

## Chapter 2

# The Intestinal Microbiota and Probiotics

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### 2.1 Introduction

The human gastrointestinal (GI) tract consists of the upper and lower GI tracts. The stomach and the small and large intestines are the main organs of the GI tract, which is about 7 m long in adults. Approximately a total of  $10^{14}$  bacteria ( $10^{11}$  cells per gram of faeces) are estimated to be present in the gut microbiota of an adult individual (Savage 1977). The microbial richness is maximal in the colon, where hundreds of different bacterial species present contain 100-fold more genes than the human genome. It is estimated that the microbial population within a human outnumbers the human cells by a factor of ten (O'Hara and Shanahan 2006). Most of the bacterial taxa present in the GI tract have not yet been successfully cultured, identified, or otherwise characterized (Hayashi et al. 2002). Normally, the majority of bacteria in the GI tract are beneficial or harmless to the host health, while only a minority represents potentially harmful bacteria. The GI microbiota is a complex system that has a major influence on human health. It is known to contribute to e.g. maturation of the gut, nutrition of the host, resistance to pathogens and the maintenance of host health (Stecher and Hardt 2008).

### 2.2 Intestinal Microbiota in Newborns, Children, Adults and Elderly

The microbiota in the GI tract undergoes changes throughout life. The foetus is sterile, but during delivery it is colonized by bacteria from the birth canal, and subsequently exposed to bacteria from other humans as well as the surroundings. The upper GI tract consisting of the stomach, duodenum, jejunum, and upper ileum

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contains a sparse microbiota with bacterial concentrations less than  $10^4$  organisms per ml of digesta. Most of the bacteria in the upper GI tract are transient species that pass through the gut via food. The microbial concentrations increase towards the end of the small intestine, reaching levels ranging between  $10^7$  and  $10^8$  cell per gram, while concentrations of  $10^{11}$  bacteria per gram of stool are present in the large intestine. Humans have to some extent a stable microbiota, and the host's individuality has the largest effect on the structure of the microbial community. The stable part of the intestinal microbiota is referred to as the individual core microbiota (Zoetendal et al. 2008). It is also known that age-related changes occur in the abundance of GI bacteria in healthy humans.

Directly after a vaginal delivery, the sterile upper GI tract of the newborn begins to be colonized by vaginal and faecal microbiota of the mother. Infants that are born by caesarean section are exposed to the microbiota of the mother. However, the primary microbial exposure of infants delivered by caesarean section probably originates from the surrounding environment, for example from the air and from the nursing staff (Schwartz et al. 2003). For vaginally born infants it takes about one month before their primary intestinal microbiota is established, while for infants born by cesarean delivery the GI microbiota may be disturbed for up to 6 months post-birth. After birth, oral and cutaneous bacteria are transferred from the mother to the infant but also bacteria from the nosocomial environment. The initial bacterial count is rather unstable during the first week after birth but is thereafter in most infants in the range of  $10^9$ – $10^{10}$  per gram of faeces (Mackie et al. 1999; Palmer et al. 2007; Schwartz et al. 2003). The first colonizers are aerobes such as enterobacteria and streptococci, while strict anaerobes such as eubacteria and clostridia represent the late colonizers (Favier et al. 2002; Orrhage and Nord 1999; Palmer et al. 2007; Park et al. 2005). The bacterial colonization seems to be host-specific (Mackie et al. 1999) and has been shown to fluctuate; *Bacteroides* might dominate the microbiota in some babies, and be nearly absent in others. Other taxa like *Prevotella*, *Acinetobacter*, *Desulfovibrio* and *Veillonella* tend to appear transiently within the first year of life of a baby. Lactobacilli have been shown to reach a peak at the age of 6 (Adlerberth et al. 2010) months and *Lactobacillus rhamnosus* and *Lactobacillus gasseri* being among the most common species (Ahre et al. 2005; Mitsou et al. 2008).

Differences in the species composition of the GI microbiota of formula-fed infants and breast-fed infants have been detected. The GI microbiota of breast-fed babies is dominated by bifidobacteria and lactobacilli (Chen et al. 2007; Orrhage and Nord 1999; Satokari et al. 2002), while the microbiota of formula-fed infants is more diverse with high numbers of *Enterobacteriaceae*, enterococci, bifidobacteria, *Bacteroides*, and clostridia (Fanaro et al. 2003; Harmsen et al. 2000). Staphylococci on the other hand are recognized as characteristic first-colonizers of the newborn gut, regardless of the mode of delivery (Adlerberth et al. 2010). Differences between formula-fed infants and breast-fed infants may also exist at species-level, for example among *Bifidobacterium* spp. The microbiota of breast-fed infants becomes similar to that of formula-fed infants after introduction of solid food and weaning.

When a child is about two years old the faecal microbiota is similar to the microbiota of adults. The majority of the microbiota is believed to be obligate anaerobes, and dominated by species from the phylum *Firmicutes* (mostly *Clostridium*; 50–70% of total bacterial count), *Bacteroidetes* (10–30%), *Proteobacteria* ( $\leq 10\%$ ), and *Actinobacterium* ( $\leq 5\%$ ) (Eckburg et al. 2005; Lay et al. 2005). Regarding the microbial concentrations in children, it was reported by Enck and co-workers that the total microbial count and individual microbial species were highest during the first year of life. In infants, the total microbial count rose during the first five months and then declined with weaning, and peaked at the age of 9–10 months. The microbial count then decreased within the first two years, while remaining stabilized for the rest of childhood. *Bacteroides* sp. and *Lactobacilli* increased with age, while *Enterococci* and *Escherichia coli* decreased, and bifidobacteria remained stable (Enck et al. 2009). Geographical variations in the composition of the human gastrointestinal microbiota can be seen. For example, considerable differences in the composition of species in Estonian and Swedish one-year-old infants have been reported (Sepp et al. 1997). While the Estonian children were intensively colonized with lactobacilli and eubacteria, the Swedish infants had increased numbers of clostridia, particularly *Clostridium difficile*, as well as bacteroides and other anaerobes.

In adults, aerotolerant bacteria such as lactobacilli and streptococci dominate the small intestinal microbiota, while the microbiota is more complex in the junction (the ileocaecal valve) of the small intestine (ileum) and the large intestine. In the large intestine, anaerobes like bifidobacteria, *Bacteroides*, *Clostridium* (clusters XIVa and IV), *Eubacterium*, and *Ruminococcus* are dominant. Facultatives which principally include the *Enterobacteriaceae* family (e.g. *E. coli*, *Klebsiella* and *Enterobacter*) and enterococci represent less than 1% of the total microbiota (Finegold et al. 1983; Gavini et al. 2001). Low numbers ( $10^3$ – $10^6$  cells per gram) of transient staphylococci can be found (Finegold et al. 1983). Fungi and yeasts such as *Candida albicans* can also be isolated from healthy individuals, but in low numbers.

In a microarray study of the faecal microbiota, clostridia and *Bacteroidetes* were the major classes identified (Paliy et al. 2009). In addition, the gut microbiota of healthy adults contained more clostridia and less *Bacteroidetes* and *Proteobacteria* compared to children.

The composition of the intestinal microbiota in elderly is changed by factors related to ageing, like mobility, nutrition and reduced intestinal functionality. Changes of the small bowel motor pattern, together with a decreased degree of mobility may influence the motility of the bowel and have a negative effect on digestion, causing constipation, and may hence be associated with changes in the gut microbiota (Holdeman et al. 1976). Furthermore, reduced secretion of saliva, gastric and pancreatic juice may lead to an increased influx of exogenous bacteria and nutrients through the digestive tract. A decrease in bifidobacteria counts is one of the most commonly mentioned changes in the composition of the intestinal microbiota of elderly, together with the shifts observed in the genus *Bacteroides* (Gavini et al. 2001; Hopkins et al. 2001; Woodmansey et al. 2004). In particular in studies in which culture based techniques have been used, the faecal *Bifidobacterium* levels seem to be reduced compared to younger adults. However, studies that have used

culture based techniques do often not observe this reduction. This discrepancy can be explained by a difference in composition of the bifidobacterial microbiota rather than a difference in numbers (Lahtinen et al. 2009; Tiihonen et al. 2010). Whilst the genus *Bifidobacterium* may contain up to four or five different species continuously in adult humans, the diversity of bifidobacterial species in the elderly is reduced to one or two dominant organisms. *Bifidobacterium adolescentis* or the phenotypically similar *Bifidobacterium angulatum* and *Bifidobacterium longum* have been reported to be the dominant species in elderly (Gavini et al. 2001; Hopkins et al. 2001; Hopkins and Macfarlane 2002). A high individual variation of *Bacteroides* species in the elderly has been reported, although *Bacteroides thetaiotaomicron* can be found in all individuals (Layton et al. 2006). A decrease in the diversity of *Bacteroides* species has been reported with an increasing age (Bartosch et al. 2004; Hopkins and Macfarlane 2002). Higher *Bacteroides* levels have been detected in males than females (Mueller et al. 2006). *Clostridium* counts have been found to be significantly higher in elderly compared with younger subjects. Conversely, levels of *Clostridium* cluster XIVa seem to decrease in elderly compared to younger persons (Hayashi et al. 2003). Furthermore, *C. difficile* is more frequently isolated from elderly than younger adults, partly as a result of hospitalization (Ljungberg et al. 1990). An increased diversity of *Eubacteria* (Hopkins and Macfarlane 2002) as well as an increase of some eubacteria that are phylogenetically related to clostridia (Woodmansey et al. 2004) have also been observed in elderly.

Lower levels of *Lactobacillus* have also been observed in elderly subjects compared to healthy adults (Hopkins et al. 2001). In a study by Mitsuoka et al. (1996), the levels of *Bacteroides*, enterococci, *Enterobacteriaceae* and clostridia in elderly were not different from adults, while an increase of *Lactobacillus* and *Enterococci* was detected. Another study reported higher levels of *Ruminococcus* and *Enterobacteriaceae*, as well as lower levels of *Eubacterium* and *Bacteroides* in elderly compared to younger adults (He et al. 2001). A higher level of aerobes in the microbiota of elderly than among the microbiota of adults has also been observed (Guigoz et al. 2008; Tiihonen et al. 2008). Similarly, a higher frequency of gammaproteobacteria, such as *Klebsiella* spp. was found in a study by Hayashi et al. (2003). In a study of elderly aged 70–100 years (with a median of 86 years) by van Tongeren et al. (2005) the prevalence of *Bacteroides/Prevotella*, *Eubacterium rectale/Clostridium coccoides*, and *Ruminococcus* were rather similar to the levels in healthy adults aged between 20 and 55 years in a study by Harmsen et al. (2002). However differences in the composition of the faecal microbiota can be observed between e.g. low and high frailty elderly (van Tongeren et al. 2005). Of the most predominant bacterial groups, the percentages of *Bacteroides/Prevotella* (from 11.0% to 4.5%), *E. rectale/C. coccoides* (from 10.6% to 6.7%) and *Faecalibacterium prausnitzii* (from 1.2% to 0.3%) decreased in the high-frailty elderly, while *Ruminococcus* (from 6.3% to 12.7%) and *Atopobium* (from 2.1% to 4.3%) increased in the high-frailty volunteers.

Through the use of molecular analysis such as rDNA gene based analysis in addition to conventional culture-based methods, more detailed information on both cultured and uncultured microorganisms can be obtained. Depending on the

method, 10–50% of the microbes in the human GI tract have been reported to be cultivable (Lay et al. 2005; Zoetendal et al. 2004). With the current large research projects on human microbiome, in which include metagenomic and genomic DNA sequencing studies of human-associated microbial populations, it is possible to investigate whether most individuals share similar core human microbiome. In addition, changes in the human microbiome and correlations with changes in human health can be recognized and understood.

Thus far, it has been shown that the microbiota of the GI differs substantially between individuals at species level. Recent studies suggest that at the level of microorganisms, it appears that there is no core microbiome that is shared between most individuals; Turnbaugh and co-workers reported that no single bacterial phylotype was present at abundant frequency in all of the 154 sampled individuals within the study (Turnbaugh et al. 2009). Instead, a core gut microbiome may exist at the level of shared genes. By analyzing 16S rRNA the microbiota has been reported to be quite stable over time of an individual with only minor changes occurring over time (Zoetendal et al. 1998). However, since the stability has been examined from samples collected within weeks, months or at most few years, the variability over a longer timescale is still unknown (Franks et al. 1998; Lofmark et al. 2006; Matto et al. 2005; Nyberg et al. 2007; Vanhoutte et al. 2004; Zoetendal et al. 1998). The microbiota responds to physiological, dietary, and environmental factors (Bartosch et al. 2004), and the response has an effect on the diversity and the individuality of the intestinal microbiota.

## 2.3 Environment Factors

In addition to intrinsic factors, such as aging and non-infectious diseases, also external events can influence the composition and activity of the intestinal microbiota. As discussed above, a well known example of this is the difference in microbiota composition between breast fed and formula fed infants, although continuous product development of infant formulas makes the difference smaller (Boehm et al. 2005).

Major environmental factors affecting the composition and activity of the intestinal microbiota are diet; e.g. consumption of pre- and/or probiotics, stress, infectious diseases and medication.

### 2.3.1 *The Influence of Prebiotics on the Intestinal Microbiota*

Prebiotics are defined as ‘non-digestible food ingredients that, when consumed in sufficient amounts, selectively stimulate the growth and/or activity of one or a limited number of microbes in the colon resulting in documented health benefits’ (Ouwehand et al. 2006). Updates of the definition have been suggested; but the essence of prebiotics remains the same. Although prebiotics have much in com-

**Table 2.1** Examples of prebiotic and proposed prebiotic dietary components

	Monomer	Degree of polymerisation	Linkage
Galacto-oligosaccharides (GOS)	Glucose-Galactose <sub>n</sub> , Galactose <sub>n</sub>	1–4	β(1→6); β(1→3); β(1→4)
Fructo-oligosaccharides (FOS)	Glucose-Fructose <sub>n</sub> , Fructose <sub>n</sub>	1–9	β(2→1)
Inulin	Glucose-Fructose <sub>n</sub>	10–60	β(2→1)
Xylo-oligosaccharides (XOS)	Xylose	1–8	β(1→4)
Lactitol	Galactose-Glucitol	2	β(1→4)
Lactulose	Galactose-Fructose	2	β(1→4)
Polydextrose	(Sorbitol)-Glucose	2–30	β(1→6); β(1→4); β(1→3); β(1→2)
Partially hydrolysed guar gum	Mannose-Galactose	2–7	β(1→4), α(1→6)

mon with dietary fiber, an important difference is the selective fermentability by the colonic microbiota. Most prebiotics would classify as dietary fiber, but not all fiber has prebiotic properties. Examples of prebiotics are presented in Table 2.1. Different production processes and sources may yield prebiotics with a different degree of polymerization, different distribution of linkages etc. It is currently not known whether these variants of the same prebiotic class have similar functional properties.

For the functionality of prebiotics, in particular the ability of the component to increase the level of faecal bifidobacteria has received much attention. In particular inulin and fructo-oligosaccharides (FOS) (Kelly 2008) and to a lesser extend galacto-oligosaccharides (GOS) (Playne and Crittenden 2008) have been documented to increase the level of faecal bifidobacteria. But also other oligosaccharides have been reported to increase faecal bifidobacteria (de Vrese and Schrezenmeir 2008), Table 2.1. The increase in *Bifidobacterium* levels is very much dependent on initial levels and an inverse correlation exists between initial *Bifidobacterium* levels and the increase due to prebiotic consumption (Tuohy et al. 2001). However, bifidogenic activity is not a health benefit *per se*; instead, improvement levels of bifidobacteria may serve as an indicator for intestinal health. A reduction in the levels of pathogenic and opportunistic microbes, and/or improved resistance against pathogenic and opportunistic microbes maybe more important for the host health. In this respect, it is important to realize that many pathogens have their target in the upper gastro-intestinal tract; such as *Helicobacter pylori* (stomach), enterotoxigenic *Escherichia coli* (small intestine) etc. Prebiotics are, in general not expected to be active at these sites. However, colonic pathogens may provide a potential target for prebiotics and protection from antibiotic associated diarrhoea with FOS has been reported (Lewis et al. 2005a) although failures have been reported as well (Lewis et al. 2005b). GOS has been reported to reduce the incidence and duration of traveler's diarrhoea, although the drop-out rate was high in this study (Drakoularakou et al. 2010). Under certain circumstances, prebiotics have been observed to enhance the coloniza-

tion of pathogens. FOS has been reported to increase translocation of *Salmonella* in rats (Bovee-Oudenhoven et al. 2003), and FOS and xylo-oligosaccharides have been observed to increase *Salmonella* translocation in mice, while apple pectin was found to increase *Salmonella* colonization in mice (Petersen et al. 2009).

Prebiotics in general also change the metabolism of the intestinal microbiota; this may have more relevance from a health perspective than a change in its composition. Increases in acetic, propionic and butyric acid have been frequently observed and result in a lowering of the faecal pH. This is thought to suppress the growth and activity of potential pathogenic members of the microbiota and may improve the solubility of minerals such as calcium; enabling an increased absorption of these minerals (de Vrese and Schrezenmeir 2008). The organic acids serve further more as energy sources; acetic acid is metabolised by the liver and muscle tissue, propionic acid is metabolized by the liver as part of gluconeogenesis and butyric acid is the main energy source for colonocytes (Scheppach and Weiler 2004).

By serving as a source of fermentable energy, prebiotics and also other fermentable fibers, direct the metabolism of the colonic microbiota towards so-called saccharolytic direction, instead of proteolytic one. The latter, also referred to as putrefaction, leads to the formation of biogenic amines, phenols and indoles, with potentially detrimental effects on the host. This is particularly true for long chain and complex prebiotics such as inulin and polydextrose (Mäkeläinen et al. 2007).

Health benefits related to the consumption of selected prebiotics include improved mineral absorption and improved bowel function. Other, as yet, less well established health benefits are: modulation of the immune system, reduction of certain markers for colorectal cancer, and positive influences on lipid metabolism. The amounts of prebiotics that need to be consumed in order to obtain a physiological effect are in general at least 4 g (de Vrese and Schrezenmeir 2008). Prebiotics are very safe; they are intrinsic components of the normal diet. Overconsumption of purified prebiotics may lead to diarrhoea which subsides as soon as consumption is stopped.

### ***2.3.2 The Influence of Probiotics on the Intestinal Microbiota***

Probiotics have been defined in many ways over the years. The most widely accepted definition is currently: 'Live microorganisms which when administered in adequate amounts confer a health benefit on the host' (FAO/WHO 2002). Probiotics are thus not restricted to food or feed applications. It is, however important to realise that health benefits cannot be extrapolated from one probiotic strain to another, not even when they are strains of the same species. Examples of probiotics are given in Table 2.2. The selection of probiotics will be described in Sect. 2.4.

Considering the amount of microbes present in the intestine, see above, it is almost surprising that consumption of probiotics (normally at a dose of  $10^9$ – $10^{10}$  CFU/day) affects the composition and activity. Nevertheless, a number of strains have been shown to increase the faecal levels of endogenous bifidobacteria and/or lac-



**Table 2.2** Examples of probiotics

Genus	Species	Strain
<i>Lactobacillus</i>	<i>acidophilus</i>	NCFM, La-5, R-52, La-14
	<i>casei</i> <sup>a</sup>	Lc-11, DN-114 001, Shirota
	<i>rhamnosus</i>	GG, Lr-32, GR-1, Lc-705, R-11, HN001
	<i>johnsonii</i>	La-1
	<i>paracasei</i>	Lpc-37, F-19
	<i>plantarum</i>	299v, Lp-115
	<i>salivarius</i>	Ls-33, UCC-118
<i>Bifidobacterium</i>	<i>reuteri</i>	RC-14
	<i>animalis</i> subsp. <i>lactis</i>	Bb-12, 420, BI-04, Bi-07, HN019, DN-173 010
<i>Bacillus</i>	<i>longum</i>	BB536, 2C, 46, BI-05
	<i>coagulans</i> <sup>b</sup>	BC30, or usually not referred to by strain
<i>Propionibacterium</i>	<i>freudenreichii</i> subsp. <i>shermanii</i>	JS
<i>Saccharomyces</i>	<i>cerevisiae</i> ( <i>boulardii</i> ) <sup>c</sup>	Usually not referred to by strain
<i>Escherichia</i>	<i>coli</i>	Nissle 1917

<sup>a</sup> Many stains that are referred to as *L. casei* are actually *L. paracasei*

<sup>b</sup> Often, incorrectly, referred to as *Lactobacillus sporogenes*

<sup>c</sup> *S. boulardii* is taxonomically considered to belong to the species *S. cerevisiae*

tobacilli (Gopal et al. 2003; Tannock et al. 2000). As with prebiotics, this does not necessarily imply a clinical health benefit, but may rather serve as a marker for improved gut environment.

In contrast to prebiotics, however, substantial indications and proof exists that specific strains of prebiotics are able to protect against or reduce the level and/or activity of pathogens and opportunistic pathogens. Several probiotic strains; e.g. *L. rhamnosus* GG, *B. lactis* Bb-12, *L. reuteri*, etc. have been documented to shorten the duration of rotavirus diarrhoea (Szajewska and Mrukowicz 2001). A number of strains have been shown to reduce the risk and duration of antibiotic associated diarrhoea. In particular *Saccharomyces cerevisiae* (*boulardii*) has been shown to shorten *C. difficile* diarrhoea (McFarland 2006). A potential mechanism of the observed effects is the stabilisation of the endogenous microbiota during antibiotic treatment (Engelbrektson et al. 2005). *Helicobacter pylori* is a pathogen of the stomach and thought to be the aetiological agent of gastric ulcer and carcinoma. In general, probiotics have been unsuccessful in the eradication of *H. pylori*. But, some strains have been shown to reduce the activity of the pathogen. Whether that is sufficient to control disease risk is not known. Better substantiated, however, is the positive effect several strains of probiotics can have on reducing the side effects of anti-*Helicobacter* treatment (Zou et al. 2009). For traveller's diarrhoea, the effects of probiotics are less clear. The reason for this may be the different aetiologies of this type of diarrhoea and the, some times high drop out rate (Sazawal et al. 2006).



In addition to their effects on the microbiota and pathogens of the gastrointestinal tract, selected probiotic strains have been suggested to affect also pathogens of the oral cavity and reduce the colonisation level and activity of cariogenic streptococci and reduce levels of *Candida* (Meurman 2005). There is also ample evidence that selected probiotics may reduce the incidence and/or duration of upper respiratory tract infections (Leyer et al. 2009). It is not clear through what mechanism this effect is obtained. Probiotics have been shown to modulate the microbiota of the upper respiratory tract (Glück and Gebbers 2003), but it cannot be excluded that a modulation of the immune system is a contributing factor. In particular two strains; *L. rhamnosus* GR-1 and *L. reuteri* RC-14 have been investigated for their effects on vaginal infections (Reid 2008).

### 2.3.3 *The Influence of Medication on the Intestinal Microbiota*

Medication may have a substantial effect on the intestinal microbiota and *vice versa*. The clearest example of this is of course alterations in the microbiota caused by antibiotics. Antibiotics, especially those that are poorly absorbed from the gastrointestinal tract can cause substantial disturbance of the microbiota. This disturbance may lead to an increase of antibiotic resistant strains that arise among the bacteria of normal microbiota. This may in turn lead to diarrhoea and infections of other organs (Sullivan et al. 2001).

Also other medication may influence the composition of the intestinal microbiota. Proton pump inhibitors (PPI) disable the antimicrobial capacity of normal gastric secretions. Not surprisingly, their use leads to substantial changes in the gastrointestinal microbiota composition. Organisms originating from the oral cavity, e.g.  $\alpha$ -haemolytic streptococci and corynebacteria, are normally killed by the action of gastric juice; but with the use of PPI they survive and colonise the stomach (Karmeli et al. 1995). This has been hypothesised to lead to increased risk for upper respiratory tract infections and gastroenteritis (Canani et al. 2006). Non-steroidal anti-inflammatory drug (NSAID), have been observed to influence the composition of the intestinal microbiota in elderly subjects. Moreover, use of NSAID by elderly subjects has been found to reduce the level of iso-butyric, iso-valeric and L-lactic acid (Tiihonen et al. 2008).

### 2.3.4 *The Intestinal Microbiota and Stress*

Psychological and physical stress is accompanied with a number of substantial changes in the gastrointestinal tract physiology; inhibition of gastric acid release, alterations in gastrointestinal motility (and thus in transit time), changes in duodenal bicarbonate release, hormonal changes and reduction in mucosal immune function. These changes have mainly been observed to reduce faecal *Lactobacillus* levels

and increases in *Bacteroides* (Hawrelak and Myers 2004). It is, however, likely that more changes in the microbiota take place during stress, but in the past the focus has been limited mainly to these genera.

## 2.4 Selection of Probiotics for Gut Health

The rapid growth of probiotic market, the growing understanding of the importance of gut microbes on human health, and the new discoveries of the health benefits of probiotics have inspired many researchers to search for new potential probiotic strains. In addition, existing strains of probiotics are finding new areas of application, with new targeted health benefits and novel probiotic product applications. Not all probiotics are equal and therefore the suitability of probiotic strains needs to be assessed separately for each new application. In the field of gut health, several widely used and established selection criteria are available. These tools are also applicable for the selection of probiotics against enteric infections. Here, commonly used selection criteria for probiotics are reviewed.

### 2.4.1 Viability During Storage in Probiotic Products

According to the current definition, probiotics need to be viable (FAO/WHO 2002). Therefore, viability of probiotics is of technological, economic, regulatory and biological importance. In products, probiotics are often stored in unfavorable conditions, for example in the presence of acids in fermented products. Good resistance to environmental stresses is therefore required to ensure that probiotics remain viable in the products through-out the shelf-life. Unfortunately this is not always the case, as there are reports suggesting that in some commercial products, the level of viable probiotics does not meet the regulatory criteria in the end of the storage (Gueimonde et al. 2004; Hamilton-Miller et al. 1999).

Stability tests in different product matrices are carried out routinely during the screening of new probiotics and when testing the suitability for established probiotics for new product applications (Laine et al. 2003; Madueira et al. 2005). The tests applied include normal stability tests as well as accelerated stability tests e.g. in elevated storage temperatures. Normally, the viability of probiotics during these tests is monitored using the traditional culture methods, usually plate counting. Recent studies have shown however that plate counting may not always be the optimal choice for determining probiotic viability in food products. Several studies have shown that during storage, probiotics may enter a state in which they cease to grow on plates yet remain viable (Amor et al. 2002; Lahtinen et al. 2006). New culture-independent methods have been developed which are very useful in the viability assays of such probiotics. However, currently the traditional plating techniques dominate the field. Despite that, according to the current definition, probiotics need

to be viable to even be considered as probiotics. Interestingly it appears that also non-viable probiotics may have certain health benefits (Ouweland and Salminen 1998). However, as long as the definition of probiotics includes the requirement for viability, the viability of probiotics during storage will remain an important screening step for the selection of new probiotics.

#### **2.4.2 Resistance to Acid and Bile and Survival in the GI Tract**

For probiotics intended for gut health applications and against enteric infections, the behavior and the fate of the probiotics during the GI passage is particularly important. The mechanisms of the probiotic action against enteric pathogens may involve production of antimicrobial agents locally in the gut—a task which can only be carried out by viable and active probiotics. *In vitro* tests assessing the effect of exposure to different pH values and to the presence of bile, typically mimicking the conditions during the GI transit, are routinely used as a screening tool for potential probiotics (Jacobsen et al. 1999). The results of these *in vitro* screenings usually correlate relatively well with *in vivo* survival of probiotics (Dunne et al. 2001). In addition to simple survival tests, the fate of probiotics in the GI tract can also be assessed with more elaborate *in vitro* assays. Several *in vitro* simulators of the GI tract (Kontula et al. 1998) or the colon (Mäkeläinen et al. 2009) have been developed, and these have been utilized in the analysis of the fate of the probiotic bacteria during simulated GI passage. In addition to *in vitro* testing, probiotic viability during GI transit can also be assessed *in vivo*. In these assessments, probiotics are administered orally and later re-isolated from faecal samples in order to demonstrate that the cells, or at least a proportion of them, have survived during the GI transit. To this regard, several animal models have been utilized (Kaplan et al. 2001; Pavan et al. 2003; Wagner et al. 1997). The gold standard for demonstrating the survival during GI passage is a clinical intervention trial, where faecal colonization of probiotics can be used to assess the survival of the probiotic in the gut, as well as the compliance of the volunteers participating in the study (Fujiwara et al. 2001; Tannock et al. 2000). In some cases, survival of probiotics in the gut has also been assessed with intestinal biopsies (Alander et al. 1997; Valeur et al. 2004).

#### **2.4.3 Adhesion to Host Tissues**

Adhesion of probiotics to host tissues, in particular to intestinal mucus and epithelial cells, is an important selection criterion for probiotics. It is believed that adhesion is important to ensure efficient host-microbial interactions, for example direct interactions between the bacteria and the cells of the host immune system. It has also been suggested that adhesion to host tissues may prolong the persistence of the probiotics in the gut, although it is generally accepted that orally administered pro-

biotics do not colonize the host permanently, and continuous intake of probiotics is required to ensure prolonged presence of the cells in the gut. While it may be postulated that good adhesion properties are needed to ensure direct interactions with the bacteria and the host immune cells, the adhesion to host mucus may not be a strict requirement for probiotic efficacy, as demonstrated by the probiotic strain *L. casei* Shirota. This strain has been linked to different gut health benefits although it does not seem to be a particularly adherent strain (Juntunen et al. 2001). Certain health benefits of probiotic bacteria may be mediated through mechanisms independent of mucus adhesion. Such mechanisms may involve direct cell-to-cell interactions between the gut bacteria, see below. In addition, the health effects may be mediated by the metabolites produced by the probiotic bacteria. In healthy colon, a direct interaction with host epithelial cells and gut microbes, including ingested probiotics, seems unlikely, as it is currently believed that in a healthy colon, a tight inner mucus layer covering the epithelial cells, impassable to microbes due to its tight structure, isolates the host epithelial cells and the bacteria attached to the loose outer mucus layer of the colon (Johansson et al. 2008). In the colon, the probiotic interactions may therefore be mediated mainly by the metabolites of the bacteria. Similar tight inner mucus layer is not present in the small intestine, where direct associations between the microbes and the epithelial cells are plausible.

#### **2.4.4 Interactions with the Host Immune System**

Probiotic bacteria, like other bacteria in the gut, may interact with the cells of the host immune system. For example, dendritic cells have been described to sample the contents of the gut lumen by extending the dendrites past the epithelial cells into the lumen, while M-cells of the Peyer's patches are specialized in antigen presentation from the gut (Mowat 2003). Moreover, bacteria may elicit immune signaling by interacting with the epithelial cells. By interacting with the host cells in the gut, probiotic bacteria may modulate the host immune responses for example by enhancing host innate immune functions (Gill et al. 2001) or by modulating the inflammatory status of the host (Foligne et al. 2007). Immunomodulatory properties are therefore an important selection criterion for probiotic bacteria. Several models for screening of the immunomodulatory properties of probiotics are available. Cell culture studies with epithelial cells (Wong and Ustunol 2006) or immune cells (Braat et al. 2004; Haller et al. 2000; Konstantinov et al. 2008) can be used to give first indication of the immunomodulatory properties of probiotics, as well as to investigate the potential mechanisms behind the host-bacterial interactions. *In vivo* animal models have also proved to be useful in this regard (Daniel et al. 2006; Gill et al. 2000; Kirjavainen et al. 1999; Madsen et al. 2001; Wagner et al. 1997). In addition to pre-clinical trials, clinical interventions have also been used as a selection for probiotics aimed at immune modulation (Paineau et al. 2008; Schiffrin et al. 1997). Immune cells and the epithelial cells are not the only host cells which can be directly affected by gut microbes and probiotics; probiotic strain *L. acidophilus* NCFM has recently

been shown to be able to interact directly also with host pain receptors (Rousseaux et al. 2006).

#### **2.4.5 Interactions Among Gut Bacteria**

Production of anti-microbial compounds has been a popular screening tool for probiotics for gut health (Bevilacqua et al. 2003). In addition to the production of metabolites such as organic acids, probiotic bacteria may also produce specific antibacterial agents, such as bacteriocins (Dobson et al. 2007; Kwak et al. 2001; Tahara et al. 1996). While specific anti-pathogenic action has been demonstrated for many strains, the antimicrobial agent responsible for the activity has not always been characterized in detail, especially in the case of bacteriocins produced by bifidobacteria (Yildirim and Johnson 1998). In addition to production of bacteriocins and bacteriocin-like compounds, and by reducing intestinal pH, probiotic bacteria may also elicit anti-pathogenic effects by blocking the adhesion of the pathogenic strains (prevention or displacement) and by co-aggregating with the pathogens (Collado et al. 2007). Screening of anti-pathogenic actions of probiotics can also be carried out in animal models of pathogen challenge (Shu et al. 2001; Wagner et al. 2000). Pre-clinical trials predominate the screening of anti-pathogenic probiotics. Although clinical intervention trials are the gold standard for documenting probiotic efficacy, human trials are not usually used as screening step for identification of anti-pathogenic strains. While effects of probiotics on the resident beneficial or commensal bacteria have been studied in clinical studies as well as in laboratory fermentations and simulations, such effects have not been used as commonly as a screening tool for probiotics. In the case of prebiotics, the effects on beneficial microbes are used routinely as a screening tool.

#### **2.4.6 Anti-carcinogenic and Anti-toxic Effects**

Effects of probiotics on colon enzyme activity and faecal water genotoxicity have been among the first health effects of probiotics investigated (Goldin and Gorbach 1984). It is postulated that by improving colonic microbiota and metabolic activity, probiotics may reduce the level of genotoxic and carcinogenic compounds in the gut. *In vitro* assessments of anti-carcinogenic effects relying on cell cultures have been utilized as a screening tool for probiotics (Burns and Rowland 2004; Choi et al. 2006). *In vitro* binding tests of mutagenic compounds such as heterocyclic amines (Orrhage et al. 1994) and mycotoxins (El Nezami et al. 1998; Haskard et al. 2001) by probiotic bacteria have also been used in the screening. Moreover, screening of anti-genotoxic and anti-carcinogenic properties of probiotics with animal models has shown that the effects observed *in vitro* may also be seen *in vivo* (Pool-Zobel et al. 1996). Human trials in this area have mainly focused on biomarker analyses

rather than clinical end-points. In addition to mycotoxins, the potential of probiotics to remove other toxins such as cyanotoxins (Nybom et al. 2008) or heavy metals (Halttunen et al. 2007) has been used as a screening tool for probiotics.

#### 2.4.7 *Origin of the Probiotic Strains*

An old dogma of probiotic selection has been that the probiotic strains should be of “human origin”. One may argue that from evolutionary point of view, describing bacteria to be of human origin does not make much sense at all. The requirement for probiotics to be of human origin relates actually to the isolation of the strain rather than the “origin” itself. Usually, the strains claimed to be “of human origin” have been isolated from faecal samples of healthy human subjects, and have therefore been considered to be “part of normal healthy human gut microbiota”. In reality the recovery of a strain from a faecal sample does not necessarily mean that this strain is part of the normal microbiota of this individual, since microbes passing the GI tract transiently can also be recovered from the faecal samples. This can be demonstrated also by the established probiotic strains: if an individual consumes a yoghurt with a certain probiotic strain, and this strain is later isolated from a faecal sample from the same individual, this does not mark a discovery of a “new strain of human origin”, but rather a re-isolation of the original probiotic strain transiently passing through the GI tract. In practice it is impossible to know the actual origin of the probiotic strains, regardless of whether they have been isolated from faecal samples, fermented dairy products or any other source for that matter. Isolation of a strain from faeces of a healthy individual is also not a guarantee of the safety of the strain—such a sample will also always contain commensal microbes which can act as opportunistic pathogens, or even low levels of true pathogens, which are present in the individual at sub-clinical levels. The authors therefore recommend that instead of concentrating on the first point of isolation, the selection processes for new potential probiotic strains should mainly focus on the functional properties of the probiotic strains rather than the “origin” (Ouwehand and Lahtinen 2008).

#### 2.4.8 *Safety*

Safety is a critically important feature of probiotics. Most commonly used probiotics belong to *Lactobacillus* spp. or *Bifidobacterium* spp., which have a long history of safe human consumption and are generally considered as safe (EFSA 2007). Despite the excellent safety record of probiotics, safety remains to be an important selection criterion for probiotics. Especially in the case of probiotics which do not belong to *Lactobacillus* spp. or *Bifidobacterium* spp., demonstration of safety is of particular importance. For example, in some cases bacteria belonging to *Enterococcus* spp. are used as probiotics, which has raised concerns of potential spread of

antibiotic resistance genes and presence of virulence determinants (Eaton and Gasson 2001; Vankerckhoven et al. 2008). Also strains belonging to *Bacillus* spp. and strains of *E. coli* have been used as probiotics. *In vitro* safety screenings of probiotics may include, among others, antibiotic resistance assays, screenings for virulence factors, resistance to host defence mechanisms and induction of haemolysis (Asahara et al. 2003; Eaton and Gasson 2001; Tompkins et al. 2008; Vankerckhoven et al. 2008; Vesterlund et al. 2007). Several different animal models have been utilized in the safety assessment of probiotics. These include models of immunodeficiency (Wagner et al. 1997), endocarditis (Asahara et al. 2003), colitis (Daniel et al. 2006) and liver injury (Osman et al. 2005). In some cases even acute toxicity of probiotics has been assessed (Zhou et al. 2000). Last but not least, also clinical intervention trials have yielded evidence on the safety of probiotics for human consumption (Dekker et al. 2009; Laitinen et al. 2005; Mäkeläinen et al. 2003; Saarela et al. 2007; Wolf et al. 1995).

#### **2.4.9 Suitability for Commercial Production**

A critically important and sometimes overlooked aspect of probiotic selection is the suitability of the strain for large-scale industrial production. While a new probiotic candidate strain may have interesting properties in laboratory analyses and in pilot trials, it can never become a commercial probiotic consumed daily by customers, if it cannot be produced cost-efficiently in an industrial scale. Discovery of such strain will be of academic interest only. Strains which have too stringent requirements for rapid growth cannot be produced efficiently in large scale and their cost-in-use may therefore be too high. In addition to growth requirements, technological properties such as stability during processing and in finished formulations are of utmost importance.

### **2.5 Conclusions**

The intestinal microbiota has a major role in regulation of GI function and host health. The sterile GI tract in a foetus is rapidly colonized after birth by opportunistic bacteria originating from the mother and the surrounding environment. The microbiota develops in a predictable fashion into a complex system with increasing age, determined by internal and external factors. This complex system is quite stable with a majority of obligate anaerobes, and dominated by species from the phylum *Firmicutes* and *Bacteroidetes*. However, the composition and activity of the intestinal microbiota can be influenced by external or environmental factors such as, medication or food. The probiotic market has increased rapidly during the last years, and a large variety of probiotic foods and supplements are available. With probiotics it is possible to alter the intestinal microbiota in order to improve the



health of the host or the gut microbiota. For example, the endogenous microbiota can be stabilised during an antibiotic treatment with probiotics, and the incidence and/or duration of infections may be reduced. The growing understanding of the importance of gut microbes on human health leads to new application areas for probiotics, with new targeted health benefits and novel probiotic product applications. However, it is still important to ensure the safety and the suitability for commercial production when selecting probiotics for gut health.

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