 years, the Society of Dairy Technology (SDT) has sought to provide education and training in the dairy field, disseminating knowledge and fostering personal development through symposia, conferences, residential courses, publications, and its journal, the International Journal of Dairy Technology (previously known as Journal of the Society of Dairy Technology).

In recent years, there have been significant advances in our understanding of milk systems, probably the most complex natural food available to man. Improvements in process technology have been accompanied by massive changes in the scale of many milk/dairy processing operations, and the manufacture of a wide range of dairy and other related products.

The Society has now embarked on a project with Blackwell Publishing to produce a Technical Series of dairy-related books to provide an invaluable source of information for practising dairy scientists and technologists, covering the range from traditional to modern large-scale operations. This, the first volume in the series, on 'Probiotic Dairy Products', under the editorship of Dr Adnan Tamime, complements the second volume on 'Fermented Milks' in providing a wide-ranging review of this group of micro-organisms, which are increasingly recognised as playing a vital role in the maintenance of our health while also contributing to the microbiology of many fermented dairy products.

Andrew Wilbey
President, SDT
February 2005

Preface to the Second Edition

Since the publication of the first edition of this book in 2005, we have witnessed incredible advances in our knowledge and understanding of the human microbiota, mainly due to the development and use of new molecular analysis techniques. One example is the new 'omic' technologies that have been used to detect and analyse all the genes, proteins and metabolites of individuals' gut microbiota. Studies investigating different population groups in various states of health that have used such methods have given a better overall picture of the composition and functions of the gut microbiota. This new edition of 'Probiotic Dairy Products' reflects this scientific interest by incorporating a new chapter on the human gut microbiota (see Chapter 1), which reviews current knowledge.

The vast amount of research that has been conducted in this field, which has included several multi-national projects, has resulted in numerous high-profile scientific papers that have helped to drive medical and consumer interest in probiotics, because of their influences on the gut, its microbiota and overall health. Another new chapter for this edition describes the history of probiotics (see Chapter 2), reminding us of the origins of these products and the early pioneers in this field. It is generally acknowledged that the probiotic concept started with Metchnikoff's idea that a long healthy life could be promoted by increasing numbers of lactic acid bacteria in the colon at the expense of 'putrefying' bacteria that were injurious to health. In the twenty-first century, probiotic benefits have been reported for an extraordinary range of health and disease areas (see Chapter 8), and it is important to note that clinical studies have been conducted not just with tablets or powders but also with probiotic dairy products, in the form of fermented milk drinks and yoghurts. One great advantage of dairy products over pharmaceuticals is that the former can be incorporated readily into one's daily diet, and thus can quite easily be part of a proactive strategy for health maintenance.

It is an absolute requirement that manufacturers can assure product quality and safety. Probiotic products must contain adequate numbers of live microbial strains, and other chapters in this book provide valuable updates on genomic analysis of probiotic strains (Chapter 3) and aspects of probiotic products' production and quality control (Chapter 4). The new molecular technologies can now be applied for the identification and enumeration of the live probiotic strains in dairy products, although culture methods remain important. These methods are reviewed in Chapter 6.

Since the first edition of the book, the sale and marketing of probiotics have expanded to around the world, which has led to regulatory changes to ensure that, among other

things, probiotic health claims are substantiated by scientific evidence. This is reviewed in Chapter 5. Probiotics are sometimes combined with prebiotics to make synbiotic products, and the research behind prebiotics is discussed in Chapter 7, whilst Chapter 9 gives an overview of the different metabolites that can be produced by probiotic strains that have potential health benefits. Finally, Chapter 10 speculates on the future for probiotic dairy products, and the current barriers to progress.

A.Y. Tamime and Linda V. Thomas
December 2016

Preface to the First Edition

Fermented foods, including milk and dairy products, have played important roles in the diet of humans worldwide for thousands of years. Since the mid-1950s, there has been increasing knowledge of the benefits of certain micro-organisms, such as lactic acid bacteria (LAB) and probiotic gut flora, and their impact on human biological processes and, at the same time, of the identity of certain dairy and non-dairy components of fermented milks and their role in human health and body function. The purpose of this book, which is written by a team of international scientists, is to review the latest scientific developments in these fields with regard to the 'functional' aspects of fermented milk products and their ingredients.

Some scientific aspects reviewed in this publication are: (a) the latest knowledge regarding the gut microflora (e.g. identifying the beneficial microbiota in terms of probiotic and health aspects); (b) the use of a wide range of probiotic micro-organisms during the manufacture of different dairy products that have dominated the global markets for the past decades and are used as vehicles to increase the probiotic gut flora of humans; (c) the genomic sequences of certain strains of LAB; and (d) the use of prebiotic ingredients, such as galacto- and fructo-oligosaccharides, to enhance the viable count of probiotic microflora in humans.

Furthermore, numerous related topics – for example, the current statutory regulations (national and international), analytical methods to enumerate these beneficial organisms, sensory profiling to improve the quality of the product and enhance consumer acceptability, bioactive components produced by the probiotic microflora, and the treatment of certain human diseases – are also reviewed. It is of interest to note that the current research work on probiotic dairy products, which aims to understand the role of the intestinal microbiota, will underpin new strategies to improve the health status of consumers, and will contribute to a reduction in healthcare costs, particularly in ageing populations.

A.Y. Tamime
February 2005

1 Microbiota of the Human Gut[1]

H.B. Ghoddusi and L.V. Thomas

1.1 Background

The human gastrointestinal (GI) tract has been the subject of intense research over the past decade, since the publication of the first edition of this book. Notably, the Human Microbiome Project in the United States of America (USA) (http://hmpdacc.org) (Turnbaugh *et al*., 2007) and the Metagenomics of the Human Intestinal Tract consortium in Europe (MetaHIT; www.metahit.eu) (Qin *et al*., 2010) have been two major initiatives, but very many other research groups have published their findings. Scientists can get qualitative and quantitative information about all the microbes present in the gut (the gut microbiota) in the context of their habitat, genomes and surrounding environment (the gut microbiome), as well as cataloguing all the metabolites in the gut (metabolomics) and getting an overview of microbial functions in the gut based on analysis of all their genes (metagenomics), the genes' activity (transcriptomics) and proteins present (metaproteomics) (Marchesi *et al*., 2016). Such work has amassed a vast amount of data and helped improve our understanding of microbial communities in the human body. Although the main target of this research has been the human intestinal tract, other body parts, including the skin and the nasal, oral and urogenital tracts, have not been overlooked. Apart from finding an answer to the 'What is there?' question, the main purpose of this research has been to look for associations between any observed changes in the microbiome and the prevalence of certain diseases (Korecka & Arulampalam, 2012). One clear outcome, however, has been the confirmation of the key influence of the human gut microbiota on health, not just of the gut but of the whole body, because of the gut microbiota's influence on different systems in the body (Rooks & Garrett, 2016). In fact, many scientists and medics are now of the opinion that the gut microbiota should be considered equivalent to a body organ (Marchesi *et al*., 2016).

The highly specialised ecosystem that is the human gut microbiota has evolved to achieve a symbiotic homeostatic relationship with the host (Bäckhed *et al*., 2005; Flint *et al*., 2012). The GI tract and its microbiota cannot be really considered as separate

[1] In the book's first edition, this chapter was authored by Dr B. O'Grady and Professor Glenn Gibson of the University of Reading. The current chapter constitutes a major update of that work to reflect the significant advances in this field since 2005.

Probiotic Dairy Products, Second Edition. Edited by Adnan Y. Tamime and Linda V. Thomas.

entities because together they represent a dynamic biological system that has developed together from birth. The human GI tract is composed of highly adapted regions for mediation of its diverse functions, many of which impact markedly upon host health and welfare. Physiological considerations in each unique region influence the degree and type of colonisation, and initial colonisers also modify the physiological conditions therein. This results in the development of distinct microhabitats along the length of the GI tract, which influence metabolism, protection and immune stimulation (Flint *et al*., 2012; Thomas *et al*., 2014; Honda & Littman, 2016). Such effects are both local and systemic, as the GI tract is connected to the vascular, lymphatic and nervous systems. The ability of the gut to sustain a microbiota that is supportive of health is critical for host health and reduction of disease risk.

1.2 The human GI tract and its microbiota

It has long been thought that colonisation of the GI tract begins immediately after birth (Castanys-Muñoz *et al*., 2016), but although this is certainly when the primary colonisation process occurs, recent studies have reported the detection of micro-organisms in meconium, placenta, umbilical cord and amniotic fluid (Thomas, 2016). Micro-organisms have also been detected in breast milk (Fernández *et al*., 2013).

Microbial colonisation of the neonate mainly occurs during the delivery process. The inoculum may be largely derived either from the mother's vaginal and faecal microbiota (in a conventional birth) or from the environment (in a Caesarean delivery); hence, the micro-organisms that colonise the new-born tract are primarily acquired postnatally. The delivery method is key, as new-borns delivered by Caesarean section are exposed to a different microbiota compared to that found in the vagina. In a recent pilot study, Dominguez-Bello *et al*. (2016) demonstrated that by exposing infants delivered by Caesarean section to maternal vaginal fluids at birth, not only the gut but also the oral and skin bacterial communities of these new-borns were partially altered to become more like those of a naturally delivered infant during the first 30 d of their life. The potential long-term health effects of Caesarean delivery remain unclear, although microbial differences may last for at least one year (Rutayisire *et al*., 2016), and links to health risks such as childhood obesity (Blustein *et al*., 2013) and allergic disease (Brandão *et al*., 2016) have been reported.

Bacterial populations in the gut develop progressively during the first few days of life; facultative anaerobes predominate initially and create a reduced environment that allows for the growth of strict anaerobes (Rodríguez *et al*., 2015). The choice of diet for the new-born is also of importance as the microbiota of breast-fed infants is predominated by bifidobacteria, whereas formula-fed infants have a more complex microbiota that resembles the adult gut, in that *Bacteroides*, clostridia, bifidobacteria, lactobacilli, Gram-positive cocci, coliforms and other groups are all represented in fairly equal proportions (Lozupone *et al*., 2012; Ghoddusi & Tamime, 2014). Breastfeeding promotes a more beneficial microbiota; the presence of certain oligosaccharides in human breast milk, for instance, promotes the growth of beneficial bifidobacteria (Smilowitz *et al*., 2014). During weaning, the microbiota becomes more complex, and the ecosystem is thought to become fairly stable at around two years of age. The prevalence of

Table 1.1 The change in the gut microbiota through life.

Stage of life	Intestinal microbiota profile
Foetus	Usually sterile
Baby	Immediately after birth, there is rapid colonisation of the gut with micro-organisms from the immediate surroundings; the gut microbiota composition is influenced by mode of delivery and type of feeding: • *Breast-fed*: low diversity, dominated by bifidobacteria. • *Formula-fed*: a more diverse microbiota with more Bacteroidetes and fewer bifidobacteria.
Child	The gut microbiota becomes more stable and complex over the first three years (particularly after weaning), so that it becomes much more diverse in its composition and more like that of an adult.
Adults	A diverse composition; dominant phyla are Firmicutes, Bacteroidetes and Actinobacteria.
Old age	The microbiota changes to become less diverse and resilient; there are fewer Firmicutes and bifidobacteria and more Bacteroidetes and Proteobacteria.

bifidobacteria in breast-fed infants is thought to confer protection by improving the colonisation resistance of the gut; among other mechanisms, bifidobacteria exert directly antagonistic activities against gut pathogens. New-borns are susceptible to intestinal infections and atopic diseases as their immune system and GI tract develop. The mode of delivery and subsequent diet, therefore, have important implications, both at birth and later in life, as the initial colonisation process has a strong influence on the development of the GI tract and its microbiota, and in the maturation of the immune system. During the first few years of life and after weaning, the infant microbiota normalises to a composition that remains relatively stable throughout most of adult life (Thomas, 2016). Table 1.1 summarises how the intestinal microbiota develops with age.

In recent years, the development of next-generation sequencing (NGS) techniques has played a major role in revealing that the human body harbours more than 1000 phylotypes, although intestinal bacteria mainly belong to just a few phyla (Tojo *et al*., 2014). Most of this work comes from analysis of faecal samples; these best represent the distal portion of the gut. Due to the difficulties in obtaining samples higher in the gut, it has proved more difficult to get a true picture of the microbial communities in the small and proximal large intestines (Li *et al*., 2015; Marchesi *et al*., 2016).

The GI tract begins with the oral cavity (the mouth, nose and throat), where a complex microbiota exists that comprises viruses, bacteria, archaea and protozoa. Bacterial species cause dental caries and periodontal species, but many bacteria in the oral microbiome remain uncultured (Wade, 2013). Bacteria are found on the posterior and anterior tongue, sub- and supra-gingival plaque, buccal mucosa and vestibular mucosa (Willis *et al*., 1999). These include members of the *Prevotella*, *Porphyromonas*, *Peptostreptococcus*, *Bacteroides*, *Fusobacterium*, *Eubacterium* and *Desulfovibrio* genera. Bacterial numbers drop dramatically to $<10^3$ colony forming units (cfu) mL^{-1} of gastric contents as they encounter the stomach, which provides a highly effective barrier against invading micro-organisms, both pathogenic and benign. Few micro-organisms, with the exception of acid-tolerant lactobacilli, yeasts and notably *Helicobacter pylori*, can survive the harsh, strongly acidic and peristaltic nature of the stomach.

There is a high degree of variability between the stomach, small intestine and colon in terms of numbers and bacterial population types, due predominantly to different transit times, secretions and nutrient availability (Lambert & Hull, 1996; Guilliams, 1999). Micro-organisms themselves are also determinants because they interact with and influence their surroundings to ensure their survival against competitors. This is achieved through many mechanisms, such as increasing aerobic conditions in the gut or producing inhibitory compounds, such as bacteriocins or short-chain fatty acids (which also lower the pH of the gut milieu). Such compounds may also affect the host with positive or negative consequences (Fooks & Gibson, 2002; Fuller & Perdigón, 2003).

The rapid transit time, low pH and presence of bile associated with the small intestine do not provide an environment that encourages the growth of bacteria. The duodenum also has low microbial numbers due to its short transit time and the secretion of intestinal fluids, which create a hostile environment (Sanford, 1992); however, there is a progressive increase in both numbers and species along the jejunum and ileum. The small intestine harbours enterococci, enterobacteria, lactobacilli, *Bacteroides* and clostridia. These rapidly increase in numbers from 10^4–10^6 cfu mL^{-1} in the small intestine to 10^{11}–10^{12} cfu mL^{-1} in the large intestine, as the flow of intestinal chyme slows upon entry into the colon (Salminen *et al*., 1998).

The large gut is favourable for bacterial growth with its slow transit time, ready availability of nutrients and more favourable pH. Several hundred culturable species may be present here, although a significant proportion is not cultivable by conventional methods. The proximal colon is the site of saccharolytic fermentation, due to its high substrate availability (Scott *et al*., 2012; Russell *et al*., 2013; Shanahan, 2013). Organic acids produced from fermentation result in a lower pH (of 5.5–6.0) compared to the more neutral pH found in the distal colon. Transit in the distal colon is slower and nutrient availability is minimised, producing slower growing populations that tend towards more proteolytic fermentations.

An intriguing question about the human microbiota is the relevance of microbial variations in healthy and diseased individuals, and whether microbial mapping could help predict specific conditions (Knights *et al*., 2014). Despite the diverse range of micro-organisms found in the human digestive tract, it has been suggested that just five or six genera and two phyla shape the mainstream biomass. Numerically dominant genera include *Bacteroides*, *Bifidobacterium* and *Eubacterium* and, to a lesser extent, although still important, *Clostridium*, *Enterobacteriaceae* and *Streptococcus* (Gibson & Roberfroid, 1995; Salminen *et al*., 1998). Five bacterial phyla represent the bulk of the bacteria in the gut, with the two major phyla being the Gram-positive Firmicutes and the Gram-negative Bacteroidetes (LePage *et al*., 2013), which have relatively similar proportions in different individuals (Jeffery *et al*., 2012). In 2011, three different profiles for the human gut microbiota were proposed, termed 'enterotypes', that were dominated by *Bacteroides*, *Prevotella* or *Ruminococcus* (Arumugam *et al*., 2011). The situation, however, may be more complex than this, and further research is also needed to elucidate the health implications of such enterotypes (Gibson *et al*., 2016).

Table 1.2 illustrates the representation of the microbiota of the GI tract, highlighting some of the common bacteria and their abundance in different parts of the human digestive system. Yeasts, including the opportunistic pathogen *Candida albicans*, are also

Table 1.2 Representative bacteria in the gastrointestinal (GI) tract.

Bacterial family or genus	GI tract region	Microbial count (colony forming units (cfu) mL^{-1}	Function of the GI tract region
Lactobacillus *Streptococcus* *Helicobacter* *Peptostreptococcus*	Stomach	$1–10^2$	• Hydrochloric acid secretion • Macromolecule digestion • pH 2
Streptococcus *Lactobacillus*	Duodenum Jejunum Ileum	$10^1–10^3$ $10^3–10^4$ $10^7–10^9$	• Main digestion • Absorption of monosaccharides, amino acids, fatty acids and water • pH 4–5
Bacteroides *Clostridium* *Streptococcus* Actinomycineae	Caecum	NR[1]	• Absorption of fluids and salts • Mixing of the lumen contents with mucus • pH 5.7
Bacteroides *Clostridium* *Bifidobacterium* Enterobacteriaceae *Eubacterium*	Colon	$10^{11}–10^{12}$	• Microbial production of secondary bile acids and vitamin B_{12} • Water absorption • pH 7
NR	Rectum	NR	• Storage of faeces before evacuation • pH 6.7

NR = Not reported.
Adapted from Korecka and Arulampalam (2012).

present in the gut microbiota, although in healthy individuals its counts do not exceed 10^4 cfu g^{-1} in faeces (Bernhardt *et al*., 1995; Bernhardt & Knoke, 1997). The vast majority (>90%) of the total cells in the body are present as bacteria in the colon. It is thought that over 60% of the faecal mass exists as prokaryotic cells. As well as the different microhabitats along the length of the GI tract, there are other microhabitats, such as the surface of the gut epithelia, the gut lumen, the colonic mucus layers and the ileum/caecum and colon (Donaldson *et al*., 2016).

The classification of the microbiota as autochthonous or allochthonous complements the distinction between these different habitats of the GI tract (Savage *et al*., 1968). Autochthonous micro-organisms are indigenous and colonise the GI tract, whereas allochthonous micro-organisms are transient and will predictably be found in the lumen. The slow transit time of the large intestine allows multiplication of the luminal microbiota; allochthonous micro-organisms exert equally important effects on the GI tract as their autochthonous counterparts.

1.3 Functions of the GI microbiota

The GI tract along with its microbiota comprise one of the most metabolically active organs in the human body. The intestinal microbiota is involved in the fermentation of endogenous and exogenous microbial growth substrates. The metabolic end products of carbohydrate fermentation are benign or even advantageous to human health (Macfarlane

& Gibson, 1994; Flint *et al*., 2012; Rooks *et al*., 2016). Major substrates available for the colonic fermentation are starches that, for various reasons, are resistant to the action of pancreatic amylases but can be degraded by bacterial enzymes, as well as dietary fibres, such as pectins and xylans. Other carbohydrate sources available for fermentation in lower concentrations include oligosaccharides and a variety of sugars and non-absorbable sugar alcohols. Saccharolysis results in the production of short-chain fatty acids (SCFAs), such as butyrate, acetate, propionate and lactate that contribute towards the energy metabolism of the large intestinal mucosa and colonic cell growth; they can also be metabolised by host tissues, such as the liver, muscle and brain. The production of SCFAs concomitantly results in a lower pH that can protect against invading micro-organisms and also reduces the transformation of primary bile acids into secondary pro-carcinogenic bile acids (Cummings & Macfarlane, 1997; Marchesi *et al*., 2016). This is one of the mechanisms utilised by beneficial bacteria in the gut that results in protection for the host.

Proteins and amino acids can be effective growth substrates for colonic bacteria, whilst bacterial secretions, lysis products, sloughed epithelial cells and mucins may also make a contribution. However, diet provides, by far, the predominant source of nutrients, with around 70–100 g d^{-1} of dietary residues available for the colonic microbiota. These materials are degraded by a wide range of bacterial polysaccharidases, glycosidases, proteases and amino-peptidases to smaller oligomers and their component sugars and amino acids (Macfarlane & Gibson, 1994).

The gut profile of each adult represents a population of microbes that has evolved since birth and that can best cope with the physiological and microbiological pressure encountered within this ecosystem. This stability provides resistance for the host, also known as the 'barrier effect', against invading micro-organisms, both pathogenic and benign. The indigenous gut microbiota is better adapted to compete for nutrients and attachment sites than any incoming micro-organism, which it may also inhibit through the production of compounds (Alderbeth *et al*., 2000). The role of the intestinal microbiota in challenging invading micro-organisms and preventing disease through competitive exclusion is best demonstrated by the studies showing that germ-free animals are more susceptible to infection (Baba *et al*., 1991). This demonstrates the individual role of beneficial micro-organisms in preventing infection through colonisation resistance.

Another important function of the gut microbiota is the production of vitamins B and K; this is best demonstrated by studies where germ-free animals required a 30% increase in their diet to maintain their body weight, and supplementation with vitamins B and K as compared to animals with a microbiota (Hooper *et al*., 2002).

The ability of the gut microbiota, however, to utilise biologically available compounds can have negative outcomes. *Helicobacter pylori* can affect the absorption of vitamin C and important micronutrients for host health (Annibale *et al*., 2002). Moreover, the fermentation of proteins and amino acids in the distal colon can lead to the production of toxic substances such as ammonia, phenols and amines that are undesirable for host health (Mykkanen *et al*., 1998; Kim *et al*., 2013). This highlights the importance of ensuring a balance of beneficial bacteria to prevent the multiplication of pathogens or bacteria whose growth and metabolism may increase disease risk.

The GI tract is in more contact with the external environment than our skin, which exposes ~2 m^2, whereas the GI tract exposes a surface area of ~200 m^2 (Guilliams, 1999). The microbiota of the GI tract is therefore heavily involved in gut maturation. As mentioned in this chapter, exposure to the intestinal microbiota after birth plays a critical role in stimulating local and systemic responses and supporting the maturation of the immune system. The intestinal microbiota also provides a source for non-inflammatory immune stimulation, throughout life, by stimulating the production of secretory IgA, which neutralises foreign bacteria and viruses (Moreau, 2000; Mathias *et al*., 2014). The immune system–microbiota alliance provides a dynamic environment by defending the host from pathogens as well as maintaining a balanced and controlled tolerance to harmless antigens. Many factors can play a role in destabilising this coalition and disturbing this symbiotic relationship, including changes in diet and overuse of antibiotics, which in turn could allow the proliferation of a microbiota lacking in diversity or the resilience and tolerance needed for a well-functioning immune system. The rise in autoimmune diseases and inflammatory disorders has been suggested to be partly the result of this troubled reciprocal relationship. Overall, the ability of the GI tract to perform its functions of nutrient uptake in conjunction with the exclusion of foreign antigens or micro-organisms is a complex and difficult process. The interplay between the host immune response and the GI microbiota is critical to health; loss of tolerance may become clinically manifest through disorders, such as inflammatory bowel disease (IBD) (Malloy & Powrie, 2011).

The gut microbiota and host health has found a new clinical frontier in recent years, the so-called gut–brain axis (El Aidy *et al*., 2015), which is described as a two-way communication between the central and the enteric nervous systems, in which the emotional, intuitive, decision-making and cognitive centres of the brain are linked with peripheral intestinal functions (Mayer, 2011). This bidirectional interaction is believed to include signal exchange between gut microbiota and the brain through neural, endocrine, immune and humoral links (Carabotti *et al*., 2015; Kountouras *et al*., 2015). To provide evidence of these interactions, studies on germ-free animal models, probiotics, antibiotics and infection have been carried out. At a clinical level, studies have focused on central nervous disorders such as autism, anxiety-depressive behaviours and GI disorders, such as (typically) irritable bowel syndrome. It is hoped that such investigations lead to new therapeutic strategies (Distrutti *et al*., 2016).

1.4 Influences on the GI tract and its microbiota

The profile of the intestinal microbiota that develops in each individual is a result of their host genetics (as shown in twin studies in the UK) (Goodrich *et al*., 2014), environmental factors and microbiological influences. These factors result in a stable community of micro-organisms that is more unique than an individual's own fingerprint; even homozygotic twins develop distinct microbial profiles (Zoetendal *et al*., 2001). Notwithstanding this, the overall metabolism of a healthy gut ecosystem varies little from one individual to another, as evinced by the ratios of major metabolic end products. Modern living presents numerous challenges to the human GI tract, particularly in

the developed world, with often stressful lifestyles and unhealthy intake of processed foods. Antibiotics and other medications, however, can cause immediate serious disruption of the gut microbiota, and the resulting dysbiosis may be long term (Jernberg *et al*., 2010; Francino, 2015). Disturbances of the microbiota can have serious implications, and this fragility merits careful consideration of the external influences on the GI tract and how they may disrupt host health (O'Sullivan *et al*., 2013). The numerous factors which act upon the intestinal microbiota are briefly outlined in Table 1.3; some of the more relevant influences are discussed here.

The influence of diet on the neonatal intestinal microbiota has already been outlined (do Rosario *et al*., 2016; Ojeda *et al*., 2016). The GI tract of healthy humans remains relatively stable throughout life apart from later life, when a significant decrease of beneficial bifidobacteria and loss of microbial diversity have been reported. Such changes have also been linked to indications of increased risk of disease and frailty (van Tongeren *et al*., 2005; Claessen *et al*., 2012; Jackson *et al*., 2016). Diet is an effective and rapid modulator of the microbial composition and metabolic activity of the human gut, which in turn can impact health (Claesson *et al*., 2012; Conlon & Bird, 2015) with temporary and/or lasting effects. For example, the ELDERMET study in Ireland has shown clear differences between the core microbiota in older people compared to younger ones. Furthermore, clear differences were observed in the gut microbiota that correlated to these older persons' place of residence: long-term residential care, rehabilitation hospital care for less than six months, attending hospital outpatients or living in the community (Claessen *et al*., 2012). The profile of the microbiota of those living at home was the one most similar to that of healthy younger adults, whereas the gut microbiota of the older people living in long-term care was significantly different and much less diverse. These microbiota differences correlated with the different diets eaten at home or in residential care; the latter had a much lower intake of fruit, vegetables and fibre, and a higher intake of fatty, starchy and sugary foods. Whilst long-term diet clearly influences the composition of gut microbiota, even short-term dietary modifications lead to significant and relatively swift changes in the composition of the microbiota, but

Table 1.3 Influences on the composition of the gastrointestinal microbiota.

- Type of feeding
- Amount, chemical composition and availability of growth substrate
- Availability of colonisation sites
- Immunological interactions
- Individual fermentation strategies by the bacteria
- Intestinal transit time
- Gut pH
- Redox potential
- Availability of inorganic electron acceptors
- Production of bacterial metabolites
- Presence of antimicrobial compounds
- Xenobiotic compounds
- Age of the host
- Peristalsis

Adapted from Fooks *et al*. (1999).

these would not be expected to cause a lasting shift in microbiota composition or affect the core profile. Data indicate that such changes may be at genus and species level, but not at phylum level (Wu *et al*., 2011).

Type of dietary intake has consequences in the colon as carbohydrate fermentations usually result in benign end products (Wong *et al*., 2006; do Rosario *et al*., 2016). However, when carbohydrate levels become diminished, proteolytic fermentation in more distal regions produces toxic compounds that can predispose to diseases such as colorectal cancer or ulcerative colitis (Nyangale *et al*., 2012); thus, protein-based diets such as the Atkins diet could potentially have serious long-term repercussions for gut health (Russell *et al*., 2011). High intakes of processed food and other dietary aspects will reduce levels of fibre in the diet, which is of concern as dietary fibre influences stool volume, colon motility, water absorption and faecal transit time (Dhingra *et al*., 2012).

Chronic illness, immune suppression and the use of broad-spectrum antibiotics can severely compromise the crucial balance between beneficial and harmful micro-organisms in the gut microbiota. The loss of any beneficial genera sensitive to antibiotic therapy, such as lactobacilli and bifidobacteria, has implications for GI health, as opportunistic pathogens can overgrow the gut, and the host will have increased risk for iatrogenic disease. For example, the serious concerns about the risks of antibiotic-associated diarrhoea, including that caused by *Clostridium difficile*, are well documented (Burke & Lamont, 2014; Elseviers *et al*., 2015).

The increase in antibiotic resistance, the lack of progress in developing new antibiotics, concerns over (possibly long-term) adverse effects associated with antibiotic use (such as increased risk of obesity) (Reid, 2006; Langdon *et al*., 2016; Ouwehand *et al*., 2016) plus consumer interest in dietary supplements to maintain GI health have fuelled scientific research into alternative strategies. The potential for preventing dysbiosis, increasing the resilience of the gut microbiota or otherwise fortifying the GI tract through modulation of the intestinal microbiota has been widely explored. The principle of using harmless bacteria to prevent disease dates back to the suggestion of Metchnikoff at the turn of the twentieth century that ingested bacteria could promote longevity and well-being (Metchnikoff, 1907; see Chapter 2 for details). Micro-organisms associated with health benefits *in vivo* include many members of the *Lactobacillus* and *Bifidobacterium* genera, although *Escherichia coli*, streptococci, enterococci, lactococci, bacilli and yeasts, such as *Saccharomyces cerevisiae* var. *boulardii*, have also been used (Table 1.4). Such strains have been researched for their probiotic potential, and many strains (including those marketed commercially) are the focus of intense research (see Chapter 8 for further details).

1.5 Conclusions

A number of disease states have been linked to dysbiosis and/or low diversity of the gut microbiota, suggesting that its manipulation at any stage of life but particularly in infancy could have beneficial consequences in reducing the risk of both short-term and long-term disease (Thomas *et al*., 2014; Carding *et al*., 2015; Prosberg *et al*., 2016). Differences in the ratio of Firmicutes to Bacteroidetes have also been observed between

Table 1.4 Examples of microbial species that contain probiotic strains.

Microbial genus or group	Species
Bifidobacterium	*Bifidobacterium bifidum*
	Bifidobacterium longum subsp. *longum*
	Bifidobacterium breve
	Bifidobacterium adolescentis
	Bifidobacterium longum subsp. *infantis*
Enterococcus	*Enterococcus faecalis*
	Enterococcus faecium
Lactococcus	*Lactococcus lactis* subsp. *lactis*
Lactobacillus	*Lactobacillus acidophilus*
	Lactobacillus rhamnosus
	Lactobacillus reuteri
	Lactobacillus casei
	Lactobacillus gasseri
	Lactobacillus plantarum
Yeast	*Saccharomyces cerevisiae* var. *boulardii*

individuals and patient groups. Other examples include IBD, where low counts of *Faecalibacterium prausnitzii* have been associated with increased risk of ulcerative colitis (Sokol *et al*., 2009), and several species have been implicated in colorectal cancer, including *Streptococcus gallolyticus*, *Enterococcus faecalis* and *Bacteroides fragilis* (Wu *et al*., 2009; Boleij & Tjalsma, 2013; Wang *et al*., 2015).

A key question in gut microbiota research, however, is whether such microbial changes are the *cause* of the disease or are the *result* of disease (Zhang, 2013). One tactic to explore this 'correlation/causality' microbial conundrum is to conduct clinical trials in patients or people at risk of disease, investigating the health effects of modulating the microbiota. Faecal microbiota transplantation, for example, has shown strong efficacy for treatment of *C. difficile* infection (Borody *et al*., 2015). Probiotics work through multiple mechanisms of activity, including the modulation of the gut microbiota, and evidence of probiotic benefit for a broad range of disorders has accumulated. This is discussed further in Chapter 8.

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2 Probiotics: The First 10 000 Years

R. Levin

> It can only be a matter of time, we shall obtain exact information on the influence of diets which prevent intestinal putrefaction, prolong life and maintain the body's forces.
>
> Metchnikoff (1907)

2.1 In the beginning

Milk is not only an important food for humans, it is the first food of infants. It is believed humans began domesticating animals somewhere between 8000 BC and 5000 BC. Not long after, it must have been realised that the milk of other animals was just as able as human milk to satisfy the nutritional, energy and fluid needs of both adults and children. Moreover, early humans must have soon discovered that, whereas milk normally has a short life, under certain conditions, it forms curds with an extended shelf-life.

Probiotics in the form of fermented milk products have been in regular and continuous use as a source of nutrition and, unknowingly, for health and well-being, since time immemorial. Indeed, early evidence comes from a sculptured relief found at Tel Ubaid in ancient Babylon that appears to depict the production of cultured milk products for food some 8000 years ago. Milk is also mentioned in the Old Testament several times: when three angels visited Abraham, he asked Sarah to bake bread and he brought curds and milk (Genesis 18:7). This could be the first record of processed foods containing living micro-organisms, but the Vedic Hymns of India, written before 2000 BC, also reveal that Hindu people used fermented milk in their diet (Kroger *et al*., 1989). Sumerians also crossed expanses of desert with milk carried in bags made from sheep's stomach where bacteria fermented the milk to curd, improving its flavour and keeping qualities. Hippocrates named milk as both a food and a medicine for curing stomach disorders. Plinius, the Roman historian, also recorded that fermented milk was used for stomach disorders. Since earliest times, many Nomadic and semi-Nomadic tribes have produced sour milk because of its improved keeping qualities. The best known of these are Kefir, Leben, Koumiss and Matsun (known also as Mazoor, Mazun, Matsoni or Madzoon). In the eleventh century AD, Yuseuf Has Hajib recorded the use of yoghurt by ancient Turks in his book 'Kutadgu Bilig'.

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The earliest of these milk beverages were probably produced because of spontaneous fermentation by miscellaneous bacteria that contaminated the goat skin bags carried by nomadic peoples, such as the Bulgars, who migrated from Asia to Europe in the second century AD, eventually settling in the Balkans. Many of today's traditional fermented drinks came from the Asian nomads, since fermented milks (together with animal meats) comprised their main nutritional and energy source. Nomads of Central Asia produced a variety of fermented milks, influenced by the animals they bred. Milk from at least eight species of domestic mammals (cow, buffalo, sheep, goat, horse, camel, yak and zebu) has been used to make traditional fermented milk products for human consumption. The following are details of nomadic beverages taken from descriptions given by Douglas (1911).

- Kefir has been used in the Caucasus for about as long as Koumiss has been used in the steppes. It differs in that it is prepared from the milk of sheep, goat or cow. The process is started with the addition of kefir grains to the milk contained in 'leathern' bottles. The grains are small solid kernels, kept by families and handed down from one generation to the next. They were described as a 'zoogloea' composed of bacilli and yeast, the latter being *Saccharomyces cerevisiae* (Kern, 1881). After the grains were added to the 'leathern' vessels containing the milk, in summer fermentation would proceed to completion in cool chambers for 1–2 days. During winter, the vessels would be placed in the sunshine at about ~16 to 18 °C. Agitation of the process would be supplied in the form of kicks from passers-by or children at play.
- Koumiss, thought by some to be the greatest of all the fermented milks, is made from mare's milk. It has been celebrated since ancient times as the principal food of the wandering tribes of Bashkirs, Kalmucks and Tartars who inhabit the steppes of European Russia and plains of West and Central Asia. Dr John Grieve, a surgeon in the Russian Army in 1784, sent a description of it to The Royal Society of Edinburgh (of which he was a member), entitled 'Method of Making Wine called by the Tartars Koumiss, with Observations on its Use as a Medicine'. This resulted in the establishment of sanatoria at Samura and elsewhere in Russia, which 'successfully' treated pulmonary consumption.
- Leben is a soured milk product associated with Middle Eastern countries, prepared from the milk of buffalo, cow and goat. It is prepared by adding fermented milk from the previous preparation to boiled fresh milk. The fermentation is rapid, finishing in ~6 h.
- Arka is a strong alcoholic beverage prepared by the Tartar and Burgaten tribes by distillation of fermented milk. It contains 7–8 g 100 mL^{-1} alcohol and also volatile fatty acids.
- Matzun is a drink mainly found in West Asia, prepared from buffalo, goat or cow milk and partly used for butter making. It is prepared in the Caucasus, using a similar procedure as that for Kefir.
- Yoghurt is related to Matzun and Leben. After boiling to concentrate the milk, it is inoculated with a small quantity of an old culture, then allowed to ferment at a comparatively higher temperature.

These practices still continue in some isolated mountain and desert areas of Asia and Africa. It was in the fifteenth century AD that the science behind fermentation began to be elucidated. Girolamo Fracastoro (1478–1553), an Italian physician and professor at the University of Padua, was possibly the first to propose that epidemic diseases are caused by external factors. He conceived the possibility that tiny transferable particles could transmit disease by direct or indirect contact, or even without contact, over long distances, but he did not anticipate that such tiny particles would be living entities. This hypothesis persisted for three centuries until Louis Pasteur revealed their true nature (Pasteur, 1878). Meanwhile, in about 1590, two Dutch spectacle makers discovered that when two lenses were placed in a tube, nearby objects appeared greatly enlarged. One century later, Anton van Leeuwenhoek, also from Holland, while working in a store where magnifying glasses were used to count the threads on cloth, taught himself methods of grinding tiny lenses to great curvature for higher magnification. Two of these, placed in a tube, led to his first microscope and the consequent first visualisation of bacteria, yeasts and blood corpuscles upon which his fame became based. He reported his findings meticulously in more than one hundred letters to the Royal Society in London and the French Academy in Paris.

2.2 The intervention of science

The first major discovery in bacteriology was by French chemist Louis Pasteur (1822–1895) who, using a microscope, revealed that the cause of spoilage in local beer, wine and milk was microbial contamination. He and Claude Bernard went on to invent a process in which milk was heated to kill most of any bacteria and moulds present, completing its first test in April 1862. The process duly became known as pasteurisation. On becoming Professor of Chemistry at the University of Strasbourg in 1849, Pasteur married the daughter of the university's rector and together they had five children, but only two survived to adulthood, the others succumbing to typhoid. These personal losses undoubtedly inspired Pasteur to seek cures for deadly microbial diseases, such as typhoid. Convinced from his contaminated beverage studies that animals and humans could be similarly afflicted by disease causing micro-organisms, he formally presented the evidence for his Germ Theory of Disease in 1878, for which he would subsequently be awarded the Nobel Prize for Medicine. Pasteur is now recognised as one of the founders of preventative medicine.

Fresh milk can turn sour within hours, but fermented milks (e.g. yoghurt) last much longer and, moreover, are characterised by the presence of microbial metabolites that, fortunately, render the product pleasant to taste. The sensory properties of fermented milks (taste, aroma and viscosity) are all the direct result of specific bacterial action. Pasteur's publication of his Germ Theory of Disease prompted and coincided with an intensive period of progress in the scientific study of milk. The dairy industry appeared to have captured the attention of scientific investigators throughout the world, but especially in the Pasteur Institute in Paris. A wholly unexpected and very close relationship between milk, intestinal disease and longevity then began to emerge.

2.3 A remarkable sequence of important discoveries

The sequence of discoveries that ultimately led Metchnikoff to his lactic microbe hypothesis was as follows:

- Senator (1868) declared that the decomposition of protein within the alimentary tract under normal conditions results in the formation of substances toxic to the host.
- Billroth (1874) was credited with being the first to observe that the meconium of the new-born infant is sterile. This was later confirmed by other researchers between 1880 and 1900.
- Bouchard (1884) elaborated the theory of intestinal intoxication in which he claimed that the amount of putrefactive products eliminated in urine was a measure of intestinal putrefaction, calling his measurements 'urotoxic coefficients'.
- Ortweiller (1886) demonstrated that the administration of certain carbohydrates tended to lessen putrefaction in the digestive tract.
- Hirschler (1886) was the first to observe that feeding particular carbohydrates, such as sucrose, lactose, dextrin and starch, as well as alcohol and glycerol, has an inhibitory effect on intestinal putrefaction.
- Escherich (1886) was a pioneer paediatrician, who devoted himself to improving child-care, particularly with regard to infant hygiene and nutrition, and he published his extensive systematic study of the microbes in infants' 'dejecta', in both health and disease states. He noted a predominance of Gram-positive rods, but (surprisingly) failed to isolate the two species that were soon to generate considerable and continuous interest, which were then known as '*Bacillus bidifus*' (presumed to be *Lactobacillus bifidus* and later renamed as *Bifidobacterium bifidum*) and '*Bacillus acidophilus*' (presumed to be *Lactobacillus acidophilus*). Nevertheless, the quality of his study and his monograph on the relationship of intestinal bacteria to the physiology of digestion in the infant established him as the leading bacteriologist in the field of paediatrics. In 1919, *Bacterium coli* was renamed *Escherichia coli*, after its discoverer.
- Poehl (1887) noted that ingestion of soured milk tended to decrease the undesirable products of protein decomposition by bacteria. This was confirmed by other researchers between 1887 and 1903.
- Döderlein (1892) reported that vaginal lactobacilli were much depleted in numbers in women with vaginitis; he was probably the first to suggest a potentially beneficial role for lactic acid bacteria in the treatment of vaginitis.
- Grigoroff (1905), a Bulgarian postgraduate at Geneva University, was aware of the number of centenarians to be found in Bulgaria, a region in which yoghurt, a soured milk, was a staple food. Working with Professor Massol at Geneva University, he isolated several microbes from 'podkvassa' starter used for the production of Bulgarian yoghurt. Among these was a very active lactic acid-producing species that he called '*Lactobacillus bulgaricus*' (presumed to be *Lactobacillus delbrueckii* subsp. *bulgaricus*). Another species he found in the starter, *Streptococcus thermophilus*, received no attention as it was then considered to be a pathogen. Specimens of the lactic acid-producing cultures were sent, at Metchnikoff's request, to the Pasteur Institute, where they were further investigated by Döderlein and Michelson