GUT MICROBIOTA IN EARLY INFANCY: EFFECT OF ENVIRONMENT, DIET AND PROBIOTICS

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ABSTRACT

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Gut microbiota in early infancy: effect of environment, diet and probiotics
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The colonisation of the infant gut starts prior to birth and continues throughout life. Specific factors may influence the microbiota composition in infants. The main purpose here was to characterise the compositional development of the gut microbiota in early infancy. A particular focus of interest was the impact of specific factors such as mother's gut microbiota, environment, dietary habits and probiotics, which may influence this process.

Maternal *B. bifidum* and *B. breve* colonisation increased the infants' probability of being colonised by these species. High maternal levels of *Bifidobacterium* genus and *B. longum* correlated with high levels of the same bacteria in infants at 1 and 6 months of age.
Gut microbiota analyses of infants from different geographical locations revealed bifidobacteria to be dominant at 6 months of age in Malawian, Finnish and German infant populations. Distinctions were related to differences in bifidobacteria, clostridia, *Bacteroides* and *Akkermansia*, between Malawian and Finnish, and Finnish and German infants. Different feeding practices in Malawian and Finnish and German infants had an impact on microbiota composition and development in infants. Maternal and infant probiotic intervention with specific study strains modulated the gut microbiota in 6-month-old infants. The properties of the probiotic strains used in studies on human subjects were altered by the production process; the probiotic *L. rhamnosus* GG isolates obtained from different sources of probiotic products showed different properties in pathogen exclusion from the mucus.

Factors such as maternal microbiota, infant's diet, hygiene and use of probiotics guide the intestinal microbiota composition in infants and this may have an impact on early programming for later health. In addition, the quality of probiotic products should be evaluated prior to clinical intervention studies.

Keywords: gut microbiota, mother, infant, probiotic, nutrition, breast-feeding, formula-feeding, *Bifidobacterium, Lactobacillus rhamnosus* GG, adhesion

ABBREVIATIONS

AAD	antibiotic-associated diarrhoea
Bb12	*Bifidobacterium lactis* Bb12
BL999	*Bifidobacterium longum* (ATCC: BAA-999)
CI	confidence interval
DI	diversity index
ERIC-PCR	enterobacterial repetitive intergenic consensus-PCR
FAO	Food and Agriculture Organisation
FCM-FISH	flow cytometry-FISH
FISH	fluorescent *in situ* hybridisation
FOS	fructo-oligosaccharides
GALT	gut associated lymphoid tissue
GIT	gastrointestinal tract
GOS	galacto-oligosaccharides
IBD	inflammatory bowel disease
IBS	irritable bowel syndrome
IgA	immunoglobulin A
IQR	interquartile range
LAB	lactic acid bacteria
LGG	*Lactobacillus rhamnosus* GG
LPR	*Lactobacillus rhamnosus* (CGMCC 1.3724)
NEC	necrotising enterocolitis
PCR	polymerase chain reaction
PFGE	pulsed field gel electrophoresis
qPCR	quantitative-PCR
RCTs	randomised controlled trials
RAPD	randomly amplified polymorphic DNA
SCFA	short-chain fatty acids
SD	standard deviation
SI	similarity index
ssp.	subspecies
ST11	*Lactobacillus paracasei* (CNCM 1-2116)
WHO	World Health Organisation
16S rDNA	ribosomal bacterial 16S DNA coding gene of 16S rRNA
16S rRNA	ribosomal bacterial 16S RNA molecule

ORIGINAL PUBLICATIONS

I Grönlund MM, Grześkowiak Ł, Isolauri E, Salminen S. Influence of mother's intestinal microbiota on gut colonisation in the infant. Gut Microbes 2011;2:1-7.

II Grześkowiak Ł, Collado MC, Mangani C, Maleta K, Laitinen K, Ashorn P, Isolauri E, Salminen S. Distinct gut microbiota in South Eastern African and Northern European infants. Submitted.

III Grześkowiak Ł, Grönlund MM, Beckmann C, Salminen S, von Berg A, Isolauri E. The impact of perinatal probiotic intervention on gut microbiota: double-blind placebo-controlled trials in Finland and Germany. Anaerobe 2011, doi:10.1016/j.anaerobe.2011.09.006.

IV Grześkowiak Ł, Isolauri E, Salminen S, Gueimonde M. Manufacturing process influences properties of probiotic bacteria. Br J Nutr 2011;105:887-94.

In addition, some unpublished results are presented.

1. INTRODUCTION

The human gastrointestinal tract is the home of a vast community of microbes, which include bacteria, viruses and yeasts. Microbes are spread throughout the gut, the majority however being present in the colon. These indigenous microbes co-exist with the host in a beneficial mutual relationship crucial for the maintenance of health at all stages of life (Guarner and Malagelada 2003).

Diet, ethnicity, sanitation, hygiene, geography and climate have been found to have a long-term impact on the succession of microbes in infants. Differences in microbiota composition characterise infants living in different parts of the world, but bifidobacteria dominate in the infant microbial ecosystem (Favier et al. 2002, Fanaro et al. 2003). Differences in bifidobacterial composition and level, again, may appear in infants living in different countries (Fallani et al. 2010).

Aberrancies in the compositional development of the microbiota have been documented as increasing the risk of immunological and inflammatory diseases (Kalliomäki et al. 2001a, 2008) both in children and in adults. This increase may in part be attributable to changes in host-microbe interactions caused by implementation of different antimicrobial strategies (i.e. excessive use of antibiotics, improved hygiene and Western diets). Moreover, it is increasingly evident that gut microbes may influence energy harvesting and storage and shape the host metabolic network, thereby impacting on the growth pattern and risk of obesity and related disorders (Ley et al. 2006).

During recent years beneficial bacteria, so-called probiotics, have been suggested to play an important role in gut microbiota modulation and activity. Probiotics may thus maintain the health of the host, contributing to immune function and defence against certain disorders (Collado et al. 2009). Specific probiotics with health benefits are selected based on their *in vitro* and *in vivo* properties. However, such properties may in fact be influenced by the production process and this may have an impact on their efficacy in human studies.

The aim of the present work was to characterise the compositional development of the gut microbiota in early infancy, in particular the microbial link between mother and infant during the perinatal period and the role of diet, environment and specific probiotic use, which influence the microbiota colonisation process.

2. REVIEW OF THE LITERATURE

2.1 Microbial community in the gastrointestinal tract

The condition and function of the gastrointestinal tract (GIT) are crucial to our wellbeing. The GIT constitutes one of the largest body surface areas, estimated to be approximately 250 to 400 m^2 in area and 7 m long in adults. The GIT harbours a microbiota of at least 500-1000 different bacterial species comprising nearly 2 million genes. It is populated with up to 10^{14} microbial cells, which is estimated to be 10 times the number of human cells. This rich microbiota is regarded as an active "organ" of the human body (Magalhaes et al. 2007, Artis et al. 2008, Ley et al. 2008, Zoetendal et al. 2008).

Recent reports indicate that 80-90% of the bacterial phylotypes are members of *Bacteroidetes* phylum, including *Bacteroides* and *Prevotella* genus, and *Firmicutes* phylum, including *Clostridium*, *Enterococcus*, *Lactobacillus* and *Ruminococcus* genus. Other phyla present in the gut belong to *Actinobacteria,* including the *Bifidobacterium* genus, and *Proteobacteria,* including *Helicobacter* and *Escherichia* (Eckburg et al. 2005, Turroni et al. 2008, Tap et al. 2009).

The bacteria of the human gut include indigenous (native) species which permanently colonise the GI tract, and a variable set of living microorganisms which transit temporarily through the tract. The indigenous bacteria are mainly acquired at birth and during the first years of life and have sometimes been classified as either potentially harmful or health-promoting; most of them, however, constitute part of the commensal microbiota. Transient bacteria are continuously being ingested from the environment, this including food.

The number and composition of microbes vary along the GIT and concomitant with environmental conditions and the age, gender, country and health status of the host. The dominant microbial genera inhabiting the human GIT are presented in **Figure 1.** The mouth is colonised by facultative and strict anaerobes including streptococci, *Bacteroides*, lactobacilli and yeasts. The oesophagus has no microbiota of its own; bacteria originate from the oral cavity, the respiratory system and food. Microbiota numbers in the stomach and duodenum are low due to flow and low pH in the stomach and secretion of bile and pancreatic juice in the duodenum; the colonisation is mainly represented by facultative anaerobes and yeasts. The resident found in the stomach is *Helicobacter pylori*. In the

jejunum and ileum bacterial numbers gradually increase, reaching their highest level and diversity in the colon. The microbiota of the jejunum and ileum is represented by facultative anaerobes and anaerobes. The majority of species in the colon are obligate anaerobic microorganisms forming a complex and dynamic ecosystem (Bik 2009, Tan et al. 2011).

The gut microbiota may exist in the lumen or be associated with the intestinal mucosa, forming a biofilm. *Bacteroides, Eubacterium, Bifidobacterium, Lactobacillus*, Gram-positive cocci and *Clostridium* represent lumenal (planktonic) bacteria, whose number and composition are affected by the diet. The mucosally-associated bacteria, for example *Bacteroides, Fusobacterium, Spirochaetes, E. coli, Helicobacter, Bifidobacterium* and Gram-positive cocci, are less affected by the diet, remain more stable and have the ability to regulate the lumenal bacteria (Probert and Gibson 2002, Ouwehand et al. 2004).

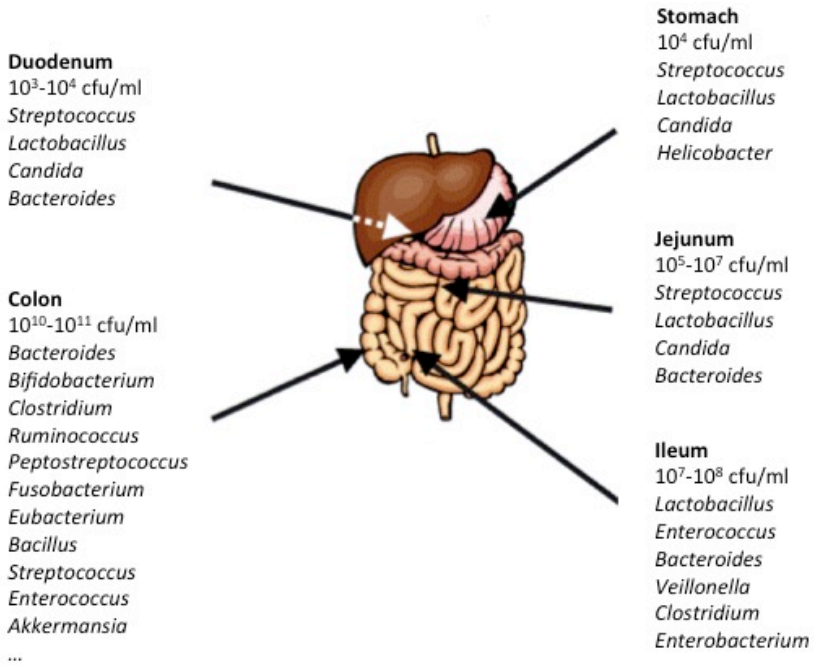

Figure 1. The human gastrointestinal tract and its dominant microbial genera (modified after Ouwehand and Vesterlund 2003).

The gastrointestinal tract is covered by epithelial cells and mucus, which form a barrier between the external and internal environment of the human body. The mucus is a viscous gel composed mainly of high-molecular-mass-glycoproteins called mucins; water, lipids,

other proteins and salts. The mucus layer has a very large surface area (300 m^2) forming an ecological niche for intestinal microbiota. The major function of the mucus is to protect the epithelial cells from damage caused by chemical, enzymatic and mechanical activity in the GIT. The mucus also forms a barrier to protect the underlying epithelium from the attachment of pathogens and serves as a source of nutrients for some commensal bacteria such as *Bacteroides* and *Akkermansia* (Salyers et al. 1988, Guarner and Malagelada 2003, Derrien et al. 2004).

The activity of the intestinal microbiota is high, involving metabolic processes such as fermentation of exogenous and endogenous carbon and energy sources. Fermentation of different oligosaccharides results in the production of short-chain fatty acids (SCFA), which provide the host with additional energy and maintain mucosal health (Mortensen and Clausen 1996). Some members of the intestinal microbiota, for example bifidobacteria, lactobacilli and *Bacteroides* are involved in the production of vitamins B and K (Strozzi and Mogna 2008). Additionaly, intestinal microbes are able to utilise other substrates such as proteins and amino acids, which may lead to the production of a variety of toxic substances such as tumour inducers. The intestinal microbiota is considered an important constituent in the mucosal defence barrier. It provides protection against incoming microbes via competition for the same attachment sites as these, uses the same nutrients as pathogens, and produces numerous compounds inhibiting the growth of undesirable bacteria (Magalhaes et al. 2007). The gut microbiota also stimulates the maturation of the immune system. Exposure to microbes increases the number of specific immune components, which promote the immunological barrier of the gut mucosa (Grönlund et al. 2000).

2.2 Development of gut microbiota

The newborn child is continuously exposed to new environmental microbes which enter the gastrointestinal tract with food and drink (**Figure 2**).

The development of the gut microbiota passes through the different life stages, especially infancy and weaning.

2.2.1 Infancy

The gastrointestinal tract of a foetus has been generally considered microbiologically sterile, but there are indications of exposure to bacteria already prior to delivery (Jiménez et al. 2005, Onderdonk et al. 2008, Satokari et al. 2009).

After birth, the infant gut begins to be gradually colonised by a rapidly diversifying microbiota. The first microbes to colonise are facultative anaerobes such as enterobacteria, coliforms, lactobacilli and streptococci, followed on the 2^{nd}-3^{rd} day by anaerobes such as bifidobacteria, *Bacteroides*, clostridia and eubacteria as the main microorganisms in 1-2-week-old infant faeces (Benno and Mitsuoka 1986, Park et al. 2005). Molecular methods have also demonstrated the presence of other types such as *Ruminococcus* and *Akkermansia*-like bacteria at an early stage in the infant gut (Favier et al. 2002, Collado et al. 2007a).

The most common bifidobacteria present in infant faeces are *B. longum, B. infantis* and *B. breve* (Roger et al. 2010). Among lactobacilli the most abundant species found are *L. gasseri* and *L. johnsonii,* both belonging to the *L. acidophilus* group (Morelli et al. 1998).

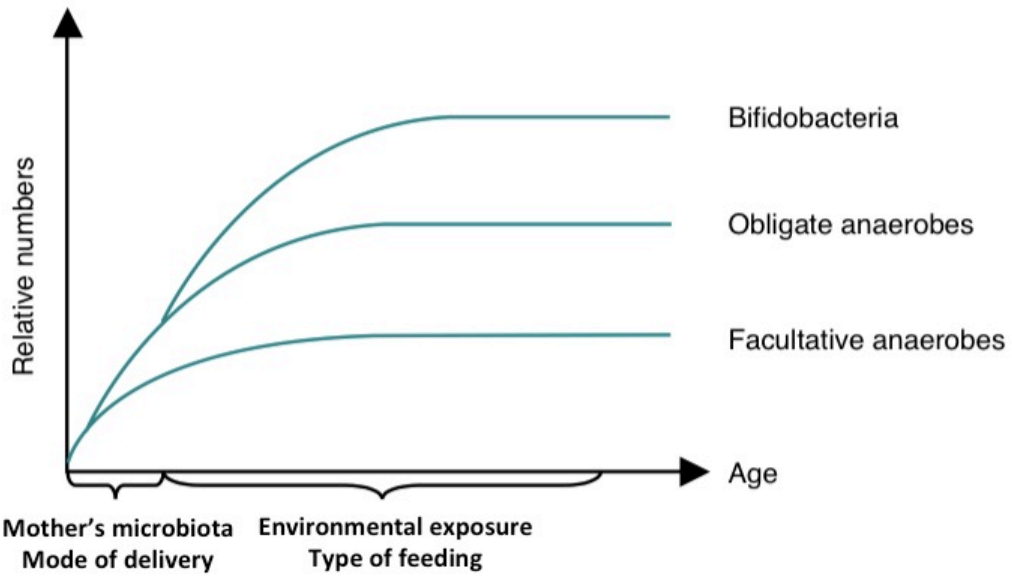

Figure 2. The establishment of the gut microbiota – a step-wise process (modified after Benno and Mitsuoka 1986).

2.2.2 Early life

After the first 6 months of life, when solid food is introduced to the diet, the gut microbiota becomes more diverse. The succession of *Bacteroides*, *Clostridium* and anaerobic cocci increases rapidly while the proportion of bifidobacteria decreases and becomes more stable. The changes in microbiota composition during weaning are more drastic in breast-fed than in formula-fed infants. After weaning the gut microbiota continues to develop towards the adult status and after 2 years of age the composition is practically identical to that of an adult (Magne et al. 2006, Morelli 2008).

2.3 Factors affecting gut microbiota development and composition in infants

Microbiota development in the infant is rapid and depends on the first inoculum, the mother's microbiota, mode of delivery, type of feeding and the environment, including weaning food practices and the use of antimicrobials (**Figure 2**), (Favier et al. 2002, Adlerberth and Wold 2009).

Maternal microbiota

It has been hypothesised that the maternal intestinal bacteria or their components are present in the placenta and that the foetus may be exposed to them. Recent studies have shown that horizontal transfer of bacterial DNA from mother to foetus may occur via placenta (Satokari et al. 2009).

In a study conducted by Gueimonde and collaborators (2006) administration of specific probiotics to the mothers during pregnancy was seen to influence microbiota establishment in newborn infants. This suggests that specific changes in the transfer and initial establishment of microbiota in neonates take place as a consequence of the consumption of probiotics by the mothers.

Mode of delivery

The mode of delivery has a significant effect on the microbial colonisation of the infant gut. Vaginally delivered infants come into contact with the maternal vaginal and faecal

microbiota. This results in gut colonisation by microbes originating from the mother's birth canal. The maternal gut is the most important source of *E. coli,* bifidobacteria and *Bacteroides* in the early colonisation of the infant gut. Colonisation with these bacteria is delayed in infants born by caesarean section. These infants are more often colonised with clostridia and less with bifidobacteria and lactobacilli (Grönlund et al. 1999, Penders et al. 2006, Marques et al. 2010).

Type of feeding

The type of feeding, together with the above-mentioned mode of delivery, exert the most significant influences on the development of the microbiota in the infant. The preferred food for the infant is breastmilk, which in healthy mothers contains from 10^5 to 10^9 bacteria per mL (Morelli 2008). Breast-feeding is strongly recommended, as many protective factors are transmitted via breastmilk. Breastmilk delivers numerous bioactive components to the infant's gut, which influence microbial colonisation and maturation of the intestinal mucosa and immune system. Breastmilk is also an important source of bifidobacteria and lactic acid bacteria, which further facilitate the impact of the mother in gut colonisation of the infant (Gueimonde at al. 2007, ESPGHAN 2009, Beattie and Weaver 2011).

The intestinal microbiota of healthy breast-fed infants is composed mainly of bifidobacteria (Grönlund et al. 2007, Roger et al. 2010), which may reach up to 60-90% of the total faecal microbiota (Favier et al. 2002). Other microbes include *Staphylococcus, Streptococcus, Corynebacterium, Lactobacillus, Micrococcus* and *Propionibacterium,* originating from the nipple, milk ducts and the surrounding skin.

The profile of formula-fed infants is more complex and similar to that of adults, predominant facultative anaerobes being *Bacteroides* and *Clostridium,* followed by *Staphylococcus, Streptococcus* and *Enterobacteriaceae.* Colonisation by bifidobacteria is delayed (Matsuki et al. 1999, Harmsen et al. 2000, Grönlund et al. 2007, Marques et al. 2010). The target of infant formula research has been to develop formulas to closely resemble breastmilk (Rinne et al. 2005).

Lifestyle

Lifestyle, comprising such factors as family structure and home environment, may have a significant impact on gut microbiota composition. A recent study reports that infants

without older siblings have more non-*E. coli* enterobacteria and clostridia and a lower ratio of anaerobic to facultative bacteria by one year of age compared to children with siblings (Adlerberth et al. 2007). In another study, conducted by Penders and collaborators (2006), single children tended to have lower counts of bifidobacteria in the gut at one month of age than infants with older siblings. A recent study conducted by Ege and coworkers (2011) shows that children living on farms are exposed to a wider range of microbes than those who are not living on farms. This would suggest a more mature colonisation pattern in children with older siblings and those living on a farm and may prove relavant to later health.

Geographic location

Several groups have reported that geographic location may affect the composition of the gut microbiota. A recent study conducted by Fallani and coworkers (2010) shows a "geographical gradient" in the microbiota composition across young Europeans. The 6-week-old infant faeces from northern European locations (Glasgow and Stockholm) contained higher proportions of *Bifidobacterium*, *Atopobium, Clostridium perfringens, C. difficile* than in infants from the south (Granada and Reggio Emilia) at the same age, containing higher proportions of *Bacteroides*, *Eubacterium* and *Lactobacillus*. Based on numerous reports, it seems that geographical differences in microbiota composition result from different dietary habits in the region or country in question. In a recent study by De Filippo and collaborators (2010), again, it was demonstrated that children living in rural villages in Burkina Faso are characterised by an enrichment of *Bacteroides* as against *Enterobacteriaceae* in Italian children. It is noteworthy that the above-mentioned Burkina Faso subjects were characterised by a higher intake of dietary fibre such as GOS (galacto-oligosaccharides) and FOS (fructo-oligosaccharides) in their diet, which may have significantly guided microbiota composition. Differences in microbiota composition have also been reported between infants from two northern European countries. Sepp and coworkers (1997) found high counts of lactobacilli, eubacteria and enterococci in Estonian infants whereas Swedish infants had high numbers of clostridia and bacteroides in their faeces.

Dietary strategies: antibiotics, probiotics and prebiotics

The development of the infant's gut microbiota can be influenced by the use of antibiotics, probiotics and prebiotics, which can shape the microbiota composition. Antibiotics are very commonly used to treat specific infections, often however without considering their impact on the gut. Antimicrobials are not only selective for pathogens, but fundamentally affect all members of the commensal gut microbial ecosystem, especially reducing the levels of anaerobic bacteria, *Bacteroides*, *E. coli* and beneficial bifidobacteria, and increasing the levels of potentially harmful clostridia and *Klebsiella* (Penders et al. 2006). Infants treated with antibiotics during hospitalisation may develop complications such as antibiotic-associated diarrhoea (AAD). Such symptoms may be due to the disturbances in the intestinal microbiota composition and activity followed by an overgrowth and adhesion of pathogens such as *C. difficile* on the intestinal epithelium (McFarland 1998, Sun et al. 2011).

Probiotics and prebiotics have been proved to beneficially modulate the gut microbiota and restore the disturbed microbial ecosystem after antibiotic therapy (Sartor 2004, Engelbrektson et al. 2009).

Other factors

The bacterial composition in the gut may also be affected by other factors which in fact conceivably play a role in the development of the gut microbiota. Gestational age or whether the birth was full- or pre-term have been found to have a prominent role in determining the colonisation of the gut (Penders et al. 2006). If infants are separated from their mothers for long periods after birth, being for example hospitalised, the environment becomes an important source of colonising bacteria (Murono et al. 1993, Savino et al. 2011). Other factors include pH in the stomach, peristaltis in the intestines, the gut immune system and stress (Kirjavainen and Gibson 1999, Hawrelak and Myers 2004).

Taken together, the basis of a healthy gut microbiota lies in early infancy and the initial process of intestinal colonisation, this forming an important prerequisite for later health. The original inoculum and early feeding are thus of interest in defining early development. Furthermore, such development should be compared in different countries.

2.4 Host-microbe interactions

2.4.1 Host-microbe cross-talk

At birth, the immune system is immature and develops upon exposure to microbes. The intestinal microbiota has a significant impact on the intestinal mucosal barrier function and intestinal maturation. It is also necessary for full development of the body's largest collection of lymphoid tissue, the gut-associated lymphoid tissue (GALT) (Vael and Desager 2009). The initial neonatal microbial colonisers are also important in stimulating immune development. It has been shown that the absence of early microbial stimuli in germ-free mouse models causes defects in intestinal barrier function and reduced inflammatory responses (Sudo et al. 1997, Sellon et al. 1998). Inoculation of germ-free mice with an intestinal microbiota leads on the other hand to a rapid expansion of the immune system. The maintenance of immune homeostasis takes place through the constant interactions between commensal bacteria in the intestinal lumen and the epithelial and immune cells within the gut.

Probiotic bacteria are able to modulate immune responses, enhance epithelial barrier function and inhibit pathogen growth. The immunomodulatory properties of commensal bacteria and probiotics appear to be markedly bacteria-specific and environment-dependent (Christensen et al. 2002, Perdigon et al. 2002).

2.4.2 Bacterial adhesion and interaction with pathogens

Adherence of bacteria to the intestinal mucus is known to be a prerequisite for colonisation and infection of the gastrointestinal tract by many pathogens. Adhesion is also one of the bacterial virulence factors (Finlay and Falkow 1997, Juntunen et al. 2001). The mechanism of adhesion is complex and involves interactions between microbe and surface. The structures responsible for bacterial adhesion, adhesins (for example flagella, fimbriae, and cell wall proteins, carbohydrates and lipoteichoic acids), are associated with the cell surface and have been suggested to be involved in the adhesion of bacteria to intestinal surfaces.

Adhesins have the ability to recognise oligosaccharide residues of glycoproteins or glycolipids on the surface of host cells (MacKenzie et al. 2009).
The attachment of bacteria to intestinal surfaces is mediated by numerous factors, including those related to gastrointestinal conditions such as pH, digestive enzymes and the presence of bile, calcium, magnesium and zinc, and the mucin concentration (Ouwehand et al. 2001a, Ouwehand and Salminen 2003, Lahtinen and Ouwehand 2009, Sanchez et al. 2010). Also the age and the health status of the host both affect the adhesive potential of bacteria (Ouwehand et al. 1999, He et al. 2001a, b).

The adhesion of probiotics to intestinal surfaces has been widely studied. Adherent probiotic strains may at least temporarily colonise the gastrointestinal tract and compete with pathogens for binding sites (Vesterlund et al. 2006). Several reports have described the inhibitory effects of probiotic strains on model pathogen adhesion to the human mucus (Ouwehand et al. 2001b, Collado et al. 2007c, e).
The ability to inhibit the adhesion of pathogens by probiotics shows high specificity and depends on both the probiotic and the pathogen strains. It has been recognised that the exclusion of pathogens is never related to the adhesive ability of the probiotic tested (Bibiloni et al. 1999, Collado et al. 2009). Also different mechanisms are involved in the inhibition, displacement and competition of pathogens with probiotics for the sites on the mucus.

2.5 Probiotics

Currently, a probiotic is defined as a “live microorganism which when administered in adequate amounts confers a health benefit on the host” (FAO/WHO 2002).

Most probiotics are bacteria and belong to the *Lactobacillus* and *Bifidobacterium* genera, for example *Lactobacillus rhamnosus, L. acidophilus, L. casei, L. gasseri, L. reuteri* and *Bifidobacterium lactis, B. longum, B. breve, B. bifidum*. Other bacteria considered as probiotics include enterococci such as *Enterococcus faecalis, E. faecium,* and propionibacteria such as *Propionibacterium freudenreichii* ssp. *shermanii*, and also

Streptococcus thermophilus, Lactococcus lactis, Clostridium butyricum, E. coli and others. Yeasts such as *Saccharomyces boulardii* and *Aspergillus oryzae* are also regarded as probiotics (Seki et al. 2003, Saxelin et al. 2005, Gupta and Garg 2009).

Lactobacilli and bifidobacteria are Gram-positive, non-motile, non-spore forming, catalase-negative rods. Lactobacilli form the major part of the lactic acid bacteria (LAB) group and comprise more than 120 species. As members of LAB, they produce lactic acid as the main or one of the main end products of carbohydrate metabolism. The genus *Bifidobacterium* includes 29 species. Lactic acid is the end product of bifidobacterial fermentative metabolism; however, bifidobacteria differ from LAB phylogenetically and by a different sugar fermentation pathway (Axelsson 1998, Tannock 1999, Ballongue 2004).

2.5.1 Selection criteria and safety assessment

The guidelines for the evaluation and selection of probiotics for food use were established by the Food and Agriculture Organisation of the United Nations (FAO) and World Health Organisation (WHO) groups in 2002 (FAO/WHO 2002).

It was established that probiotics should be characterised up to strain level, as probiotic effects are strain-specific. Identification of a strain should link it to a specific health effect and enable accurate surveillance and epidemiological studies.

It was recommended that a probiotic strain should be identified by the most up-to-date, valid methodology such as combination of phenotypic and genotypic tests, and that all strains should be deposited in an internationally recognised culture collection.

Hitherto, specific probiotics have been selected and characterised based on their *in vitro* properties, for example tolerance to gastrointestinal conditions (resistance to gastric acidity, bile acid resistance), adhesion to the intestinal mucus or epithelial cells and competitive exclusion of target pathogens, bile salt hydrolase activity and resistance to spermicides (applicable to probiotics for vaginal use) (Tuomola et al. 2001, FAO/WHO 2002, Ouwehand et al. 2002, Collado et al. 2007d).

The efficacy of probiotics for humans should be proved in human trials and benefits should be reflected in improvements in condition, symptoms, signs, well-being or quality of

life, reduced risk of disease or longer time to next occurrence, or faster recovery from illness.

Standard methods for clinical evaluations involve 4 phases: Phase 1 is concerned with safety, Phase 2 efficacy (form of randomised, double-blind, placebo-controlled design, measuring efficacy compared with placebo; also assesses adverse effects), Phase 3 effectiveness and Phase 4 surveillance (FAO/WHO 2002).

The use of probiotics has increased significantly; new and more effective probiotic strains are constantly appearing on the market. It is recommended that probiotic foods be properly labeled with strain designation, minimum numbers of viable bacteria and the end of shelf-life, storage conditions and manufacturer's contact details, and importantly – health claim(s) (FAO/WHO 2002). However, numerous probiotic products available on the market lack this information.

In general, both LAB and bifidobacteria have a good safety record; since they are present in the normal intestinal microbiota of humans and have a long history of safe use, they are rarely associated with side-effects (Ishibashi and Yamazaki 2001).

It is of importance for novel probiotic strains to have their safety ensured and conform to all local regulations. The safety evaluation procedures proposed by FAO/WHO (2002) recommend that probiotic strains be characterised by the following tests:

1. Determination of antibiotic resistance patterns
2. Assessment of certain metabolic activities
3. Assessment of side-effects during human studies
4. Post-market monitoring
5. If the strain being evaluated belongs to a species known to produce a mammalian toxin or to have haemolytic potential, it must be tested for these characteristics.

Probiotics may theoretically exert a variety of side-effects such as infections, metabolic effects, adverse immunological events, gene transfer, and antibiotic resistance (FAO/WHO 2002, Marteau and Shanahan 2003). Reports of bacteriaemia involving bifidobacteria or *L. rhamnosus* GG are rare (Salminen et al. 2002, Mahlen and Clarridge 2009, Whelan and

Myers 2010). If infection has occurred and *L. rhamnosus* GG has been found, the subject has always been predisposed to infection by surgical trauma or underlying health disorders and the probiotics have formed part of a mixed culture bacteriaemia involving other benign commensal bacteria. However, there are reports showing other lactobacilli and also *Saccharomyces* to have been involved in several onset of bacteriaemia (Mackay et al. 1999, Salminen et al. 2004) and fungaemia (Hennequin et al. 2000) infection, but these have occurred in groups of patients whose conditions predisposed them to opportunistic infections. So far no negative side-effects of probiotics have been reported in preterm infants. In addition, in Finland, for example, *L. rhamnosus* GG has been widely used in very-low-birth-weight preterm infants since 1997 without major adverse side-effects (Luoto et al. 2010).

2.5.2 Health effect of probiotics

Probiotic microorganisms may act directly on the host or via modulation of the host intestinal microbiota.

Documented health effects of specific probiotics have been obtained in randomised controlled trials (RCTs) among children in the prevention and treatment of acute gastroenteritis (antibiotic-associated diarrhoea, rotavirus diarrhoea, respectively), prevention of allergy, prevention of necrotising enterocolitis (NEC) in very-low-birth-weight infants, strenghthening of the immune system and modulation of the immune response (**Figure 3**).

According to several studies, specific probiotic strains have been successful in the prevention and/or treatment of some types of diarrhoea in children. The beneficial effects have resulted from the exclusion of diarrhoeal pathogens from the intestinal mucosa, balancing the commensal gut microbiota and modulating the immune response.

A common complication during antibiotic treatment is antibiotic-associated diarrhoea. The risk factors include broad-spectrum antibiotics, host physiological factors and the hospital

environment. AAD results from disturbance of the intestinal microbiota, which serves as a barrier to opportunistic pathogens. Based on meta-analyses of published results of RCTs, most of the tested probiotics, for example *Lactobacillus rhamnosus* GG (LGG) (Szajewska et al. 2006, Ruszczyński et al. 2008), *B. lactis* and *S. thermophilus*, both commercial strains (Corrêa et al. 2005), and *S. boulardii*, (Kotowska et al. 2005) can significantly reduce the incidence of AAD in children.
However, there are no reports on RCTs among children treated with probiotics against AAD or *Clostridium difficile* AAD.

Acute rotavirus diarrhoea is a common paediatric disease with high mortality worldwide. In several RCTs it has been shown that probiotics such as LGG (Guandalini et al. 2000), *S. thermophilus* St065 (Thibault et al. 2004), *L. casei* DN-114 001 (Pedone et al. 2000)*, B. lactis* Bb12 *and L. reuteri* ATCC 55730 (Weizman et al. 2005), mixed with milk or infant formula or given as an oral supplement, are effective in the prevention of acute rotavirus diarrhea, reducing and shortening the episodes in children.
The role of probiotics in the treatment of acute rotavirus diarrhoea in children has been well documented; *S. boulardii* (Billoo et al. 2006) and LGG (Szymański et al. 2006) have yelded positive results, shortening the duration of acute rotavirus diarrhoea in children.

Atopic disorders such as atopic eczema, allergic rhinitis and asthma are increasing rapidly in the more developed countries. While the exact aetiology is unclear, environmental factors, aberrant gut microbiota composition and activity, impairment of the intestinal mucosal barrier and shift of the Th1/Th2 –type cytokine balance towards a Th2 response have been shown to have a crucial role in the development of allergic diseases (Guarner et al. 2006). Specific probiotics administered to allergic infants are effective in the prevention of atopic eczema (Kalliomäki et al. 2001b), acting by stabilising the intestinal barrier function and reducing gastrointestinal symptoms in children with atopic diseases as well as modulating the immune system (Isolauri et al. 2001) back to a Th1 response (Isolauri 2004). The effect of probiotics in preventing atopic dermatitis has been demonstrated in a double-blinded RCT from Finland where *Lactobacillus rhamnosus* GG was given to pregnant and lactating mothers who had a marked family history of eczema, allergic rhinitis or asthma, and to their infants for the first 6 months after birth. The frequency of atopic dermatitis developing in

the offspring was significantly reduced by 2, 4, and 7 years (Kalliomäki et al. 2001b, 2003, 2007). In a study conducted by Kukkonen and collaborators (2007), pregnant women carrying high-risk children were given a mixture of 4 probiotic strains (*Lactobacillus rhamnosus* GG, *L. rhamnosus* LC705, *Bifidobacterium breve* Bb99, *Propionibacterium freudenreichii* ssp. shermanii JS) before delivery and their infants subsequently received the same probiotics along with galacto-oligosaccharides for 6 months after birth. Probiotic treatment showed no effect on the incidence of allergic diseases but significantly prevented eczema and atopic eczema. Both studies conducted in Finland suggest an inverse association between atopic diseases and colonisation of the gut by probiotics. Numerous RCTs with probiotics aiming at the prevention of atopic diseases have failed. One example is a study conducted by a group under Kopp (2008), designed on a protocol similar to that of Kalliomäki and associates (2001b) but demonstrating no lowered cumulative risk of allergic diseases. Variation in the outcomes of clinical studies may result from host factors such as genetic susceptibility, microbiota composition and environmental factors such as geographic region and diet, and study variables including the probiotic strains and doses used.
Probiotics have not yet been proved to be effective in the treatment of allergic disorders.

NEC is the most common gastrointestinal emergency in low-birth-weight preterm infants. Delayed enteral feeding, frequent use of antibiotic therapy and altered acquisition of normal digestive microbiota are the factors most likely contributing to the development of NEC in preterm infants. Several RCTs have reported that enteral probiotic supplementation significantly reduced the incidence of NEC and mortality among premature infants. Probiotics used in these studies have included LGG (Manzoni et al. 2006), *B. breve* YIT 4010 (Kitajima et al. 1997)*, S. boulardii* (Costalos et al. 2003) and mixtures of *S. thermophilus* + B*. infantis* + *B. bifidum,* ABC Dophilus strains (Bin-Nun et al. 2005) and *L. acidophilus* NCDO 1748 + *B. bifidum* NCDO 1453 (Lin et al. 2008).

Helicobacter pylori is an inhabitant of the stomach in 70-90% of the population in developing countries and 25-50% in developed countries; in Finland however the prevalence of this microorganism is very low (Salomaa-Räsänen et al. 2010). In some cases these bacteria may become harmful for the host, causing gastric and duodenal ulcers, gastric cancer and other gastric complications (Brown 2000). The eradication treatment includes

proton pump inhibitor plus at least two antibiotics. The strain *Lactobacillus casei* DN-114001 has a high success rate in eradicating *H. pylori* in children when administered at a high daily dosage of 1 x 10^{10} cfu (Sykora et al. 2005). However, it has been found that early exposure to *H. pylori*, particularly in childhood, is associated with a lower incidence of allergic diseases (Seiskari et al. 2007, Serrano et al. 2011), supporting the hypothesis that the microbial load is an important environmental factor conferring protection against the development of allergies in childhood.

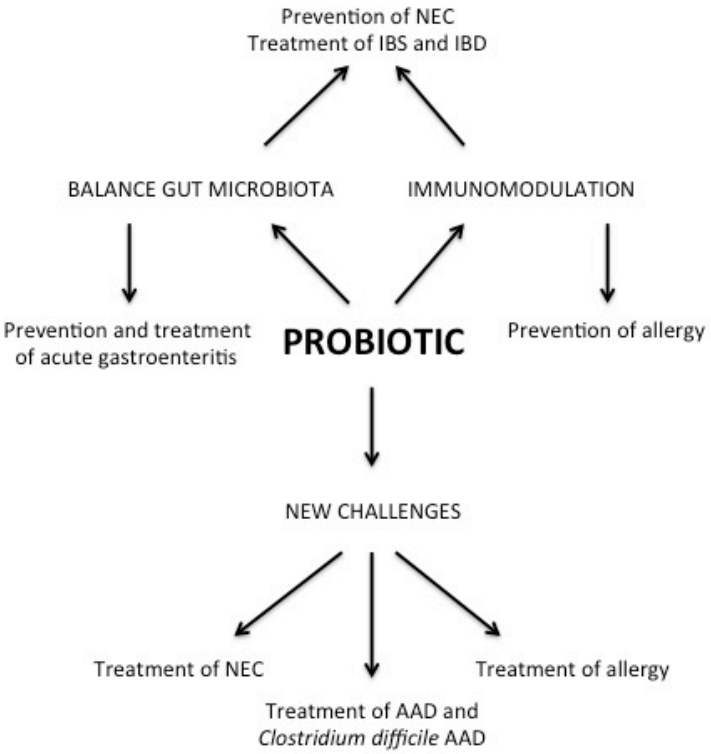

Figure 3. Suggested health effects and new challenges in specific probiotic strain use in infants.

Probiotics have also proved efficacious in the treatment of bowel disorders such as irritable bowel syndrome (IBS) and inflammatory bowel disease (IBD) in children.

IBS includes abdominal discomfort or pain, diarrhoea, constipation, bloating and flatulence, these being caused by altered gut motility, visceral hypersensitivity and dysregulation of the brain-gut axis (Alaradi and Barkin 2002). It has however been suggested that IBS is also caused by an imbalanced intestinal microbiota profile and enteric bacteria-mediated

mucosal inflammation (Bolino and Bercik 2010). Administration of specific probiotic strains can however relieve some IBS symptoms such as constipation, flatulence and borborygmi (Nobaek et al. 2000, Koebnick et al. 2003). One RCT has been published concerning probiotic treatment of children with IBS in which LGG was able to reduce abdominal distension and discomfort (Bausserman and Michail 2005).

IBD includes Crohn's disease and ulcerative colitis. The aetiology is complex and not always clear. It is suggested that IBD may be caused by a hyper-responsive cell-mediated immune response to intestinal commensal bacteria or microbiota aberrancies in genetically susceptible patients (Sartor 2004, Iltanen et al. 2006). The addition of specific probiotic strains or their combination to the diet can eliminate the inflammation-inducing bacteria and may possibly treat active IBD. The beneficial effect of probiotics in the treatment of IBD in children has been reported once; the specific probiotic preparation VSL#3 combined with standard therapy proved helpful in the treatment of chronic ulcerative colitis in children (Miele et al. 2009).

A common condition typically affecting infants in the first 4 months of life is colic. The aetiology is again complex and includes abnormal gastrointestinal function. The administration of the probiotic *L. reuteri* ATCC 55730 has been shown to be effective in the treatment of this condition (Savino et al. 2007).

Studies on the long-term health benefits of probiotics for the development of the immune system, prevention of cancer and treatment of allergy, diarrhoeas and NEC in children are still ongoing and their effects need to be proved (**Figure 3**).
Based on research reports and clinical intervention studies it has been concluded that all probiotic health effects are strain-, dose-, disease- and host-dependent.

2.6 Methods of microbiota analyses

For an investigation of the intestinal microbiota, the easiest and most readily available sample is the faeces. However, the faeces represent the intestinal microbiota in the lumen

of the sigmoid colon, while the microbial composition differs both along the GIT and between the lumen and the mucosa (Probert and Gibson 2002, Ouwehand et al. 2004). Samples taken at endoscopy or during surgery may however not well represent the intestinal microbiota as the bowel cleansing and anaesthetics used during these procedures may have an impact on the intestinal microbiota. It is of interest to study microbiota associated with the intestinal mucosa as being in close contact with the human intestinal tissue and thereby with the immune system of the host; the composition and activity of the mucosal microbiota may be more relevant to host health than the luminal microbiota.

Culture-dependent methods

Culture-dependent methods are based on isolation, identification and enumeration of bacteria using classical microbiological methods. The counts of a specific bacterial group are determined after incubation in appropriate conditions. These include media type (non-selective, selective), atmosphere (aerobic, anaerobic), temperature and other factors influencing the growth of specific bacterial groups. Bacteria can be identified using morphological examinations (colony and/or cell appearance), biochemical (biochemical functional activities, gas liquid chromatography of bacterial fatty acids) and genetic (using nucleic acid-based methods) tests. However, morphological examinations and biochemical tests lead to numerous misidentifications and should not be the sole approaches in bacterial identification, even if helpful in assessing the phenotypic characteristics of the bacteria studied.

Genetic techniques have contributed substantially to our knowledge of the intestinal microbiota. Most genetic methods are based on the use of the ribosomal bacterial 16S RNA molecule (16S rRNA). Partial amplification and sequencing of the 16S rRNA gene from previously isolated colonies are used for detection and quantification of bacteria at genus and species level. The use of 16S rRNA techniques often however requires previous construction of primers and/or probes and worldwide sequence databases (Gueimonde and Reyes-Gavilán 2009).

Culture-independent methods

Most of the bacteria inhabiting human gut have not yet been cultured in laboratory conditions. Until now, researchers have been able to characterise about 25% of the total

bacterial gut population using classical culture-based methods. These methods are thus not appropriate in describing the total microbial diversity. During the last few years, significant progress in molecular biology has led to the development of culture-independent methods allowing identification and characterisation of numerous new bacterial groups. Culture-independent techniques are based mainly on the use of the 16S rRNA molecule. The 16S rDNA gene is characterised by highly conserved and variable regions which are characteristic for specific bacteria present in the gut. Based on these characteristics, specific primers and probes can be designed to detect microbes sought. Techniques based on 16S rRNA use include polymerase chain reaction (PCR) and its variables, randomly amplified polymorphism DNA (RAPD), temperature gradient gel electrophoresis / denaturing gradient gel electrophoresis (TGGE/DGGE), terminal-restriction fragment length polymorphism (T-RFLP), oligonucleotide arrays, fluorescent *in situ* hybridisation (FISH) (Tannock 2001, Albuquerque et al. 2009, Turroni et al. 2008).

FISH and quantitative PCR (qPCR) are the methods established to determine the total amounts and/or proportions of bacterial groups/species of interest in a complex microbial community.

The FISH method is commonly used in microbial ecological studies. The technique allows visualisation of bacteria containing rRNA, which is targeted for hybridisation with a fluorescently labeled oligonucleotides probe with a sequence specific for a bacterial species or genus (directed toward the 16S rRNA gene). The FISH method allows the analysis of complex microbial communities such as the human faeces. A variety of microorganisms can be quantitatively identified (Amann et al. 1996, Albuquerque et al. 2009). The combination of FISH with flow cytometry (FCM-FISH) detection appears to be a very high throughput means to the identification and quantification of specific microorganisms.

Quantitative PCR is widely used to detect and quantify specific DNA sequences of microorganisms. The method is a particularly rapid and accurate means of determining the amount of target DNA in a sample. Bacterial DNA can be isolated from the sample and the rRNA genes can be amplified using the polymerase chain reaction (PCR). The choice of PCR primers allows a wide spectrum of specificity. If general primers are used, almost all rRNA

sequences can be amplified. In contrast, if unique PCR primers are chosen, only a single species or a small group of bacterial rRNA sequences in the sample will be amplified (Brunk et al. 2002, Albuquerque et al. 2009).

Novel techniques to study microbial communities focus on activity biomarkers such as messenger RNA, proteins or metabolites and include metagenomics, proteomics, metabolomics.

3. AIMS OF THE STUDY

The main goal in the present series was to characterise the gut microbiota in early infancy and the specific determinants influencing its composition.

The specific aims were to assess:

1. the association between the gut microbiota of mother and infant (**I**)
2. the effect of supplementation of probiotics in the last trimester of pregnancy on the maternal and neonatal gut microbiota (**I**)
3. the gut microbiota in infants living in different human populations such as Malawian and Finnish (**II**)
4. the gut microbiota in infants from Finland in comparison with infants in Germany (**III**)
5. the differences in the gut microbiota composition between breast-fed and formula-fed infants from Germany (**A**)
6. the impact of probiotic administration to mother and/or infant on gut microbiota in Finnish and German infants (**III**)
7. the specific properties of a probiotic strain, *Lactobacillus rhamnosus* GG (**IV**).

4. SUBJECTS, MATERIALS AND METHODS

4.1 Subjects

The profiles of each separate study (**I, II, III, A**) are presented in **Figures 4, 5, 6** and **7**.

4.1.1 Influence of mother's intestinal microbiota on gut colonisation in the infant

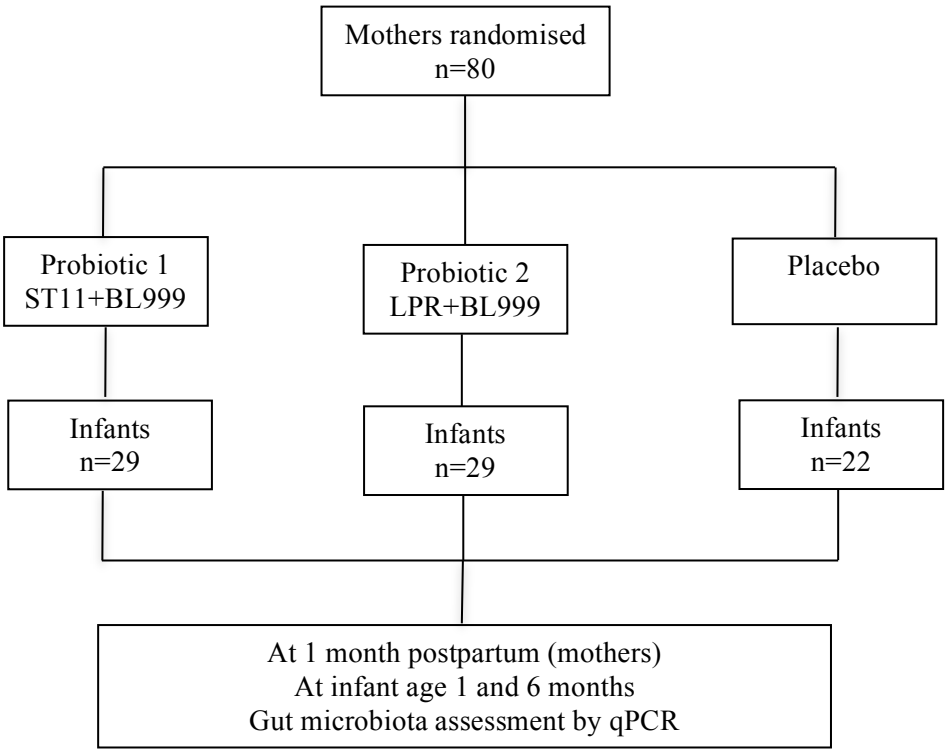

Figure 4. Study **I** design

To investigate mother-infant association in the gut colonisation of 1-6-month-old infants and to establish whether probiotics can influence this process, faecal samples from 80 mother-infant pairs were analysed at 1 month (mothers' postpartum time and infants' age) and 6 months (infants' age) (**I**), (**Figure 4**). The mother-infant pairs were selected from the main trial (Identifier: NCT00167700) on the criterion that the mother was exclusively breast-feeding the infant for a minimum of 4 months and partial / exclusive breast-feeding continued until the infant completed 6 months of age. This yielded 80 mother-infant pairs. The mothers received probiotic / placebo for 2 months before delivery, and for 2 months

after delivery or until they stopped breast-feeding. There were three intervention groups: 1. *Lactobacillus rhamnosus* LPR (CGMCC1.3724) with *Bifidobacterium longum* strain BL999 (ATCC: BAA-999) (LPR+BL999, 29 mother-infant pairs), 2. *Lactobacillus paracasei* ST11 (CNCM 1-2116) with *Bifidobacterium longum* BL999 (ST11+BL999, 29 mother-infant pairs) and 3. Placebo (22 mother-infant pairs). The dose of probiotics was 10^8 / day of each strain provided in one sachet of 7 g per day (powder form), diluted in a glass of water. Clinical data on the study infants are shown in **Table 1**.

Table 1. Clinical characteristics of the infants in Study **I.**

Clinical characteristics	ST11+BL999 (n=29)	LPR+BL999 (n=29)	Placebo (n=22)	Total (n=80)
Vaginal delivery	28 (97%)	28 (97%)	19 (86%)	75 (94%)
Male sex	14 (48%)	15 (52%)	9 (41%)	38 (48%)
Gestational age (wk)	41.0 (36-42)	40.0 (37-42)	39.5 (35-42)	40.0 (35-42)
Weight at birth (g)	3714 (525)	3614 (523)	3653 (336)	3661 (476)
Height at birth (cm)	51.7 (2.4)	50.5 (2.1)	50.8 (1.6)	51.0 (2.1)
Apgar 1 minute	9 (2-10)	9 (5-10)	9 (3-10)	9 (2-10)
Apgar 5 minute	9 (3-10)	9 (8-10)	9 (6-10)	9 (3-10)
Apgar 15 minute	9 (4-10)	9 (8-10)	9 (8-10)	9 (4-10)
Antibiotics during labour or in delivery hospital				
Mother	3 (10%)	4 (14%)	6 (27%)	13 (16%)
Infant	2 (7%)	3 (10%)	1 (5%)	6 (8%)
Antibiotics <1 month*	1 (3%)	3 (10%)	1 (5%)	5 (6%)
Antibiotics at 1-3 months	1 (3%)	0	0	1 (1%)
Antibiotics at 3-6 months	5 (17%)	4 (14%)	4 (18%)	13 (16%)

Data are shown as number (%) and mean (SD)
* Given at home, not including those in hospital

4.1.2 Gut microbiota profiles in infants residing in a South Eastern African and Northern European country

The aim of this study (**II**) was to compare the gut microbiota in 6-month-old infants living in rural Malawi with that in children of the same age living in urban Finland, both having an age-appropriate diet typical for each area (**Figure 5**).

The Malawian study population comprised 44 healthy 6-month-old rural infants, who were enrolled for an epidemiological clinical trial assessing the impact of selected dietary

interventions on early childhood growth (Identifier: NCT00524446). The Malawian infants represented a population typical of that age in Malawi. The clinical characteristics and patterns of current dietary intakes of the Malawian infants participating in the study are presented in **Table 2**.

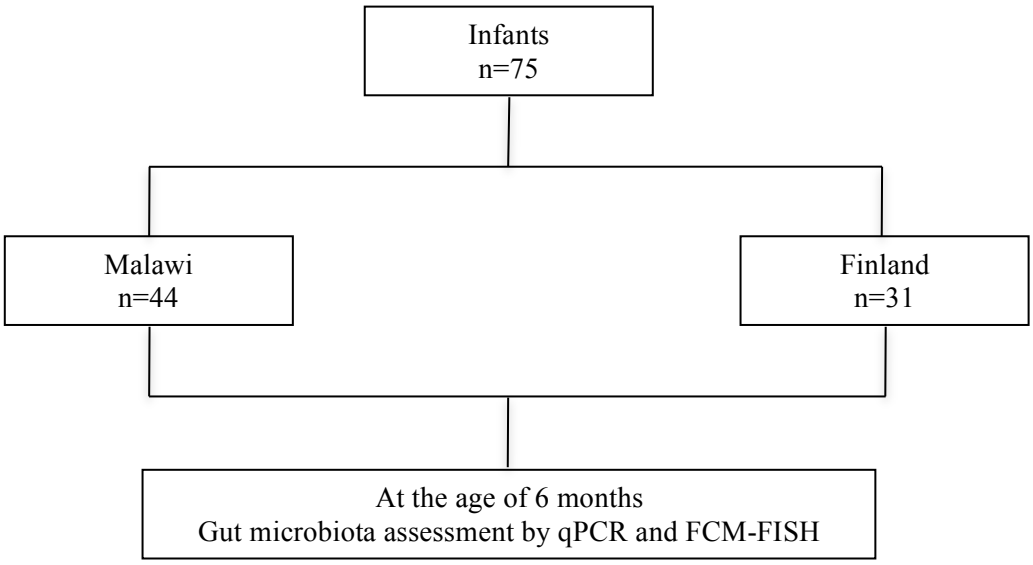

Figure 5. Study **II** design

Table 2. Clinical characteristics the infants in Study **II.**

Clinical characteristics	Malawian infants (n=44)	Finnish infants (n=31)
Gender		
Male	20/44 (45.5%)	14/31 (45.2%)
Female	24/44 (54.5%)	18/31 (54.8%)
Age (months)	6.0 (5.6-6.0)	6.1 (5.9-6.4)
Weight (kg)	6.6 (6.2-7.2)	8.1 (7.5-8.7)
Mode of delivery (% vaginal)	Not known	24/31 (77.4%)
Breastfeeding	44/44 (100.0%)	21/31 (67.7%)
Breast milk + complementary food	44/44 (100.0%)	18/31 (58.1%)
Breast milk + complementary food + formula	Non-existent	3/31 (9.7%)
Formula + complementary food	Non-existent	10/31 (32.2%)
Breast milk + complementary food + other milk*	18/44 (40.9%)	None

Data on age and weight are shown as median with IQR. *Other milk includes cow's and goat's milk

The Finnish study population comprised 31 healthy 6-month-old infants participating in an ongoing prospective randomised study in the city of Turku and neighboring areas in south-western Finland, (Identifier: NCT00167700, section 3). The 31 infants represented a population typical of that age group in Finland. The infants included in this study did not

receive probiotics or prebiotics and their mothers, if breast-feeding, did not have probiotic or prebiotic products in their diet. The mothers' diets reflected the typical Finnish diet. The clinical characteristics of the infants are presented in **Table 2.**

4.1.3 The impact of geographical location and diet on gut microbiota composition in infants

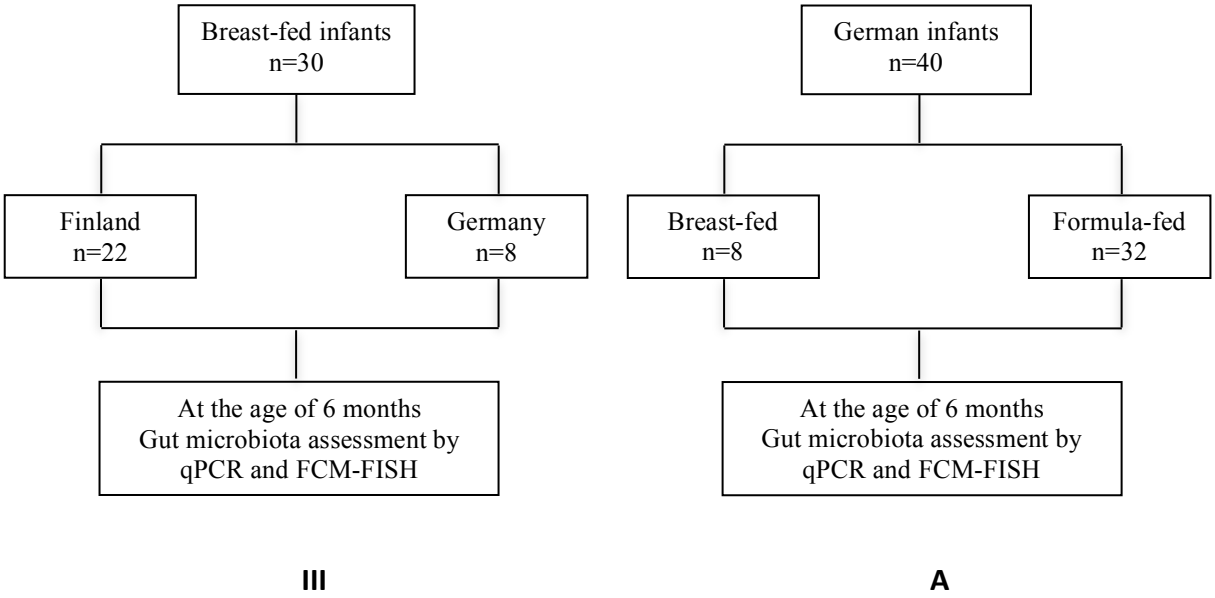

Figure 6. Study **III** and **A** design

To analyse the impact of the environment on gut microbiota composition, 22 infants from Finland (placebo group from Study **III**) and 8 from Germany (additional group), both being breast-fed, were compared, (**Figure 6 III**).

In addition, 8 German breast-fed infants (additional group) were compared with 32 German formula-fed infants (placebo group from Study **III**) in order to bring out differences in microbiota composition, if any, (**Figure 6 A**).

Detailed information on 22 infants from Finland and 32 infants from Germany is given in Study **III** (Paragraph 4.1.4).

4.1.4 Perinatal probiotic intervention in 6-month-old infants from two European locations

To analyse the effect of probiotic administration to the mother or the infant, mothers and their infants from Finland and infants from Germany were recruited (**III**), (**Figure 7**). The Finnish study cohort comprised 79 infants (Identifier: NCT00167700). The mothers in Finland were chosen on the criterion that their infants were breast-fed exclusively until 4 months of age and partially / exclusively until 6 months. Probiotic product and placebo were given to the mothers for 2 months before delivery and for 2 months after delivery during breast-feeding. Probiotics or placebo were used for a maximum of 4 months. Twenty-eight mothers received a probiotic product consisting of *L. rhamnosus* LPR (CGMCC 1.3724) with *B. longum* BL999 (ATCC: BAA-999), (LPR+BL999), 29 mothers received *L. paracasei* ST11 (CNCM 1-2116) with *B. longum* BL999 (ST11+BL999), and 22 placebo. The dose to the mother was 10^8 / day of each probiotic strain provided in one sachet of 7 g per day (powder form), diluted in a glass of water.

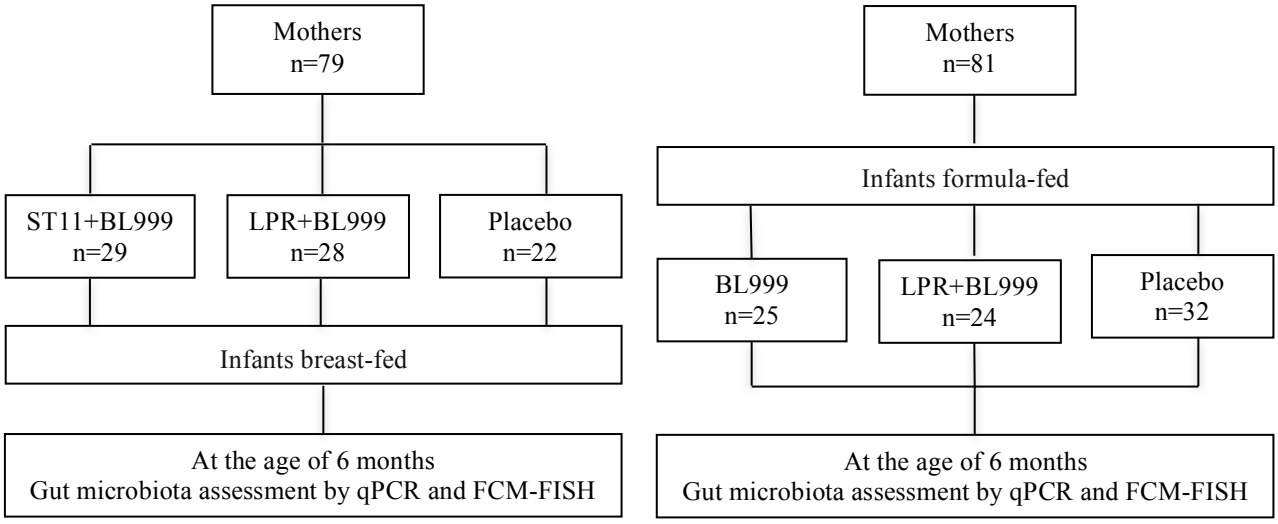

Figure 7. Study **III** design

The German study population comprised 81 infants receiving formula with or without probiotics. Twenty-four infants received partially hydrolysed formula supplemented with *L. rhamnosus* LPR (CGMCC 1.3724) and *B. longum* BL999 (ATCC: BAA-999) (LPR+BL999),

25 received partially hydrolysed formula with *B. longum* BL999 (ATCC: BAA-999), and 32 received partially hydrolysed formula. The probiotic and placebo formulas were administered to the infants when they went onto formula, at the latest at 1 month of age. The intervention lasted for 4 months. The dose of the probiotic was at least 10^9 CFU / day of each strain provided in metallic tins, each containing 400 g of the study formula.

4.2 Materials

4.2.1 Production process influences *in vitro* properties of probiotic *L. rhamnosus* GG

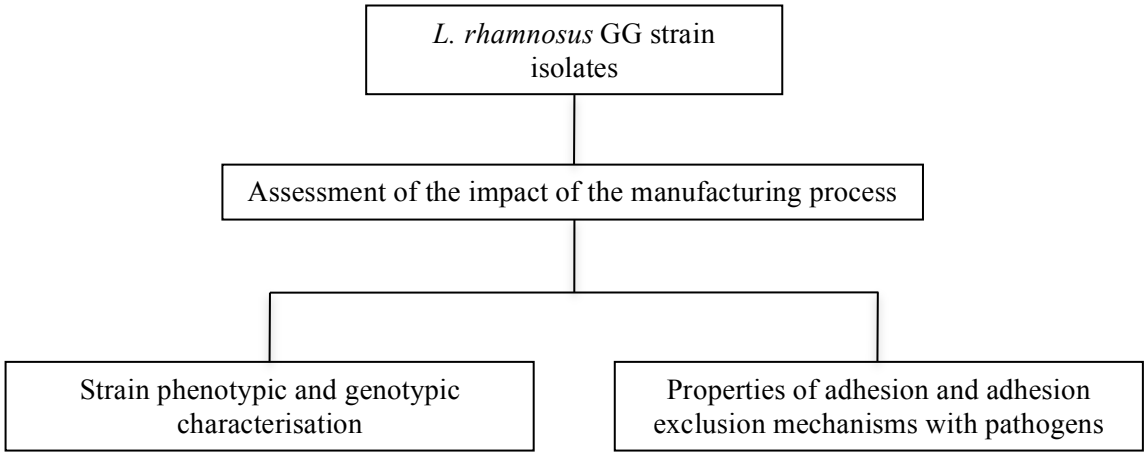

Figure 8. Study **IV** design

To investigate whether the properties of a specific probiotic strain may differ depending on the product and source of the strain (**Figure 8**), 13 *L. rhamnosus* isolates claimed to be *L. rhamnosus* GG (ATCC 53103) were isolated from different probiotic products from different countries (**Table 3**). For comparison, the original *L. rhamnosus* GG (original *L. rhamnosus* GG strain isolate donated by Professor Sherwood Gorbach of Tufts University, Boston, MA, USA) from which other cultures used in probiotic products were derived was included (**IV**).

Table 3. List of *L. rhamnosus* isolates and source of isolation.

Isolate code	Source
AL EL FL NL	capsule product
LL ML	commercial infant food
DL HL KL	freeze-dried powder
BL CL IL JL	provided in soft agar
OL	commercial food

An additional commercial probiotic *L. rhamnosus* strain (identification code OL) of the same species but known to be phenotypically different from *L. rhamnosus* GG was included as an external reference strain (**Figure 8**).

For further assessment, the bacterial pathogens used were *Cronobacter sakazakii* (ATCC 29544), *Staphylococcus aureus* (DSM 20231), *Clostridium perfringens* (DSM 756) and *Salmonella enterica* serovar Typhimurium (ATCC 12028).

4.3 Methods

4.3.1 Sample preparation and DNA extraction

Faecal samples were taken for the analysis from mothers at 1 month postpartum (**I**) and infants at 1 (**I**) and 6 months (**II, III, A**) of age and stored at -80 °C until analysis.
Faecal samples (0.5 g) were weighed, diluted 1:10 (w/v) in phosphate buffer PBS (pH 7.4) and homogenised by thorough agitation in a vortex. Aliquots of these dilutions were used for DNA extraction. DNA from both faeces and also, from pure cultures of the different bacterial strains used as reference were extracted using the QIAamp DNA stool Mini kit (Qiagen, Hilden, Germany) following the manufacturer's instructions.

4.3.2 Evaluation of prevalence of target species in faecal samples

In detecting the presence of specific gut microbiota (**I, II, III, A**) qualitative PCR was used. Amplification of the DNA was performed using a PCR iCycler (Bio-Rad, Espoo, Finland). The total volume of each PCR was 50 µL, including 1 µL of DNA extract as a template. The reaction mixture was composed of 1 x PCR buffer II (Applied Biosystems, Foster City, CA, USA), 2.5 mM $MgCl_2$, 0.2 µM of each primer, 200 µL of each dNTP (Amersham Biosciences, Helsinki, Finland) and 1.25 U AmpliTaq Gold DNA polymerase (Applied Biosystems). The thermal cycle program consisted of the following time and temperature profile: an initial cycle of 95 °C for 10 min for denaturation and polymerase activation, 30 cycles 15 s at 95 °C,

1 min at the annealing temperature of the corresponding primer pair and 45 s at 72 °C and a final extension step of 10 min at 72 °C. Amplified products were subjected to gel electrophoresis in 1% agarose gels and were visualised by ethidium bromide staining. The following reference strains were used: *Bifidobacterium* genus, *B. longum* (DSM 20219), *B. catenulatum* (JCM 7130), *B. bifidum* (DSM 20456), *B. lactis* (DSM 20606), *B. infantis* (DSM 20090), *B. adolescentis* (DSM 20083), *B. breve* (DSM 20213), *Akkermansia muciniphila* (ATTC BAA-835), *S. aureus* (DSM 20231), *C. coccoides* (DSM 935^T), *C. leptum* (DSM 753^T), *C. difficile* (DSM 1296^T) and *C. perfringens* (DSM 756). The primer sequences of the reference strains and the annealing temperatures of the primers have been published elsewhere (Matsuki et al. 1999, Penders et al. 2006, Collado et al. 2008).

4.3.3 Real-time polymerase chain reaction (qPCR) analysis of gut bacteria

Quantitative real time PCR was used to characterise the faecal microbiota (**I, II, III, A**) using group- and species-specific primers. These oligonucleotides were purchased from the Thermo Electron Corporation (Thermo Biosciences, Ulm, Germany).

Briefly, PCR amplification and detection were performed with an ABI PRISM 7300-real-time PCR system (Applied Biosystems, Foster City, California). Each reaction mixture of 25 µl was composed of Power SYBR Green PCR Master Mix (Applied Biosystems), 1 µl of each of the specific primers at a concentration of 0.2 mol/L, and 1 µl of template DNA. The fluorescent products were detected in the last step of each cycle. A melting curve analysis was made after amplification to distinguish the targeted from the non-targeted PCR product.

The bacterial concentration in each sample was calculated by comparing the C_t values obtained from standard curves. A standard curve was made from serial dilutions of DNA isolated from each pure culture of the different reference strains. A linear relationship was observed between cell number and C_t values (r^2 = 0.99-0.97).

The following reference strains were used to construct the corresponding standard curves: *B. longum* (DSM 20219) (this strain was also used as the standard strain for the quantification of *Bifidobacterium* genus), *B. catenulatum* (JCM 7130), *B. bifidum* (DSM 20456), *B. lactis* (DSM 20606), *B. infantis* (DSM 20090), *B. adolescentis* (DSM 20083), *B.*

breve (DSM 20213)*, Akkermansia muciniphila* (ATTC BAA-835), *S. aureus* (DSM 20231), *C. coccoides* (DSM 935^T), *C. leptum* (DSM 753^T), *C. difficile* (DSM 1296^T) and *C. perfringens* (DSM 756). The primer sequences of the reference strains and the annealing temperatures of the primers have been published elsewhere (Matsuki et al. 1999, Penders et al. 2006, Collado et al. 2008).

4.3.4 Flow cytometry – fluorescent *in situ* hybridisation (FCM-FISH) analysis of gut bacteria

Homogenised faecal samples (**II, III, A**) were fixed overnight in 4% paraformaldehyde and stored in PBS-ethanol in -20 ^{o}C until analysed. Fluorescent *in situ* hybridisation coupled with a flow cytometer was performed as previously described (Collado et al. 2008, Kalliomäki et al. 2008). In brief, samples of the fixed cells were hybridised overnight at specific temperatures in hybridisation buffer with specific probes at a concentration of 5 ng/µl. After overnight hybridisation the samples were washed with buffer without SDS, pelleted, and resuspended in PBS. The EUB338 probes were covalently linked at their 5´-end with fluorescein isothiocyanate (FITC) and other probes with carbocyanine 3 (Cy3) (Thermo Biosciences, Ulm, Germany). Probes included: EUB338 for the total bacteria, Bif164 for *Bifidobacterium* group, Bac303 for *Bacteroides-Prevotella* group, Chis150 for *Clostridium histolyticum* group, Lab158 for *Lactobacillus-Enterococcus* group, Muc1437 for *Akkermansia muciniphila*, with the probe sequences and references described elsewhere (Amann et al. 2001, Collado et al. 2008, Franks et al. 1998, Harmsen et al. 1999, Langendijk et al. 1995, Manz et al. 1996). Data acquisition was performed by LSR II flow cytometer equipped with a HTS 96-well plate reader (Becton Dickinson, San Jose, USA). Forty µl of samples were collected in duplicate of each. Data were analysed with BD FACSDiva™ software (Becton Dickinson).

The total amount of bacteria was determined with the EUB338-FITC probe. Determination of specific bacteria was performed by combining each of the group-specific Cy3 probes with the EUB338-FITC probe and counting double positive cells, as described elsewhere (Rigottier-Gois et al. 2003).

4.3.5 Bacteria and growth conditions

The *L. rhamnosus* isolates (**IV**) were grown in de Man, Rogosa and Sharpe broth (Oxoid Limited, Basingstoke, Hampshire, UK) and incubated at 37 °C under anaerobic conditions (10% H_2, 10% CO_2 and 80% N_2; Concept 400 anaerobic chamber, Ruskin Technology, Leeds, UK).

4.3.6 Phenotypic and genotypic characterisation of bacteria

Characterisation of the isolates by carbohydrate fermentation profiles

The fermentation ability of the *L. rhamnosus* isolates (**IV**) was obtained in API 50 CH strips (Bio-Mérieux, Marcy l'Etoile, France) following the manufacturer's instructions.

Tolerance to acid

Bacterial cultures (**IV**) (5 ml) were grown overnight, cells were harvested, washed twice with 0.85% NaCl and resuspended in 500 µl of the same solution; 100 µl of the bacterial suspensions (approximately 10^8 cells) were added to 900 µL of simulated gastric juice (125 mM-NaCl, 7 mM-KCl, 45 mM-$NaHCO_3$ and pepsin (3 g/l; Sigma)) adjusted to pH 2.0 or 2.5 with HCl. Suspensions were then incubated for 90 min. Plate counts were made at time-point 0 and after 90 min of incubation.

Species identity of isolates by partial sequence analysis of the 16S rRNA gene

The *L. rhamnosus* isolates used in the present study (**IV**) were identified at species level by partial sequence analysis of the 16S rRNA gene, followed by Blast analysis. In brief, microorganisms were grown overnight, 1 ml of cells was harvested and the DNA extracted using the GenElutee Bacterial Genomic DNA Kit (Sigma, St. Louis, MO, USA) following the manufacturer's instructions. Partial amplification of the 16S rRNA gene and species identification were carried out as previously described by Gueimonde and coworkers (2004). Amplified PCR products were purified using the GenElutee PCR Clean-Up Kit (Sigma), and automated sequencing of the amplicons was carried out at Secugen SL (Madrid, Spain) in an automated sequencer ABI Prism (Applied Biosystems). The sequences obtained were

compared with those held at the GenBank database using the Blast application at the NCBI webpage.

Randomly amplified polymorphic DNA-PCR

DNA extracts from the different *L. rhamnosus* isolates (**IV**) were used for strain typification by randomly amplified polymorphic DNA analysis using previously described conditions, the primer for *L. rhamnosus* and methodology by Tynkkynen and collaborators (1999).

Enterobacterial repetitive intergenic consensus-PCR

Enterobacterial repetitive intergenic consensus-PCR is based on the use of oligonucleotides targeting short repetitive sequences distributed throughout the bacterial genome. Bacterial DNA (**IV**) was amplified using primers 5'-ATGTAAGCTCCTGGGGATTCAC-3' and 5'-AAGTAAGTGACTGGGGTGAGCG-3' (Sigma Genosys) as previously described (Ventura et al. 2003).

DNA restriction patterns by pulsed-field gel electrophoresis

The *L. rhamnosus* isolates (**IV**) were typified using analysis of DNA restriction patterns by pulsed-field gel electrophoresis (PFGE) as described elsewhere (Tynkkynen et al. 1999).

4.3.7 Adhesion assay with radiolabelled bacteria

Mucus preparation

For the present study (**IV**) human intestinal mucus was obtained as described by Ouwehand and coworkers (2002) from the healthy part of resected human colonic tissue and the protein concentration was determined according to the method of Lowry and colleagues (Lowry et al. 1951, Miller and Hoskins 1981). Human colonic mucus was dissolved (0.5 mg/ml protein) in HEPES (*N*-2-hydroxyethylpiperazine-*N*-2-ethanosulphonic acid)–Hanks buffer (HH; 10 mM HEPES, pH 7.4), and 100 µL of this solution was immobilised in polystyrene microtitre plate wells (Maxisorp, Nunc, Roskilde, Denmark) by overnight incubation at 4 °C according to the method of Kirjavainen and colleagues (1998).

Adhesion assay

For adhesion, inhibition, displacement and competition assays isolates (**IV**) were grown for 18 h, harvested and then washed twice with phosphate-buffered saline (PBS) buffer. All microorganisms were metabolically labeled by addition to the media of 10 µL / mL tritiated thymidine (5'-^{3}H-thymidine 1.0 mCi / mL; Amersham Biosciences, Little Chalfont, Bucks, UK).

Radiolabeled bacteria were centrifuged (6000 rpm for 7 min) and washed twice with HH buffer, and the absorbance (A_{600nm}) was adjusted to 0.25 ± 0.01 to standardise the bacterial concentration (10^{8} cells/ml). Thereafter, bacteria (100 µL) were added to the wells and incubated for 1 h at 37 °C. The wells were then washed twice with 200 µL of HH to remove unbound bacteria. Adhering bacteria were released and lysed with 1% (wt/vol) sodium dodecyl sulfate (SDS) in 0.1 mol/litre NaOH (200 µL per well) by incubation at 60 °C for 1 h. The contents of the wells were transferred to microfuge tubes containing scintillation liquid (OptiPhase 'HiSafe 3', Wallac, Milton Keynes, UK), and the radioactivity measured by liquid scintillation. Adhesion was expressed as the percentage of radioactivity recovered after adhesion relative to the radioactivity of the bacterial suspension added to the immobilised mucus. Adhesion was determined in three independent experiments, and each assay was performed in triplicate to calculate intra-assay variation.

Exclusion assays

Exclusion by inhibition, displacement and competition assays was carried out as described by Collado and coworkers (2007b, c).

The percentage of adhesion inhibition, displacement and competition was calculated as the difference between the adhesion of the pathogen in the absence and presence of the different isolates. Inhibition, displacement and competition assays were each conducted in three independent experiments and each assay was performed in triplicate.

4.3.8 Statistics

Statistical analyses were performed using SPSS (SPSS Inc, Chicago, IL, USA) versions 16.0 (**I**), 17.0 (**III,A**), 18.0 (**IV**) and SAS for Windows (version 9.2, SAS Institute Inc., Cary, NC, USA) (**II**).

For Studies **I** and **III** all subjects were chosen and identified by a statistician using criteria provided. The randomisation codes (**I, III**) were available to the statistician alone in order to maintain blindness for the ongoing main studies.

Study I: The Mantel-Haenszel method was applied to estimate the odds ratio (OR) for each study group and to obtain a combined OR estimate with a 95% confidence interval. The Breslow-Day test was used to analyse homogeneity between odds ratios, i.e. the interaction between probiotic intervention and maternal colonisation. The non-parametric correlation was used to verify the results, when appropriate. Analysis of covariance (ANCOVA) was used to detect the possible interaction between probiotic intervention and maternal faecal counts, i.e. to test whether the relation between maternal and infants' faecal counts was affected by the probiotic intervention. A bifidobacterial diversity index (DI) was calculated for the mothers at 1 month after delivery and for the infant at 1 and 6 months of age as follows: DI=(A/B)x100, where A represents the number of bifidobacteria species found in a sample and B the total number of bifidobacteria species analysed (=7) (Shadid et al. 2007). A bifidobacterial similarity index (SI) in the mother-infant pairs was calculated at 1 and 6 months after delivery correspondingly: SI=(C/D)x100, where C represents the number of bifidobacteria species found in both the mother and the infant and D the number of all bifidobacteria species found in the mother, the infant or both (Shadid et al. 2007). Kruskal-Wallis 1-way ANOVA and Mann-Whitney U-test were used to compare these indices between study groups and between categories of other explaining factors, and the Wilcoxon signed-rank test was used to test changes from 1 month to 6 months. The DI and SI were further analysed using different ordinal regression models, where colonisation, use of antibiotics, mode of delivery, probiotic intervention and mother's DI were included as explaining factors. Spearman rank correlation was used to study the association between DI and SI indices in mothers and infants.

Study II: Continuous variables were compared between Malawian and Finnish children using the Kruskall-Wallis test. Continuous variables are presented using medians with inter-quartile ranges (IQR). Fisher's exact test was used to establish the percentages of positive values for qPCR.

Study III, A: The bacterial counts and percentages detected by qPCR and FCM-FISH analysis were the primary variables. The counts were not normally distributed and are expressed as medians with inter-quartile ranges (IQR) and analysed using non-parametric methods. In the FCM-FISH results the bacterial counts as percentages of total bacterial counts were normally distributed, and were thus expressed as means with 95% confidence interval (95% CI). In qPCR results the Chi-squared test was used to compare the percentages of infants positive for the specified bacteria and the Kruskal-Wallis test to compare counts of bacteria in those cases where at least 50% of the infants were positive in all study groups. Analysis of variance (ANOVA) was used to analyse differences between intervention groups. All post-hoc comparisons were Bonferroni-corrected.

Study IV: The adhesion (%) measured in different conditions was here the primary endpoint. One-way ANOVA was used to test the overall difference in adhesion properties between strains. When $p<0.10$, Dunnett's (two-tailed) t test was used to compare each strain with the reference strain *L. rhamnosus* GG without any other pairwise comparisons. The cut-off point to indicate statistical significance was not strictly set at 0.05 due to the small sample size and large type II error. Based on Dunnett's t test, the exact P values are given, where both 0.05 and 0.10 were used as cut-off points to indicate the degree of statistical significance in the difference between strains.

4.3.9 Ethics

The studies were approved by the Committee on Ethical Practice of Turku University Hospital in Finland **(I, II, III**), the ethical Committee of the Pirkanmaa Hospital District in Finland (**II**), the College of Medicine Research and Ethics Committee of University of Malawi in Malawi (**II**) and the Marien-Hospital Wesel in Germany (**III, A**). Infants and mothers were enrolled in the studies after written informed consent had been obtained from the mothers.

5. RESULTS

5.1 Intestinal microbial association between mother and infant during prenatal period

Association of bacterial colonisation rates between mothers and infants

The overall colonisation rates in mothers and infants with the different bacteria studied (**I**) are shown in **Table 4.**

Probiotic intervention had no effect on the colonisation rates or faecal bacterial counts in mothers (data not shown).

When all mother-infant pairs were analysed together, it emerged that the colonisation rate of *B. bifidum* was significantly associated in the mother-infant pairs at 1 month and 6 months after delivery (**Table 5**). Thus, if the mother was positive for *B. bifidum* the likelihood of the child being positive was 19 times higher (95% CI 5.18-69.69, p<0.001) at 1 month and 23 times higher (95% CI 5.36-100.0, p<0.001) at 6 months of age compared to infants whose mothers were negative for *B. bifidum*. Likewise colonisation by *B. breve* was significantly associated between mothers and infants at 1 month after delivery (OR 6.89, 95% CI 1.62-29.29, p=0.009), but not at 6 months.

Table 4. Colonisation rates of faecal microbiota in mothers 1 month postpartum and in infants at 1 and 6 months of age.

Bacteria	Prevalence of positive samples (%)		
	Mother (n=76)	Infant, 1 month (n=80)	Infant, 6 months (n=79)
Bifidobacterium genus	100	96.3	100
B.longum	98.7	88.8	94.9
B.catenulatum	61.8	31.3	51.9
B.lactis	60.5	17.5	38.0
B.adolescentis	57.9	6.3	13.9
B.bifidum	38.2	42.5	51.9
B.infantis	11.8	6.3	29.1
B.breve	14.5	28.8	64.6
C.coccoides	100	25.3	83.3
C.leptum	100	25.0	68.4
C.perfringens	8.0	11.3	12.7
C.difficile	2.7	0	21.5
A.muciniphila	85.3	2.5	12.7
S.aureus	21.3	23.8	32.9

The colonisation rates of *S. aureus* tended to be associated between mothers and infants at 1 month after delivery; if the mother was colonised with *S. aureus* the likelihood of the child being colonised was 3.23 times higher (95% CI 0.93-11.18, p=0.064) than if the mother was not colonised. At 6 months no such association was observed.

No other significant or marginally significant associations were detected between mothers and infants in the colonisation rates of other bacteria (data not shown).

There was no evidence that probiotic intervention could modulate the level of association in faecal bacterial colonisation rates between mothers and infants either during probiotic supplementation (1 month after delivery) or thereafter (6 months after delivery). Substantial differences in odds ratios were observed between the study groups, but due to the small sample size, the interactions were not significant (Breslow-Day test, **Table 5**).

Correlation of bacterial counts between mothers and infants

When all mother-infant pairs were assessed together (**I**), a positive correlation was found between maternal and infant faecal counts of the genus *Bifidobacterium* during supplementation (1 month after delivery) (R=0.31, p=0.006) as well as a trend towards correlation after supplementation (6 months after delivery) (R= 0.20, p=0.089). The counts of *B. longum* did not correlate between mothers and infants at 1 month after delivery (R=0.14, p=0.22), while a significant positive correlation was detected at 6 months (R=0.24, p=0.038).

Interactions were tested in quantitative analysis to establish whether the correlations between faecal bacterial counts of mothers and infants were affected by the probiotic intervention (**I**). The interactions were non-significant during supplementation (1 month after delivery) (p=0.11 for the *Bifdobacterium* genus and p=0.40 for *B. longum*) but significant after conclusion of supplementation (6 months after delivery) (p=0.043 for *Bifidobacterium* genus and p=0.023 for *B. longum*).

Table 5. Association between maternal colonisation with *Bifidobacterium bifidum*, *B. breve* and *Staphylococcus aureus* one month after delivery and colonisation in infants at 1 and 6 months of age

		Maternal colonization				Likelihood of infant being positive at 1 month if the mother is positive			Maternal colonization				Likelihood of infant being positive at 6 months if the mother is positive		
		Posit.		Negat.					Posit.		Negat.				
		Posit. infants at 1 mo		Posit. infants at 1 mo		OR	95% CI	p*	Posit. infants at 6 mo		Posit. infants at 6 mo		OR	95% CI	p*
		n	%	n	%				n	%	n	%			
*B. bifidum***	Total	23/29	79.3	9/47	19.1	19.0	5.18-69.69	<0.001	25/29	86.2	13/46	28.3	23.15	5.36-100.0	<0.001
	ST11+BL999	6/8	75.0	1/19	5.3	54.0	4.12-707.1	0.001	8/8	100.0	6/19	31.6	NA	NA	0.002
	LPR+BL999	9/10	90.0	6/18	33.3	18.0	1.83-177.2	0.006	9/10	90.0	5/17	29.4	21.6	2.14-218.6	0.004
	Placebo	8/11	72.7	2/10	20.0	10.67	1.39-82.03	0.030	8/11	72.7	2/10	20.0	10.67	1.39-82.03	0.030
B. breve **	Total	7/11	63.6	15/65	23.1	6.89	1.62-29.29	0.009	8/11	72.7	40/64	62.5	1.69	0.42-6.85	0.462
	ST11+BL999	3/3	100.0	6/24	25.0	NA	NA	0.029	2/3	66.7	17/24	70.8	0.82	0.06-10.62	1.000
	LPR+BL999	2/5	40.0	4/23	17.4	3.17	0.39-25.58	0.285	4/5	80.0	11/22	50.0	4.00	0.38-41.74	0.342
	Placebo	2/3	66.7	5/18	27.8	5.20	0.38-70.90	0.247	2/3	66.7	12/18	66.7	1.00	0.07-13.37	1.000
*S. aureus***	Total	6/16	37.5	9/59	15.3	3.23	0.93-11.18	0.064	5/16	31.3	19/58	32.8	0.92	0.28-3.04	0.887
	ST11+BL999	1/4	25.0	4/23	17.4	1.58	0.13-19.42	1.000	2/4	50.0	7/23	30.4	2.29	0.27-19.66	0.582
	LPR+BL999	1/6	16.7	3/21	14.3	1.20	0.10-14.19	1.000	1/6	16.7	6/20	30.0	0.47	0.04-4.90	1.000
	Placebo	4/6	66.7	2/15	13.3	13.00	1.36-124.30	0.031	2/6	33.3	6/15	40.0	0.75	0.10-5.47	1.000

n indicates the number of infants with positive bacteria/total number of infants; **%** indicates percentage of infants with positive bacteria; **OR**, odds ratio, indicates the likehood of infants of positive mothers being colonised by the bacteria compared to infants of negative mothers; **95% CI**, confidence interval; ***** Mantel-Haenszel method was applied to estimate OR; ****** the test of homogeneity (Breslow-Day test) was non-significant for all three bacteria, showing that the interaction between maternal colonisation and probiotic intervention was not significant: *B. bifidum* p=0.617 at 1 month and p=0.522 at 6 months, *B. breve* p=0.402 at 1 month and p=0.602 at 6 months, *S. aureus* p=0.286 at 1 month and p=0.581 at 6 months; **NA**, not analysed; could not be estimated since all infants of positive mothers were positive

In sub-group analysis at 6 months after delivery the only positive correlation in faecal counts of *Bifidobacterium* between mothers and infants was found in the placebo group (R=0.48, p=0.028), whereas in the faecal counts of *B. longum* a positive correlation between mothers and infants was found in the placebo (R=0.50, p=0.022) and the ST11+BL999 groups (R=0.41, p=0.032).

Bifidobacterial diversity indices and mother-infant similarity indices

The median (IQR) bifidobacterial diversity index (DI) was 50% (28.6%-57.1%) in the mothers at 1 month after delivery and 28.6% (14.3%-42.9%) and 42.9% (28.6%-71.4%) in the infants at 1 and 6 months of age, respectively, without any significant differences between the intervention groups (**I**). In infants, the change in DI from 1 to 6 months was significant (p<0.001). The bifidobacterial DI correlated significantly between mothers and infants at 1 month (rho=0.358, p=0.001). Also, infants' DI at 1 and 6 months of age correlated (rho=0.356, p=0.001).

It was further explored whether maternal intestinal colonisation with some specific bifidobacteria species could be associated with the bifidobacterial DI of the infants at 1 and 6 months of age. Comparisons between infants of colonised and non-colonised mothers showed that detection of *B. bifidum* in the mothers' intestinal microbiota was associated with significantly higher faecal bifidobacterial DI in the infants both at 1 and 6 months of age (p<0.001 and p=0.03, respectively, **Table 6**). Also, mothers' colonisation with *B. breve* was associated with higher bifidobacterial DI in infants at 1 and 6 months of age (p=0.004 and p=0.072, respectively, **Table 6**).

In addition, the effect of other background factors (mode of delivery, use of antibiotics, mother's DI, probiotic intervention), together with mother's bifidobacterial colonisation status, on the infants' bifidobactrial DI at 1 and 6 months of age was analysed by ordinal regression analysis, but no single factor other than maternal *B. bifidum* and *B. breve* colonisation affecting the infants' DI was found (data not shown).

Table 6. Association between maternal bifidobacteria colonisation one month after delivery and the diversity index of the infants at 1 and 6 months of age.

Maternal species	Infant's diversity index at 1 month of age							Infant's diversity index at 6 month of age						
	Mother negative			Mother positive				Mother negative			Mother positive			
	n	Median	IQR	n	Median	IQR	p*	n	Median	IQR	n	Median	IQR	p*
B. longum	1	28.6		76	28.6	14.3-42.9	NA	1	28.6		75	42.9	28.6-71.4	NA
B. catenulatum	29	28.6	14.3-42.9	48	28.6	14.3-42.9	0.820	28	42.9	28.6-64.3	48	50.0	28.6-71.4	0.688
B. bifidum	48	28.6	14.3-28.6	29	42.9	28.6-57.1	<0.001	47	42.9	28.6-57.1	29	57.1	42.9-71.4	0.030
B. lactis	31	28.6	14.3-42.9	46	28.6	14.3-42.9	0.680	31	42.9	42.9-71.4	45	57.1	28.6-71.4	0.901
B. infantis	68	28.6	14.3-42.9	9	28.6	14.3-57.1	0.695	67	42.9	28.6-71.4	9	42.9	28.6-57.1	0.755
B. adolescentis	32	28.6	14.3-28.6	45	28.6	14.3-57.1	0.060	32	57.1	35.7-71.4	44	42.9	28.6-71.4	0.698
B. breve	66	28.6	14.3-42.9	11	42.9	28.6-57.1	0.004	65	42.9	28.6-57.1	11	71.4	42.9-71.4	0.072

* Mann-Whitney U-test was used to compare the diversity indices between infants of colonisation-positive and -negative mothers; **IQR**, interquartile range; **NA**, not analysed since only 1 infant whose mother was negative

Table 7. Association between maternal bifidobacteria colonisation one month after delivery and the similarity index of the infants at 1 and 6 months of age.

Maternal species	Infant's similarity index at 1 month of age							Infant's similarity index at 6 months of age						
	Mother negative			Mother positive				Mother negative			Mother positive			
	n	Media n	IQR	n	Median	IQR	p*	n	Median	IQR	n	Median	IQR	p*
B. longum	1	33.3		76	40.0	25.0-50.0	NA	1	33.3		75	40.0	28.6-50.0	NA
B. catenulatum	29	33.3	25.0-50.0	48	40.0	25.0-60.0	0.562	28	33.3	25.0-45.0	48	40.0	33.3-57.1	0.050
B. bifidum	48	33.3	25.0-50.0	29	50.0	40.0-60.0	0.028	47	33.3	25.0-50.0	29	42.9	40.0-57.1	0.031
B. lactis	31	33.3	25.0-50.0	46	40.0	25.0-50.0	0.921	31	33.3	25.0-40.0	45	42.9	33.3-60.0	0.033
B. infantis	68	40.0	29.2-60.0	9	28.6	20.0-42.9	0.083	67	40.0	28.6-50.0	9	40.0	33.3-50.0	0.650
B. adolescentis	32	36.7	25.0-50.0	45	40.0	28.6-60.0	0.576	32	40.0	33.3-50.0	44	40.0	28.6-57.1	0.911
B. breve	66	36.7	25.0-50.0	11	60.0	33.3-66.7	0.075	65	40.0	33.3-50.0	11	57.1	28.6-80.0	0.190

* Mann-Whitney U-test was used to compare the diversity indices between infants of colonization-positive and -negative mothers; **IQR**, interquartile range; **NA**, not analysed since only 1 infant whose mother was negative

The similarity index (SI) between infants' and mothers' bifidobacterial microbiota was analysed at 1 and 6 months after delivery (**I**). Median (IQR) SI between mothers and infants was 40% (25.5%-50.0%) at 1 month and 40% (28.6%-50.0%) at 6 months after delivery, without significant differences between intervention groups or between 1 and 6 months. Detection of *B. bifidum* in the mother's faecal microbiota was associated with higher SI at 1 and 6 months after delivery (p=0.028 and p=0.031, respectively, **Table 7**). Further, detection of *B. lactis* in the mothers' faecal microbiota was associated with infants' higher SI at 6 months after delivery (p=0.033, **Table 7**).

According to univariate and multivariate analysis, other background factors (mode of delivery, use of antibiotics, mother's DI, probiotic intervention) had no effect on the infant SI at 1 or 6 months of age (data not shown).

5.2. Effect of environmental factors in shaping the gut microbiota in infants

In Study **II**, differences in the gut microbiota composition of Malawian and Finnish infants were observed in most bacteria assessed. Percentages of *Bifidobacterium* group, *Bacteroides-Prevotella* group, *Clostridium histolyticum* group and the total number of bacteria detected by FCM-FISH were significantly higher in Malawian than in Finnish infants (70.8% vs. 46.8%, p<0.001; 17.2% vs. 4.7%, p<0.001; 4.4% vs. 2.8%, p=0.01; 90.9% vs. 58.6%, p<0.001; respectively), (**Figure 9**).

Likewise the counts of *Bifidobacterium*, *Bacteroides-Prevotella, Clostridium histolyticum* groups and the total number of bacteria detected by FCM-FISH were significantly higher in the Malawian infants (9.2 log cells/g, IQR 8.4-9.6 vs. 8.4 log cells/g IQR 8.0-8.7, p<0.001; 8.6 log cells/g, IQR 8.1-8.9 vs. 7.5 log cells/g IQR 6.9-8.0, p<0.001; 8.0 log cells/g, IQR 7.6-8.4 vs. 7.3 log cells/g, IQR 6.2-7.6, p<0.001; 9.4 log cells/g, IQR 8.8-9.8 vs. 8.8 log cells/g IQR 8.4-9.0, p=0.003, respectively).

At the same time, using qPCR higher counts of the *Bifidobacterium* genus and the species *B. longum* and *B. bifidum* were detected in higher amounts in Malawian compared to Finnish infants (10.4 vs. 10.0 log cells/g, 10.3 vs. 9.1 log cells/g, 10.1 vs. 5.9 log cells/g, respectively).

The *B. catenulatum*, *A. muciniphila* and *C. difficile* species were rare in Malawian as compared to Finnish infants. Bacteria belonging to the *B. adolescentis, S. aureus* and *C. perfringens* species, present in Finnish, were not detectable in Malawian infants (**Table 8**).

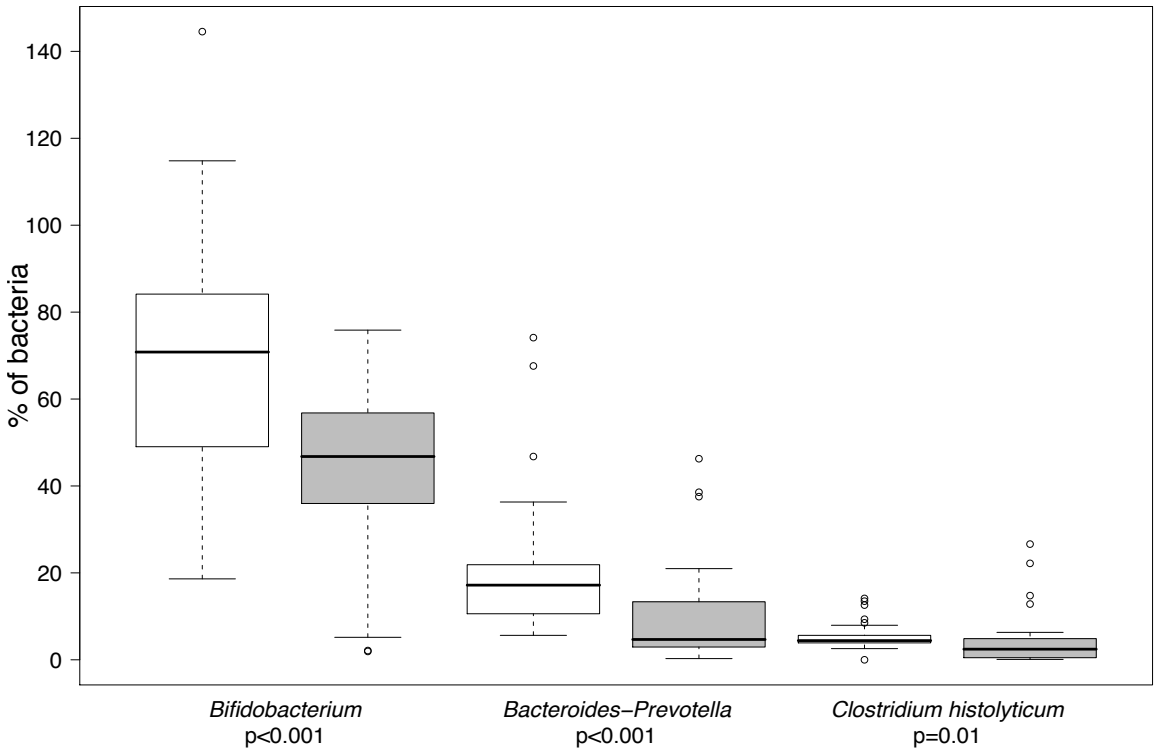

Figure 9. Percentages of the major bacterial groups as compared to the total amount of bacteria in faecal samples from 6-month-old breast-fed Malawian and Finnish infants in Study **II**, analysed by FCM-FISH. The white bars indicate Malawian and grey bars Finnish subjects. The line in the box represents the median (50th percentile), the lower line the 25% border (25th percentile) and the upper line the 75% border (75th percentile). The end of the upper vertical line represents the maximum data value, outliers not considered. The end of the lower vertical line represents the lowest value, outliers not considered. The separate dots indicate outliers.

Table 8. Bacterial prevalences and counts (log cells/g) in faecal samples from 6-month-old Malawian and Finnish infants in Study **II**, analysed by qPCR.

	Malawian infants (n=44)		Finnish infants (n=31)		Comparison of groups	
Bacteria	**%**	**Median (IQR)**	**%**	**Median (IQR)**	**P[1]**	**P[2]**
Bifidobacterium genus	100.0	10.4 (10.0-11.2)	100.0	10.0 (9.4-10.4)	ND	0.04
B.longum	100.0	10.3 (9.8-10.8)	100.0	9.1 (7.3-9.8)	ND	<0.001
B.catenulatum	47.7	0 (0-7.5)	83.8	5.8 (4.9-9.5)	0.017	0.056
B.bifidum	97.8	10.1 (9.0-10.8)	58.1	5.9 (0-9.2)	1.666E-04	<0.001
B.adolescentis	0	0	32.3	0 (0-4.8)	5.351E-04	<0.001
B.breve	93.2	7.8 (6.9-8.7)	83.9	7.2 (6.7-10.1)	2.629	9.356
C.coccoides	65.9	6.0 (0-7.4)	100.0	9.08 (8.2-9.6)	1.917E-03	<0.001
C.leptum	40.9	0 (0-6.4)	87.1	6.7 (5.8-7.7)	9.083E-04	<0.001
C.difficile	9.1	0	45.2	0 (0-8.9)	6.545E-03	0.004
C.perfringens	0	0	19.4	0	0.037	0.025
A.muciniphila	2.3	0	38.7	0 (0-3.9)	5.068E-04	<0.001
S.aureus	0	0	54.8	5.4 (0-7.1)	8.937E-08	<0.001

P[1] – Fisher's exact test was used to test the percentages of positive values; **P[2]** – The Kruskal-Wallis test was used to test counts of bacteria; **ND**, not defined

5.3 Gut microbiota in relation to geographical location and diet

In Study **III**, the percentage of the *Bifidobacterium* group among the total number of bacteria detected by FCM-FISH tended to be higher in Finnish than in German infants (68.1% vs. 58.3%, p=0.142). Similarly, qPCR identified a trend towards higher counts of bifidobacteria in Finnish infants (10.68 log cells/g, IQR 10.20-11.35 vs. 9.90 log cells/g, IQR 9.40-10.78, p=0.097). A significantly higher percentage of the total bacterial count comprised *Akkermansia muciniphila* in German as compared to Finnish breast-fed infants (8.6% vs. 4.7%, p=0.004, **Table 9**) and the *Akkermansia muciniphila* counts were also higher (7.81 log cells/g, 95% CI 7.49-8.14 vs. 7.21 log cells/g, 95% CI 6.99-7.43, p=0.003;). Likewise *Clostridium histolyticum* and *Bacteroides-Prevotella* group counts were significantly higher in German compared to Finnish infants (7.67 log cells/g, 95% CI 7.16-8.17 vs. 7.26 log cells/g, 95% CI 7.08-7.44, p=0.035; 7.84 log cells/g 95% CI 7.47-8.21 vs. 7.42 log cells/g 95% CI 7.23-7.60, p=0.027, respectively).

Table 9. Percentages of the major bacterial groups as compared to the total amount of bacteria in faecal samples from 6-month-old breast-fed Finnish and German infants analysed by FCM-FISH.

	Finnish infants (n=22)		German infants (n=8)		
% Bacteria	**Mean**	**95% CI**	**Mean**	**95% CI**	**P#**
Bifidobacterium	68.1	57.0-79.3	58.3	39.5-77.0	0.142
Bacteroides-Prevotella	7.4	6.0-8.9	8.7	5.0-12.4	0.504
Clostridium histolyticum	5.3	4.5-6.1	5.6	4.2-7.0	0.447
Lactobacillus-Enterococcus	6.1	5.2-7.0	6.7	4.9-8.5	0.597
Akkermansia muciniphila	4.7	4.3-5.1	8.6	3.7-13.4	0.004
Total	91.7	79.9-103.4	87.8	68.8-106.9	0.447

Mann-Whitney U test

When the gut microbiota was compared between breast-fed and formula-fed infants from Germany (**A**), it was noted that the percentage of the *Bifidobacterium* group among the total number of bacteria detected by FCM-FISH tended to be higher in breast-fed than in formula-fed infants (58.3% vs. 43.4%, p=0.06), (**Table 10**). Using qPCR, the *Bifidobacterium* group was found to be dominant, with a 100% colonisation rate and the highest counts in both cohorts studied.

Table 10. Percentages of the major bacterial groups as compared to the total amount of bacteria in faecal samples from 6-month-old breast-fed and formula-fed German infants analysed by FCM-FISH.

	Breast-fed infants (n=8)		Formula-fed infants (n=32)		
% Bacteria	**Mean**	**95% CI**	**Mean**	**95% CI**	**P#**
Bifidobacterium	58.3	39.5-77.0	43.4	36.62-50.10	0.060
Bacteroides-Prevotella	8.7	5.0-12.4	10.7	7.92-13.42	0.489
Clostridium histolyticum	5.6	4.2-7.0	6.0	5.01-6.96	0.695
Lactobacillus-Enterococcus	6.7	4.9-8.5	6.0	5.37-6.56	0.294
Akkermansia muciniphila	8.6	3.7-13.4	6.5	5.39-7.56	0.178
Total	87.8	68.8-106.9	72.5	64.1-80.9	0.105

Mann-Whitney U test

Significantly lower counts of *B. catenulatum, C. coccoides, C. leptum, C. difficile, A. muciniphila* and *S. aureus* were found in breast-fed than in formula-fed infants (p=0.045, p=0.001, p=0.005, p=0.016, p=0.003, p=0.045, respectively). Further, the colonisation rates of *B. catenulatum C. coccoides, C. leptum, A. muciniphila* and *S. aureus* (p=0.038, p=0.004, p=0.004, p=0.001, p=0.038, respectively) were lower in breast-fed when compared to formula-fed infants (**Table 11**).

Table 11. Bacterial prevalences and counts (log cells/g) in faecal samples from 6-month-old breast-fed and formula-fed German infants, analysed by qPCR.

	Breast-fed infants (n=8)		Formula-fed infants (n=32)		Comparison of groups	
	%	Median (IQR)	%	Median (IQR)	P^1	P^2
Bifidobacterium genus	100.0	9.90 (9.40-10.78)	100.0	10.31 (9.94-11.01)	ND	0.182
B.longum	87.5	10.02 (8.86-11.05)	96.9	10.17 (7.69-10.91)	0.277	0.906
B.catenulatum	0	5.14 (5.14-5.14)	37.5	5.14 (5.14-7.71)	0.038	0.045
B.bifidum	50.0	2.72 (0-8.59)	78.1	8.71 (5.69-9.85)	0.111	0.081
B.lactis	75.0	6.08 (1.27-6.23)	59.4	7.36 (2.88-9.57)	0.414	0.266
B.infantis	25.0	6.46 (6.46-7.61)	25.0	6.46 (6.46-6.46)	1.000	0.756
B.adolescentis	0	5.00 (5.00-5.00)	34.4	5.00 (5.00-6.75)	0.051	0.059
B.breve	87.5	8.44 (6.19-8.65)	53.1	5.70 (4.38-8.22)	0.076	0.090
C.coccoides	75.0	9.59 (2.36-9.87)	100.0	10.86 (10.49-11.40)	0.004	0.001
C.leptum	75.0	5.51 (1.08-6.43)	100.0	6.96 (6.11-7.95)	0.004	0.005
C.difficile	25.0	5.15 (5.15-5.09)	62.5	8.18 (5.15-9.45)	0.057	0.016
C.perfringens	0	6.43 (6.43-6.43)	15.6	6.43 (6.43-6.43)	0.232	0.239
A.muciniphila	0	1.88 (1.88-1.88)	65.6	4.04 (1.88-5.26)	0.001	0.003
S.aureus	0	4.87 (4.87-4.87)	37.5	4.87 (4.87-5.08)	0.038	0.045

P^1 – Chi-squared test was used to test the percentages of positive values; **P^2** – Kruskal-Wallis test was used to test the counts for bacteria having ≥ 50% positive values in all study groups; **ND**, not defined

5.4 Impact of specific probiotic administration on the gut microbiota composition

In Study **III**, in Finnish infants who received probiotics bifidobacteria were the most common bacteria, this group's means being between 63.8% and 69.5% of the total bacteria. The percentages of *Bacteroides-Prevotella, Clostridium histolyticum, Lactobacillus-Enterococcus* groups and *Akkermansia muciniphila* were relatively small among the gut microbiota of all groups studied. The percentages of the major bacterial groups as compared to the total bacteria in infants assessed by FCM-FISH are presented in **Table 12.**

Using qPCR, the *Bifidobacterium* group was found to be the dominant bacteria, with a 100% colonisation rate and the highest counts. Among bifidobacteria, *B. longum* numbers were the highest. In all study groups qPCR identified high levels of *C. coccoides*. The colonisation rates and numbers of bacteria are shown in **Table 13.**

Table 12. Percentages of the major bacterial groups as compared to the total amount of bacteria in faecal samples from 6-month-old Finnish infants from Study **III**, whose mothers were treated with probiotics (ST11+BL999 or LPR+BL999) or placebo 2 months prior to and 2 months after delivery.

	ST11+BL999 (n = 29)		LPR+BL999 (n = 28)		Placebo (n = 22)		
% Bacteria	**Mean**	**95% CI**	**Mean**	**95% CI**	**Mean**	**95% CI**	**P[1]**
Bifidobacterium	63.8	54.6-72.9	69.5	61.0-77.9	68.1	57.0-79.3	0.642
Bacteroides-Prevotella	9.9	7.1-12.7	11.8	8.5-15.0	7.4	6.0-8.9	0.097
Clostridium histolyticum	6.6	4.9-8.2	6.0	5.2-6.9	5.3	4.5-6.1	0.361
Lactobacillus-Enterococcus	7.3	6.4-8.3	9.0	7.5-10.4	6.1	5.2-7.0	0.003[2]
Akkermansia muciniphila	5.2	4.7-5.8	5.4	4.8-6.1	4.7	4.3-5.1	0.163
Total	92.8	84.6-101.0	101.6	91.4-111.9	91.7	79.9-103.4	0.280

Faecal microbiota analysed by FCM-FISH; **ST11+BL999** (*L. paracasei* ST11 and *B. longum* BL999); **LPR+BL999** (*L. rhamnosus* LPR and *B. longum* BL999); the figures in the table represent the percentages of the named bacterial group as against total count detected by FCM-FISH; [1]ANOVA was used to detect the overall difference between the study groups; [2]Bonferroni-adjusted post hoc comparisons: ST11+BL999 vs. LPR+BL999, p=0.105; ST11+BL999 vs. placebo, p=0.438; LPR+BL999 vs. placebo, p=0.003

There was a significant difference in the percentage of the *Lactobacillus-Enterococcus* group (p=0.003): in STT11+BL999 this group comprised 7.3% of the total bacteria, in LPR+BL999 9.0% and in the placebo group 6.1% (**Table 12**). Post-hoc comparisons revealed that the difference between the LPR+BL999 vs. placebo group was statistically significant (p=0.003). In addition, there was a trend towards divergent percentages of the *Bacteroides-Prevotella* group between the intervention groups (p=0.097), with a significant difference in post-hoc comparison between LPR+BL999 vs. placebo group (11.8% vs. 7.4%, p=0.003). Also, *Bifidobacterium* counts at genus level were significantly different among the study groups (p=0.017); the counts were lower in the LPR+BL999 group when compared to placebo (10.03 log cells/g vs. 10.68 log cells/g, p=0.018), which later had the highest counts among the intervention groups (**Table 13**).

Further, the colonisation rates of *B. longum* and *B. infantis* differed between the study groups (p=0.026 and p=0.039, respectively). The *B. longum* rate was lower in the ST11+BL999 group (86.2%) when compared to the other two groups (both 100%). *B. infantis* was lower in LPR+BL999 (14.3%) as against other groups (ST11+BL999 44.8%, placebo 27.3%).

Table 13. Bacterial prevalences and counts (log cells/g) in faecal samples from 6-month-old Finnish infants from Study **III**, whose mothers were treated with probiotics (ST11+BL999 or LPR+BL999) or placebo, 2 months prior to and 2 months after delivery.

	STT11+BL999 (n=29)		LPR+BL999 (n=28)		Placebo (n=22)		Comparison of groups	
	%	Median (IQR)	%	Median (IQR)	%	Median (IQR)	P^1	P^2
Bifidobacterium genus	100.0	10.61 (9.80-11.04)	100.0	10.03 (9.52-10.57)	100.0	10.68 (10.20-11.35)	-	0.017
B.longum	86.2	9.87 (7.40-10.48)	100.0	9.67 (8.44-10.12)	100.0	10.02 (8.92-10.79)	0.026	0.325
B.catenulatum	65.5	5.79 (5.14-8.54)	42.9	5.14 (5.14-7.44)	45.5	5.14 (5.14-5.85)	0.179	ND
B.bifidum	51.7	5.44 (5.00-7.93)	53.6	6.66 (5.00-8.76)	50.0	5.69 (5.00-9.60)	0.969	0.605
B.lactis	37.9	2.88 (2.88-6.35)	28.6	2.88 (2.88-4.68)	50.0	4.10 (2.88-6.63)	0.301	ND
B.infantis	44.8	6.46 (6.46-7.73)	14.3	6.46 (6.46-6.46)	27.3	6.46 (6.46-6.48)	0.039	ND
B.adolescentis	17.2	5.00 (5.00-5.00)	14.3	5.00 (5.00-5.00)	9.1	5.00 (5.00-5.00)	0.705	ND
B.breve	69.0	7.19 (4.38-9.42)	57.1	5.34 (4.38-7.39)	68.2	8.63 (4.38-9.80)	0.593	0.101
C.coccoides	79.3	9.08 (6.28-10.51)	85.2	8.92 (6.19-9.76)	86.4	9.00 (7.29-10.30)	0.760	0.580
C.leptum	72.4	5.64 (3.50-6.33)	78.6	5.76 (3.74-6.52)	50.0	3.63 (3.50-6.13)	0.082	0.175
C.difficile	27.6	5.15 (5.15-6.18)	14.3	5.15 (5.15-5.15)	22.7	5.15 (5.15-5.62)	0.468	ND
C.perfringens	13.8	6.43 (6.43-6.43)	11.1	6.43 (6.43-6.43)	13.6	6.43 (6.43-6.43)	0.947	ND
A.muciniphila	20.7	1.88 (1.88-1.88)	3.6	1.88 (1.88-1.88)	13.6	1.88 (1.88-1.88)	0.149	ND
S.aureus	34.5	4.87 (4.87-5.31)	28.6	4.87 (4.87-4.89)	36.4	4.87 (4.87-5.46)	0.823	ND

Faecal microbiota analysed by qPCR; **ST11+BL999** (*L. paracasei* ST11 and *B. longum* BL999), **LPR+BL999** (*L. rhamnosus* LPR and *B. longum* BL999). **%**, percentage of positive values; **IQR**, interquartile range (25th, 75th percentiles); P^1 – Chi-squared test was used to test the percentages of positive values; P^2 – Kruskal-Wallis test was used to test the counts for bacteria having ≥ 50% positive values in all study groups; Bonferroni-adjusted post hoc comparisons for: *B. genus* (counts): ST11+BL999 vs. LPR+BL999, p=0.143; ST11+BL999 vs. placebo, p=1.000; LPR+BL999 vs. placebo, p=0.018. *B. longum* (%): ST11+BL999 vs. LPR+BL999, p=0.125; ST11+BL999 vs. placebo, p=0.209. *B. infantis* (%): ST11+BL999 vs. LPR+BL999, p=0.035; ST11+BL999 vs. placebo, p=0.597; LPR+BL999 vs. placebo, p=0.763; **ND**, not defined

In Study **III**, in German infants who received probiotics, bifidobacteria were the most common bacteria, with intervention group means between 43.4% and 46.6% of the total bacteria. The proportions of the major bacterial groups as compared to the total amount of bacteria were not influenced by probiotic treatments (**Table 14**).

Using qPCR, bifidobacteria and clostridia were the most frequently detected bacteria and *B. longum* and *C. coccoides* reached the highest counts in all intervention groups (**Table 15**). Probiotic treatment had an impact on the colonisation rate (p=0.029) and levels (p=0.044) of *B. bifidum* (**Table 15**); the lowest colonisation rate of *B. bifidum* was in the BL999 group (44.0%) compared to other groups (LPR+BB999 58.3%, placebo 78.1%), while post-hoc comparisons showed that infants receiving formula with LPR+BL999 tended to have lower counts of *B. bifidum* as compared to the placebo group (5.76 log cells/g vs. 8.71 log cells/g, p=0.078).

Table 14. Percentages of the major bacterial groups as compared to the total amount of bacteria in faecal samples from 6-month-old German infants from Study **III**, treated with probiotics (BL999 or LPR+BL999) or placebo at weaning at the latest at 1 month of age and continued for 4 months.

	BL999 (n = 25)		LPR+BL999 (n = 24)		Placebo (n = 32)		
% Bacteria	**Mean**	**95% CI**	**Mean**	**95% CI**	**Mean**	**95% CI**	**P[1]**
Bifidobacterium	44.2	33.18-55.30	46.6	37.14-56.09	43.4	36.62-50.10	0.863
Bacteroides-Prevotella	10.1	7.08-13.16	10.2	6.64-13.73	10.7	7.92-13.42	0.958
Clostridium histolyticum	6.0	4.78-7.24	6.1	4.26-7.87	6.0	5.01-6.96	0.996
Lactobacillus-Enterococcus	6.0	4.91-7.02	5.8	4.38-7.27	6.0	5.37-6.56	0.975
Akkermansia muciniphila	7.0	4.51-9.40	6.3	3.70-8.96	6.5	5.39-7.56	0.907
Total	73.3	61.3-85.3	75.0	63.5-86.5	72.5	64.1-80.9	0.939

Faecal microbiota analysed by FCM-FISH; **BL999** (*B. longum* BL999); **LPR+BL999** (*L. rhamnosus* LPR and *B. longum* BL999); the figures in the table represent the percentages of the named bacterial group as against total count detected by FCM-FISH; [1]ANOVA was used to detect the overall difference between the study groups

Table 15. Bacterial prevalences and counts (log cells/g) in faecal samples from 6-month-old German infants from Study **III**, treated with probiotics (BL999 or LPR+BL999) or placebo at weaning at the latest at 1 month of age and continued for 4 months.

	BL999 (n=25)		LPR+BL999 (n=24)		Placebo (n=32)		Comparison of groups	
	%	**Median (IQR)**	**%**	**Median (IQR)**	**%**	**Median (IQR)**	**P[1]**	**P[2]**
Bifidobacterium genus	100.0	10.57 (9.73-10.99)	100.0	10.25 (9.99-10.50)	100.0	10.31 (9.94-11.01)	ND	0.719
B.longum	100.0	10.59 (9.60-11.19)	100.0	10.34 (9.75-10.80)	96.9	10.17 (7.69-10.91)	0.461	0.452
B.catenulatum	44.0	5.14 (5.14-6.23)	37.5	5.14 (5.14-7.66)	37.5	5.14 (5.14-7.71)	0.858	ND
B.bifidum	44.0	5.00 (5.00-9.34)	58.3	5.76 (5.00-8.83)	78.1	8.71 (5.69-9.85)	0.029	0.044
B.lactis	64.0	6.62 (2.88-7.46)	62.5	6.32 (2.88-9.46)	59.4	7.36 (2.88-9.57)	0.934	0.461
B.infantis	24.0	6.46 (6.46-6.56)	25.0	6.46 (6.46-7.21)	25.0	6.46 (6.46-6.46)	0.995	ND
B.adolescentis	20.0	5.00 (5.00-5.00)	29.2	5.00 (5.00-6.79)	34.4	5.00 (5.00-6.75)	0.488	ND
B.breve	72.0	7.45 (4.38-8.78)	58.3	7.06 (4.38-8.61)	53.1	5.70 (4.38-8.22)	0.340	0.349
C.coccoides	96.0	10.94 (10.35-11.34)	95.8	10.64 (9.94-11.28)	100.0	10.86 (10.49-11.40)	0.512	0.563
C.leptum	96.0	7.29 (6.87-8.03)	95.8	7.40 (6.69-8.00)	100.0	6.96 (6.11-7.95)	0.512	0.745
C.difficile	56.0	7.85 (5.15-9.14)	58.3	7.80 (5.15-8.98)	62.5	8.18 (5.15-9.45)	0.879	0.451
C.perfringens	8.0	6.43 (6.43-6.43)	12.5	6.43 (6.43-6.43)	15.6	6.43 (6.43-6.43)	0.686	ND
A.muciniphila	48.0	1.88 (1.88-5.82)	50.0	2.84 (1.88-5.63)	65.6	4.04 (1.88-5.26)	0.334	ND
S.aureus	56.0	4.87 (4.87-5.37)	41.7	4.87 (4.87-5.13)	37.5	4.87 (4.87-5.08)	0.358	ND

Faecal microbiota analysed by qPCR; **BL999** (*B. longum* BL999); **LPR+BL999** (*L. rhamnosus* LPR and *B. longum* BL999). **%**, percentage of positive values; **IQR**, interquartile range (25th, 75th percentiles); **P[1]** – Chi-squared test was used to test the percentages of positive values; **P[2]** – Kruskal-Wallis test was used to test the counts for bacteria having ≥ 50% positive values in all study groups. Bonferroni-adjusted post-hoc comparisons for: *B. bifidum* (%): BL999 vs. LPR+BL999, p=0.947; BL999 vs. placebo, p=0.024; LPR+BL999 vs. placebo, p=0.333. *B. bifidum* (counts): BL999 vs. LPR+BL999, p=1.000; BL999 vs. placebo, p=0.156; LPR+BL999 vs. placebo, p=0.078; **ND**, not defined

5.5 *In vitro* evaluation of commercially used probiotic *L. rhamnosus* GG

Identification at species level and genotypic and phenotypic characterisation of the isolates

In Study **IV** all the *L. rhamnosus* isolates used were confirmed as members of the species in question by partial sequencing of the 16S rDNA. Sequence analysis showed 100% sequence homology among the different *L. rhamnosus* isolates studied as well as with *L. rhamnosus* 16S rDNA sequences held in the databases (data not shown).

All the *L. rhamnosus* isolates except the reference strain OL showed RAPD, ERIC and PFGE profiles identical to that of the original *L. rhamnosus* GG isolate obtained from Professors Gorbach and Goldin. These three techniques allowed differentiation of the isolate *L. rhamnosus* OL from the other *L. rhamnosus* isolates.

With regard to phenotypic characterisation, none of the *L. rhamnosus* isolates fermented glycerol, erythiol, L-arabinose, D-xylose, L-xylose, D-adonitol, methyl-βD-xylopyranoside, inositol, methyl-αD-mannopyranoside, D-melibiose, D-saccharose, inulin, D-raffinose, starch, glycogen, xylitol, D-turanose, D-lyxose, D-fucose, D-arabitol, L-arabitol, 2-ketogluconate or 5-ketogluconate. All fermented D-arabinose, D-ribose, D-galactose, D-glucose, D-mannitol, D-fructose, D-mannose, dulcitol, D-sorbitol, N-acetyl glucosamine, amigdalin, arbutin, esculin, salicin, D-cellobiose, D-trehalose, D-melezitose, gentiobiose, D-tagatose, L-fucose and gluconate. All the putative *L. rhamnosus* isolates showed identical sugar fermentation profiles, being different from those of the reference strain OL, which in addition was able to ferment L-sorbose, L-rhamnose, Methyl-αD-glucopyranoside, D-maltose and D-lactose.

Tolerance to acidic pH (pH 2 and 2.5) varied from 43.0% to 79.1% and showed no significant differences ($p>0.05$) among the tested *L. rhamnosus* isolates.

Adhesion of L. rhamnosus isolates to human colonic mucus

The adherence of *L. rhamnosus* isolates varied between different isolates (**IV**). That of the original *L. rhamnosus* GG was 19% ± 7.5; that of the other isolates varied from 12.1% to 24.3%. Only the adhesion of the reference strain OL differed significantly from that of the original *L. rhamnosus* GG (p=0.01) and it also showed the lowest percentage of adhesion among all isolates tested (data not shown).

The pathogenic strains tested adhered as follows: *S. aureus* (10.2% ± 3.0), *C. perfringens* (4.0% ± 3.1), *S. enterica* serovar Typhimurium (2.4% ± 0.7), *C. sakazakii* (2.0% ± 0.7).

Properties of pathogen exclusion by L. rhamnosus isolates as compared to the original Lactobacillus rhamnosus GG

In exclusion by inhibition adherent bacteria block the adhesion of other bacteria to the mucus. In this study (**IV**) the ability of the *L. rhamnosus* isolates to inhibit the adhesion of pathogens to the colonic mucus differed significantly (**Figure 10**). The original *L. rhamnosus* GG was able to inhibit all pathogens tested and this microorganism reduced the adhesion of *C. sakazakii* a 31%, *S. enterica* serovar Typhimurium a 25%, *C. perfringens* (25%) and *S. aureus* (24%). Differences were seen between *L. rhamnosus* isolates in the adhesion of *C. sakazakii*, *S. enterica* serovar Typhimurium and *C. perfringens* ($p<0.001$ for each) but not *S. aureus* (p=0.352). Four *L. rhamnosus* isolates (AL, LL, ML, NL) showed different inhibition (p=0.083, p=0.015, $p<0.001$, p=0.025, respectively) of *C. sakazakii* compared to the original *L. rhamnosus* GG. The isolate ML, again, differed ($p<0.001$) from the original *L. rhamnosus* GG in inhibition of the adhesion of *S. enterica* serovar Typhimurium. Two *L. rhamnosus* isolates (ML, OL) differed ($p<0.001$, p=0.032, respectively) from the original *L. rhamnosus* GG in inhibiting the adhesion of *C. perfringens*. The adhesion inhibition of *S. aureus* did not differ between the tested *L. rhamnosus* isolates and the original *L. rhamnosus* GG.

In exclusion by displacement the adherent microorganisms are outnumbered by other microbes. In this experiment (**IV**) all the *L. rhamnosus* isolates were able to displace the pre-adhered model pathogens tested in the colonic mucus. Differences were seen between *L.*

rhamnosus isolates in the adhesion of *C. sakazakii*, *S. enterica* serovar Typhimurium (p=0.063, p=0.001, respectively) but not between *C. perfringens* and *S. aureus* (p=0.128, p=0.717, respectively). The original *L. rhamnosus* GG displaced 54% of adhered *S. enterica* serovar Typhimurium and it differed (p=0.084) from the *L. rhamnosus* LL isolate. *C. perfringens* was displaced 33% and *S. aureus* 20% by the original *L. rhamnosus* GG; all other isolates showed no difference in the displacement from the original *L. rhamnosus* GG. The original *L. rhamnosus* GG displaced *C. sakazakii* by 12%, differing (p=0.082, p=0.009, p=0.017, p=0.013, p=0.068, p=0.019, p=0.01, p=0.092, p=0.051, respectively) from the other study isolates AL, BL, CL, DL, EL, HL, IL, KL and NL. The results are shown in **Figure 11.**

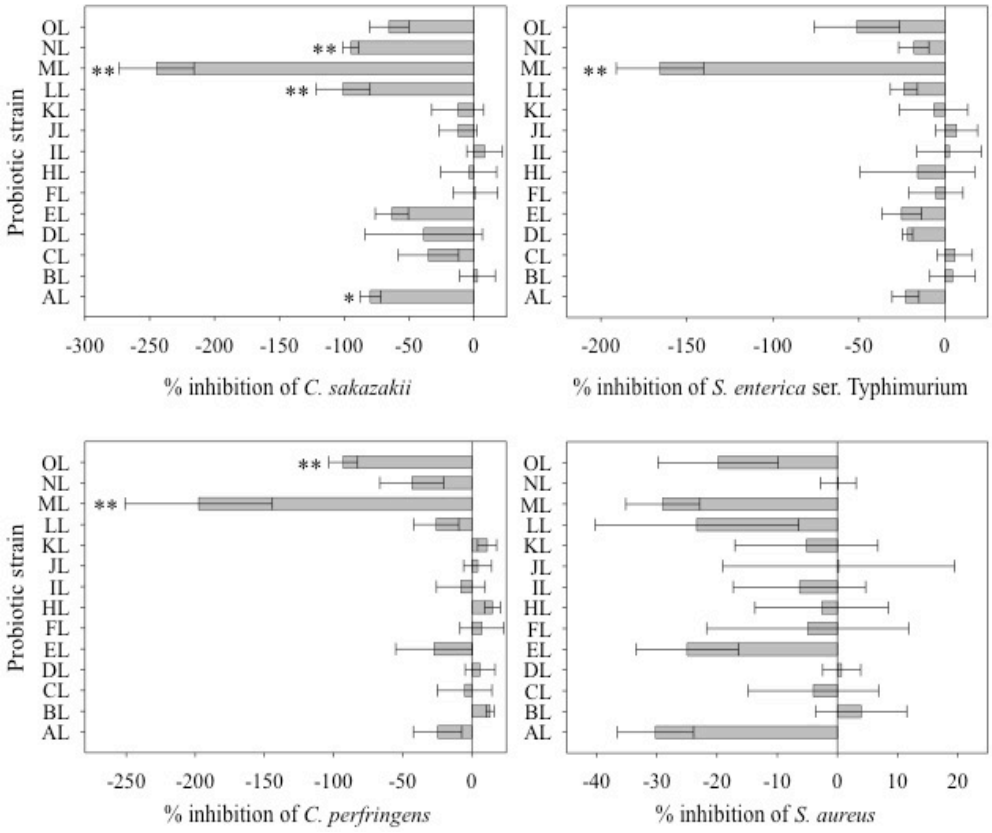

Figure 10. Inhibition of the adhesion to the colonic mucus of pathogenic bacteria by the tested *L. rhamnosus* isolates. Results are expressed as percentages with regard to the inhibition obtained with the original *L. rhamnosus* GG isolate donated by Prof. S. Gorbach. Values on the X-axis higher than 0 indicate adhesion inhibition higher than the original *L. rhamnosus* GG and those lower than 0 indicate adhesion inhibition lower than the original *L. rhamnosus* GG. Asterisks indicate adhesion significantly different from the original *L. rhamnosus* GG (* if 0.05≤ p<0.10 and ** if p<0.05). Values are means, with standard errors represented by vertical bars. The OL indicates the external reference *L. rhamnosus* strain.

In exclusion by competition microorganisms compete for adherence at the same time. In this work (**IV**) the inhibition by competition varied among *L. rhamnosus* isolates and pathogens tested. The relevant data are shown in **Figure 12.** The presence of some isolates increased the adhesion of *C sakazakii, S. enterica* serovar Typhimurium and *C. perfringens* to the colonic mucus. All but one isolate – BL were able to reduce the adhesion of *S. aureus*. Differences were noted between *L. rhamnosus* isolates in the adhesion of *C. sakazakii, S. enterica* serovar Typhimurium*, C. perfringens* and *S. aureus* ($p<0.001$, $p<0.001$, $p=0.005$, $p=0.023$, respectively).

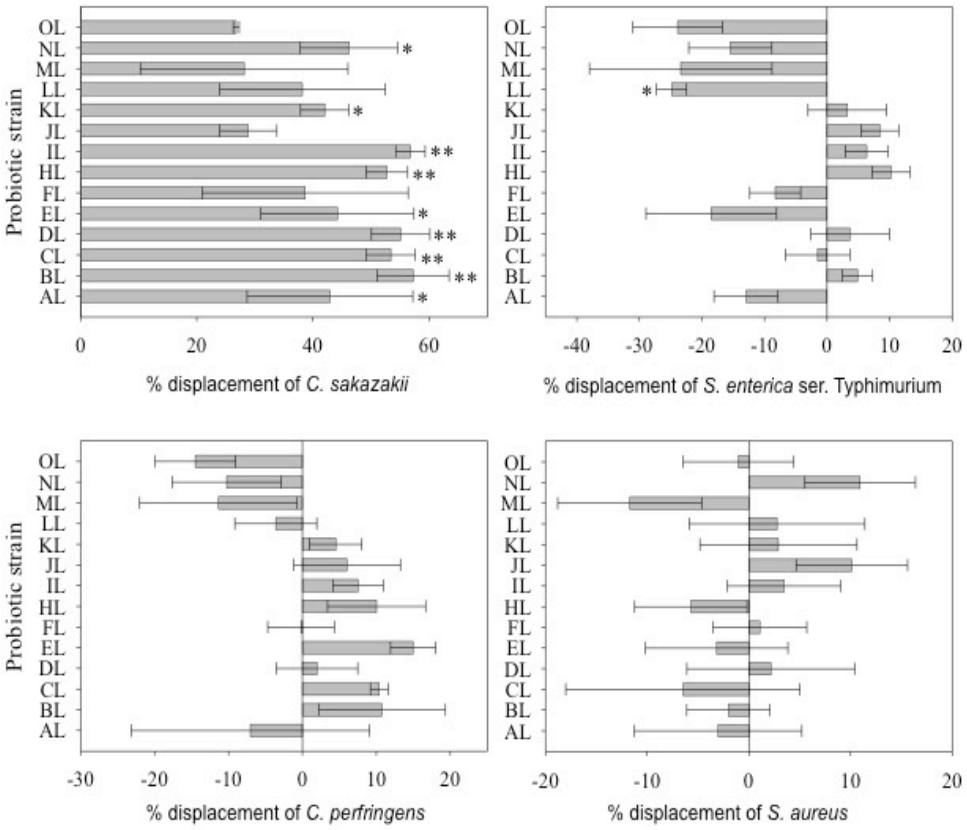

Figure 11. Displacement of adhesion to the colonic mucus of pathogenic bacteria by the tested *L. rhamnosus* isolates. Results are expressed as percentages with regard to the displacement obtained with the original *L. rhamnosus* GG isolate donated by Prof. S. Gorbach. Values on the X-axis higher than 0 indicate adhesion displacement higher than the original *L. rhamnosus* GG and those lower than 0 displacement lower than the original *L. rhamnosus* GG. Asterisks indicate adhesion displacement significantly different from the original *L. rhamnosus* GG (* if $0.05 \leq p<0.10$ and ** if $p<0.05$). Values are means, with standard errors represented by vertical bars. The OL indicates the external reference *L. rhamnosus* strain.

The original *L. rhamnosus* GG showed inhibition abilities for all pathogens tested. This microorganism inhibited the adhesion of *C. perfringens* in 47%, being different (p=0.057, p=0.059, p=0.07, p=0.091, respectively) from BL, LL, ML, NL isolates; *S. aureus* in 39%, being different (p=0.019) from BL isolate; *S. enterica* serovar Typhimurium 5%, being different (p=0.064, p=0.014, p=0.026, respectively) from IL, ML, NL isolates; and *C. sakazakii* 9%, showing a difference (p=0.001, p=0.003, p=0.048, p=0.001, p<0.001, p=0.079, respectively) from AL, DL, LL, ML, NL isolates and the reference strain OL.

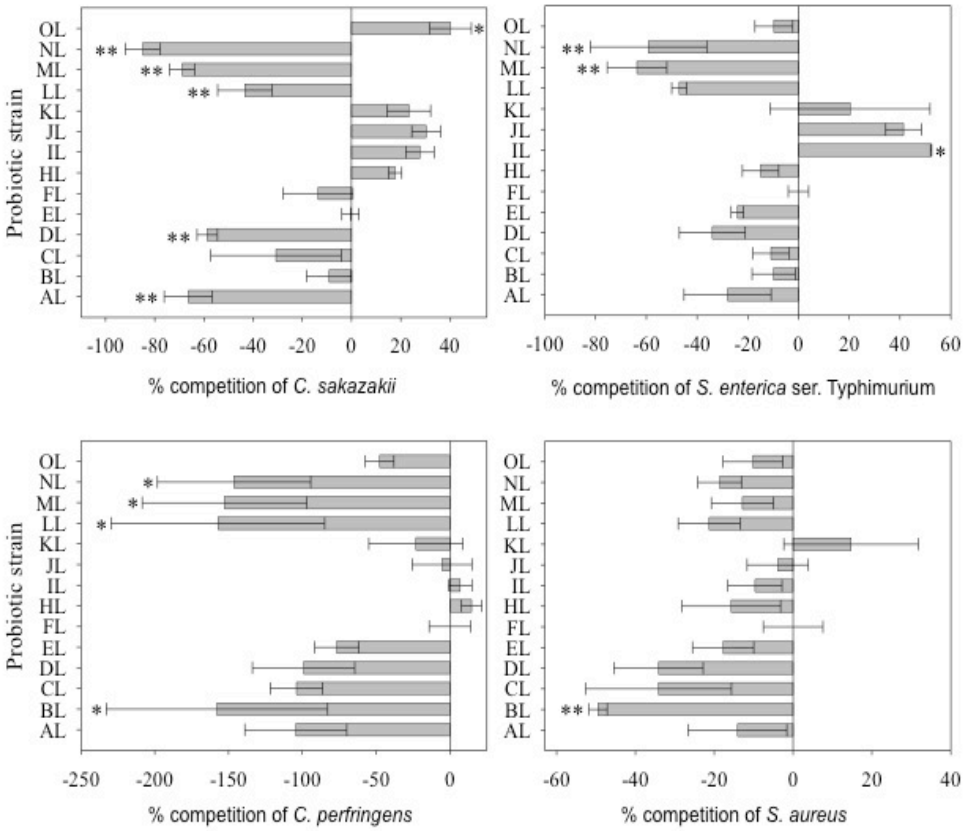

Figure 12. Competition for adhesion to the colonic mucus of pathogenic bacteria by the tested *L. rhamnosus* isolates. Results are expressed as percentages with regard to the competition obtained with the original *L. rhamnosus* GG isolate donated by Prof. S. Gorbach. Values on the X-axis higher than 0 indicate adhesion competition higher than the original *L. rhamnosus* GG and those lower than 0 competition lower than the original *L. rhamnosus* GG. Asterisks indicate adhesion competition significantly different from the original *L. rhamnosus* GG (* if 0.05≤ p<0.10 and ** if p<0.05). Values are means, with standard errors represented by vertical bars. The OL indicates the external reference *L. rhamnosus* strain.

6. DISCUSSION

6.1 Factors influencing gut microbiota composition in infants

The association between mother and infant gut microbiota during early life has been reported to be a critical factor for the subsequent succession of intestinal commensal bacteria in infancy (Grönlund et al. 1999, Adlerberth et al. 2006, Adlerberth et al. 2007). During the perinatal period an actual transmission of gut bacteria from mother to infant has been detected (Tannock et al. 1990, Murono et al. 1993). In the gut of healthy breast-fed infants, bifidobacteria are already the predominant bacterial group at 3 days of age and they continue to predominate during the first 6 months of life (Harmsen et al. 2000, Favier et al. 2002, Studies **I, II, III, A**). In Study **I**, a significant correlation in levels of *Bifidobacterium* in mothers and infants was observed at 1 month after delivery. In contrast, previous studies have found no correlation in faecal total counts of bifidobacteria between mothers and 1-month-old infants (Grönlund et al. 2007, Mikami et al. 2009). However, in the present case almost all infants were exclusively breast-fed and thus the bifidobacteria found in breastmilk could have impacted on the association detected. Additionally, the DI of bifidobacteria in infants and mothers here correlated at 1 month after delivery. Furthermore, the infants who harboured diverse bifidobacterial microbiota at 1 month of age tended to do so also later on in infancy, since the bifidobacterial DI in infants at 1 and 6 months of age correlated. These findings thus emphasise the importance of the gut microbiota association between mother and infant in early infancy. A constant association in the colonisation rates of *B. bifidum* in the mother-infant pairs was detected both 1 and 6 months after delivery. The colonisation rates of *B. breve* correlated in the mother-infant pairs only at 1 month after delivery. On the other hand, no correlation in colonisation rates between mothers and infants could be found in other bifidobacterial species assessed (*B. catenulatum, B. lactis, B. infantis, B. adolescentis*). These findings suggest that colonisation of infants by *B. bifidum* is more depedent on the mother-infant association compared to other bifidobacterial species. Along with *B. longum, B. infantis* and *B. breve*, *B. bifidum* is also one of the predominating bifidobacterial species residing in the infant gut (Harmsen et al. 2008, Matsuki et al. 1999, He et al. 2001b, Rinne et al. 2005, Grönlund et al. 2007, Studies **I, II, III, A**). Likewise, maternal *B. bifidum* was the determining bifidobacterial species

which increased the DI of bifidobacteria in the faeces of infants and the mother-infant bifidobacterial SI at both 1 and 6 months of age, while in the study by Mikami and associates (2009) *B. breve* increased both the bifidobacterial counts and the diversity of *Bifidobacterium* species in infant faeces at 1 month of age. Thus, factors which contribute to the diversity of the intestinal microbiota should be further elucidated, since diversity in the intestinal microbiota in early life has been claimed to be one important factor preventing the development of atopic diseases (Wang et al. 2008).

It is tempting to speculate that those bifidobacteria which correlate between mothers and infants are the important ones for the later health of the infants. Evolutionary vaginal delivery was the means of ensuring that maternal intestinal bacteria were transferred to the newborn. The findings here show that there is a significant correlation in the intestinal bifidobacterial microbiota between mothers and infants and that *B. bifidum* and *B. breve* constitute such maternal bifidobacterial species which might modulate the colonisation process in the newborns more markedly than others.

In addition to the mother's own microbiota, it has also been suggested that sanitation, hygiene and environment may guide the succession of microbes (Adlerberth and Wold 2009, Favier et al. 2002, Palmer et al. 2007). Distinct gut microbiota compositions in infants from different geographic areas have been reported (Adlerberth et al. 1991), as well as across Europe (Björkstén et al. 1999, Fallani et al. 2010, Sepp et al. 1997) and in Europe and Africa (De Filippo et al. 2010) respectively.

In this series of studies comparison was made between the microbiota of South Eastern African with Northern European infants (**II**) and across European infants (**III**). The gut microbiota of Malawian infants showed an abundance of *Bifidobacterium, Bacteroides-Prevotella* and *Clostridium histolyticum* as compared to the levels of these bacteria in Finnish infants (**II**). In contrast, in a study comparing gut microbiota in infants from two European cohorts (**III**), higher counts of *Clostridium histolyticum* and *Bacteroides-Prevotella* were observed in German as compared to Finnish breast-fed infants. The most likely explanation for the effect seen in African infants is the diet, this being composed of plant polysaccharides, which are introduced together with breast-feeding already in infancy. In recent studies by Ley and collaborators (2005,2006), a high *Firmicutes / Bacteroidetes* ratio has been reported in obese individuals, while an increase in *Bacteroidetes* accompanies

weight loss. These previous results are in accord with the difference observed in the numbers of *Bacteroidetes* coinciding with weight differences between Malawian and infants from south-western Finland, whether related or unrelated to the diet (**II**). In recent studies from Finland, again, higher levels of *Bacteroidetes,* clostridial species and staphylococci were seen in obese and overweight infants and adults (Collado et al. 2010, Kalliomäki et al. 2008) while at the same time *Bacteroidetes* levels were significantly higher in infants from obese mothers during the first 6 months of life (Collado et al. 2010). In addition, Malawian infants were less often colonised with specific clostridial species and staphylococci (**II**). *Bacteroides* and specific *Clostridium* species such as those found in Finnish and German infants (**II, III, A**) may often be considered as opportunistic pathogens, but they are also commonly isolated from infant faeces and are regarded as indicators of healthy microbial gut succession (Stark and Lee 1982, Wexler 2007, Tonooka et al. 2005).

Bifidobacteria have been seen to typify the gut microbiota of healthy breast-fed infants. This appears to be the case in the populations studied here (**II, III, A**). High levels of bifidobacteria in breast-fed infants have previously been reported, and this may reflect breast-feeding practices i.e. length of breast-feeding or exclusive breast-feeding, as well as the impact of the environment (Rinne et al. 2005, Grönlund et al. 2007). A more detailed breakdown of species composition reveals species differences among subpopulations of infants (Gueimonde et al. 2007, Ouwehand et al. 2001c). Such findings might be associated with the different immunomodulatory properties of specific bifidobacterial species present in the gut. In particular, *B. adolescentis* has been associated with inflammatory effects, while *B. longum* and *B. bifidum* have been shown to possess immunomodulatory properties (Collado et al. 2008, Isolauri et al. 2002). Interestingly, *B. adolescentis* was detectable only in Finnish infants and *B. catenulatum* in both groups (**II**). In Malawian (**II**) and Finnish (**III)** infants the prominent species were *B. longum*, *B. bifidum* and *B. breve*. Thus, the potential role of *Bifidobacterium* species composition and the quantity of total microbiota in infants in high-income countries may be more marked, favouring the allergy risk. In low-income countries such as Malawi, the situation may be the opposite, providing clues for measures preventive of the trend in high-income areas.

In one study in the present series (**II**) Malawian infants were less often colonised with

Akkermansia-like bacteria, which were abundant in the Finnish infants. In contrast, the findings from study **III** characterising the German breast-fed infant population demonstrated that the gut microbiota is more complex and contains significantly higher counts and percentages of *Akkermansia muciniphila* when compared to Finnish breast-fed infants. *A. muciniphila* has been shown to be a common member of the infant gut microbiota in Europe, typically beginning to colonise the intestine early in infancy and increasing in numbers again towards adulthood (Collado et al. 2007a). It has also been shown that *Akkermansia* can degrade the intestinal mucus, as this bacterium contains numerous candidate mucinase-encoding genes (Passel et al. 2011). The mucus degradation caused by *Akkermansia* may affect the innate and adaptive immune responses in that it disturbs the first-defence protection of the host mucosal surfaces. This would suggest that *Akkermansia* may have a role in the pathogenesis of inflammatory diseases (Shih et al. 2008) and even obesity (Kalliomäki et al. 2008, Collado et al. 2008, Collado et al. 2010). The absence of *Akkermansia*-like bacteria in Malawian infants could thus constitute a protective health factor against westernised diseases (**II**). This, together with the observation that *Akkermansia muciniphila* was more often detected in Finnish (**II**) and German infants (**III**), would suggest a role for this species as a marker organism for gut microbiota development and diversity.

The specific microbiota composition found in Malawian infants may constitute a barrier against some potentially pathogenic intestinal microbes constantly challenging infants in low-income areas with poor hygiene. A recent study from the Ivory Coast (Zimmermann et al. 2010) reported that anaemic African children carry an unfavourably high ratio of faecal enterobacteria to bifidobacteria and lactobacilli. This ratio is further increased by iron fortification. Thus, the specific microbiota composition in Malawian infants could be protective against potentially pathogenic microbiota.

6.2 Gut microbiota modulation by probiotics in infants and probiotic product quality measurement

Probiotics are nonpathogenic, viable microbes which exert a variety of beneficial effects on the host when consumed in adequate amounts (FAO/WHO 2002). Administration of specific probiotics perinatally and during the first months of life, but also the pre-existing microbiota and the environment, may have a long-term beneficial influence on the composition and development of the infant's microbial ecosystem, potentially leading to a reduced risk of diseases (Isolauri et al. 2001, Kalliomäki et al. 2001b).

When analysing the gut microbiota of Finnish breast-fed infants (**III**) whose mothers received the specific probiotic combination LPR+BL999, it was found that the percentages of *Lactobacillus-Enterococcus* in the total count of bacteria were significantly higher compared to those in placebo infants. The finding confirms that oral administration of a specific probiotic to the mother, for example the combination with *Lactobacillus rhamnosus* LPR and *Bifidobacterium longum* BL999, promotes the growth of lactic acid bacteria in the infant's gut, and that the impact is still in evidence 4 months after discontinuation of probiotic administration.

In contrast, species identification suggests that the LPR+BL999 combination lowered the numbers of bifidobacteria compared to the placebo group and the colonisation rate of *B. infantis* was lower than in other intervention study groups. In a recent report on *Lactobacillus rhamnosus* GG administered to mothers 4 weeks before delivery, the colonisation rate of *B. longum* in 3-month-old infants was lower as compared to that in placebo-treated infants (Lahtinen et al. 2009). This notwithstanding, in a previous study from our group *Lactobacillus rhamnosus* GG had the effect of raising the colonisation rate of *B. breve* and lowering that of *B. adolescentis* at 5 days of age, but no longer at 3 weeks of age (Gueimonde et al. 2006). Both studies, where LGG was administered via the mother, show that *Lactobacillus rhamnosus* intervention might affect the bifidobacterial colonisation process in infancy. Nonetheless, the effects at species level may be related to age, feeding regimen and country. In contrast, the probiotic combination ST11+BL999 had only a minor effect in the Finnish study infants (**III**); colonisation of *B. longum* seemed to be lower than in other groups.

The specific probiotic combinations administered to formula-fed German infants (**III**) had only minor effects on microbiota composition. The LPR+BL999 treatment affected the bifidobacterial composition by apparently lowering the bacterial counts of *B. bifidum* compared to the placebo group. The species *B. bifidum* is commonly isolated from the faeces of healthy infants and adults. Together with other bifidobacteria, *B. bifidum* plays an important role in intestinal homeostasis and strengthens gastrointestinal immunity especially in children (Lin et al. 2008). The effects of probiotic intervention in the German population were less evident than in the Finnish, this possibly depending on feeding practice. In the more complex intestinal microbiota of the German formula-fed infants the effects remained fairly small, and for example no effects on lactobacilli contents could be found.

One further cause of variation in the outcomes of clinical studies, beyond differences in populations, selection criteria and study design, may arise when different production conditions, growth media, drying conditions or cryoprotectants are used for the same strain or when a successful probiotic is combined with other bacteria or strains. For example, no clinical and immunological effects were achieved when *L. rhamnosus* GG was combined with *L. rhamnosus* LC705, *Bifidobacterium breve* Bb99 and *Propionibacterium freudenreichii* ssp. shermanii (Kukkonen et al. 2008). In addition, the adhesion properties of the *L. rhamnosus* GG strain (ATCC 53103) have been shown to depend on the composition of the growth media and the number of starter culture transfers (Elo et al. 1991). Scientifically it is not surprising that different microorganisms have different effects. Though *Lactobacillus rhamnosus* GG is one of the probiotics subjected to the largest numbers of human intervention studies, in many cases no attention has been paid to the specific properties of the strains used.

Adherence of bacteria to the intestinal mucus or epithelium is known to be a prerequisite for colonisation and infection of the gastrointestinal tract by many pathogens (Finlay and Falkow 1997). The adherent probiotic strains may at least temporarily colonise the gastrointestinal tract and inhibit or compete with pathogens (Vesterlund et al. 2006). Several reports have described the adhesion abilities of probiotic strains and also their inhibitory effects on model pathogen adhesion to the human mucus (Ouwehand et al. 2001b, Collado et al. 2005, 2007c,e). In the present series (**IV**) all tested *L. rhamnosus*

isolates showed the ability to adhere to the human colonic mucus and while the adhesion ability of different isolates varied it did not differ significantly. However, the model pathogen exclusion results varied significantly and depended on the isolate tested. These findings would indicate differences in these important properties of probiotics among different *L. rhamnosus* isolates obtained from different sources. They suggest that the *in vitro* properties of probiotics may vary depending on the production conditions used for the same strain.

The findings in this series (**IV**) indicate that different isolates of the same strain may possess different properties, which may influence their *in vivo* effects, underlining the need to control the manufacturing process and the food matrix. The *L. rhamnosus* isolates tested in this study were chosen to represent different products and origins. The manufacturing process may thus have had a significant impact on the strain properties. Often probiotics are used in fermented dairy products or as freeze-dried preparations. Both provide a matrix for the microorganisms and previous studies have indicated that the matrix may affect strain-specific properties (Kankaanpää et al. 2001,2004). This, along with the production conditions, may in part explain the divergent results obtained between strain isolates when characterising strain impact on pathogen adhesion to the mucus. Variations in these properties may have a different impact on gut microbiota modulation in individual subjects, which can partially explain the probiotic effect seen in studies **I** and **III**. They may further affect the clinical outcome of intervention studies with probiotic use.

7. SUMMARY AND CONCLUSIONS

The results of this present series suggest that the gut microbiota of 6-month-old European and African infants is dominated mainly by bifidobacteria. They further reveal that specific *Bifidobacterium* species might be present for their health-promoting role in different areas of the world.

The findings are supported by the clear association seen between the mother and the infant, reflected in gut colonisation by bifidobacteria, especially by the species *B. bifidum* and *B. breve,* of which the two latter are evidently important for the subsequent health of the infant.

Geographical differences in microbiota composition are observed in the appearance of *Bifidobacterium*, *Bacteroides, Clostridium* and *Akkermansia* in 6-month-old infants and this may have been guided by initial environmental exposures, including diet and hygiene. The role of microbiota, diet and hygiene may thus be more prominent than expected in providing protection against aberrancies and inflammatory challenges.

Probiotic administration to mothers has no major effect on the mother-infant microbiota interaction process. The gut microbiota ecosystem of the infant can be modulated by specific probiotic treatments administered during the prenatal and perinatal period. However, this process is dependent on both the mother's and the infant's own microbiota, feeding practices, and the environment. Another possible reason for the differences in outcomes of probiotic interventions in infants lies in the finding that the original properties used in the selection of a specific probiotic strain may be influenced by industrial production processes and conditions as well as by the food matrix used. This can lead to changes in probiotic properties, which may influence the outcome in human intervention studies.

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