

**Lactobacilli in the normal microbiota
and probiotic effects of *Lactobacillus plantarum***

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ABSTRACT

Lactobacilli colonise most adult individuals and are also frequently used as probiotics, i.e. bacteria which possibly have health promoting effects when ingested. In this thesis, the intestinal *Lactobacillus* microbiota was studied in longitudinally followed infants. The oral and intestinal *Lactobacillus* microbiota of adults with and without IgA deficiency was examined to investigate the influence of secretory-IgA (S-IgA) on mucosal lactobacilli. Probiotic effects of the strain *L. plantarum* 299v were studied in antibiotic-treated patients and in patients with salmonellosis.

In infants, colonisation by lactobacilli increased until six months of age, when 45 % were colonised, most often by *L. rhamnosus* or *L. gasseri*. Colonisation dropped and reached its lowest point by one year, to increase again by 18 months. By that time, *L. paracasei* and other food-related *Lactobacillus* species were most common. Only 30% of the infants harboured the same strain on at least two sampling occasions, indicating that stable colonisation by lactobacilli is quite uncommon in infants. Colonisation by *L. rhamnosus* was more common in breastfed than in weaned infants at six months, suggesting that breastfeeding favours this species. *Lactobacillus* colonisation was not significantly related to delivery mode, or to contact with siblings or pets.

The influence of S-IgA on the oral and faecal *Lactobacillus* microbiota was studied by comparing IgA-deficient and healthy adult individuals. Expression of mannose-specific (MS) adhesins by lactobacilli was studied since such adhesins could possibly interact with mannose-containing polysaccharide chains of S-IgA. Lactobacilli were isolated from the oral cavity and faeces of the majority of both IgA-deficient and healthy individuals. *L. paracasei* and *L. gasseri* dominated in oral samples, and *L. paracasei* was the most common species in faecal samples from both groups. The only significant difference in species distribution was a lower colonisation by *L. fermentum* in the oral cavity of IgA-deficient individuals. Thus, the presence of S-IgA seems to have little influence on the *Lactobacillus* species distribution. The expression of MS adhesins was more common in oral than in faecal lactobacilli, indicating that these adhesins may be of advantage for oral colonisation. Faecal isolates from IgA-deficient individuals more often expressed MS adhesins than faecal isolates from controls. Possibly, expression of MS adhesins is less advantageous for lactobacilli in the presence of S-IgA in the gut.

In two double-blind placebo-controlled studies we explored if intake of *L. plantarum* 299v could counteract gastrointestinal side-effects during treatment with antibiotics, and reduce time to clearance and symptoms of *Salmonella* in patients with non-typhoid salmonellosis, respectively. Intake of *L. plantarum* reduced the risk of experiencing loose stools or nausea in antibiotic-treated patients. The risk of diarrhoea, i.e. at least three loose stools a day for at least two days, was not reduced, and there was no effect on colonisation by toxin-producing *C. difficile*. In patients with salmonellosis, intake of *L. plantarum* 299v did not reduce time to clearance of *Salmonella*, or time to resolution of diarrhoea and other symptoms. After clearance of *Salmonella*, patients receiving *L. plantarum* less frequently had hard stools, but tended to have more loose stools than patients on placebo. The differences regarding effects of *L. plantarum* 299v in the two studies could relate to e.g. differences between the studies regarding doses and formulas of the probiotic strain.

Gender seemed to influence the course of salmonellosis. Women tended to clear *Salmonella* more quickly than men, but had diarrhoea for a longer period. After *Salmonella* clearance, women had more loose stools, nausea and flatulence than men. Also, effects of *L. plantarum* after clearance of *Salmonella* were influenced by gender. Women receiving *L. plantarum* had more abdominal pain than those on placebo, whereas men in the *L. plantarum* group had less hard stools, but more diarrhoea than men on placebo. The gender-related differences regarding salmonellosis and probiotic effects need to be further explored.

Key words: *Lactobacillus*, oral microbiota, gut microbiota, infants, adults, secretory IgA, IgA-deficiency, adherence, *Lactobacillus plantarum*, probiotics, antibiotics, salmonellosis, diarrhoea

ORIGINAL PAPERS

The thesis is based on the following papers, which are referred to in the text by their roman number (I-IV)

- I. Siv Ahrné, Elisabet Lönnemark, Agnes E. Wold, Nils Åberg, Bill Hesselmar, Robert Saalman, Inga-Lisa Strannegård, Göran Molin, Ingegerd Adlerberth. Lactobacilli in the intestinal microbiota of Swedish infants. *Microbes and Infection* 2005;7:1256-1262
- II. Elisabet Lönnemark, Forough Nowrouzian, Ingegerd Adlerberth, Siv Ahrné, Agnes E. Wold, Vanda Friman. Oral and faecal lactobacilli and their expression of mannose-specific adhesins in individuals with and without IgA deficiency. *In manuscript*.
- III. Elisabet Lönnemark, Vanda Friman, Georg Lappas, Torsten Sandberg, Anna Berggren, Ingegerd Adlerberth. Intake of *Lactobacillus plantarum* reduces certain gastrointestinal symptoms during treatment with antibiotics. *Journal of Clinical Gastroenterology* 2009 [Epub ahead of print]
- IV. Elisabet Lönnemark, Georg Lappas, Vanda Friman, Agnes E. Wold, Erik Backhaus, Ingegerd Adlerberth. Effects of probiotic intake and gender on non-typhoid *Salmonella* infection. *In manuscript*.

TABLE OF CONTENTS

ABBREVIATIONS	7
INTRODUCTION	8
The alimentary tract	8
Establishment of the microbiota of the alimentary tract	15
Bacterial pathogens causing gastroenteritis	16
Defences of the alimentary tract	18
Factors of importance for the composition of the microbiota of the alimentary tract	25
Effects on the host of the gut microbiota	31
Disturbances of the gut microbiota	33
Probiotics	35
AIMS	41
MATERIALS AND METHODS USED IN THE STUDIES	42
Study populations and study design (I-IV)	42
Permission from the Ethics Committee (I-IV)	44
Sampling and culture for the isolation of lactobacilli (I, II)	44
Identification of lactobacilli by PCR (I, II)	45
Adherence of lactobacilli to HT-29 cells (II)	47
Sampling for and detection of <i>C. difficile</i> toxin in faeces (III)	47
Sampling and culture for the detection of faecal bacterial pathogens (IV, III)	48
Salmonella serotyping (IV)	48
Statistical methods	48
RESULTS	50
Studies on the Lactobacillus microbiota of infants and adults (I, II)	50
Studies on probiotic effects of <i>L. plantarum</i> 299v (III, IV)	55
DISCUSSION	61
ACKNOWLEDGMENTS	79
REFERENCES	80

ABBREVIATIONS

AAD	Antibiotic associated diarrhoea
APC	Antigen presenting cell
CFU	Colony forming units
IFN	Interferon
IBS	Irritable bowel syndrome
IL	Interleukin
LPS	Lipopolysaccharide
MS	Mannose-specific
NO	Nitric oxide
NOD	Nucleotide-binding oligomerisation domain
PAMP	Pathogen-associated molecular patterns
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PRR	Pattern recognizing receptors
RAPD	Random Amplification of Polymorphic DNA
SCFA	Short-chain fatty acids
S-IgA	Secretory Immunoglobulin A
T _H cell	T helper cell
TLR	Toll like receptor
TNF	Tumour necrosis factor

INTRODUCTION

The alimentary tract

It is becoming increasingly clear that the alimentary tract is not only a tube designed for the uptake of nutrients, but an organ with many tasks. Much of the structure and function of the intestine seems to have developed to enable the host to handle the constant exposure to high loads of microorganisms and prevent their entrance into the body. Most parts of the alimentary tract harbour complex microbial communities, and we are constantly exposed to new bacteria from various sources. There are still many basic features of the gastro-intestinal tract and its commensal microbiota which are poorly understood.

The normal microbiota

Only 10 % of the cells in our body are of human origin, whereas the majority are bacteria [1] and the genomic content of all microbes colonising a human being (the microbiome) is estimated to be 100-fold greater than the human genome [2].

There is great diversity within the bacterial communities inhabiting various parts of the alimentary tract, and also great variation between different habitats. Several biotopes are devoid of oxygen under normal conditions. This is true for the colon, but also for several niches in the oral cavity, *e.g.* the subgingival crevices and the rough surfaces of the dorsal tongue. The vast majority of the bacteria living here are strict anaerobes, *i.e.* they cannot utilise oxygen, and are often killed by oxygen contact, whereas facultative anaerobes, which live in smaller numbers in these habitats and dominate in aerobic niches, grow better in oxygen, but can still grow and multiply without it.

Even under optimal conditions many of the bacterial species inhabiting the alimentary tract cannot be cultivated. The recent development of non-culture based identification methods has led to the discovery of several new species, and many more remain to be detected [3]. Culture-independent studies of the entire genome of a mixed microbial community, including bacteria, viruses, fungi, archaea and sometimes parasites, are referred to as metagenomics [2]. Estimates for the total number of species comprising the collective gut microbiome have recently been extended up to several thousand [4].

Numbers and species in the various parts of the gastro-intestinal tract

The approximate numbers and important groups of bacteria inhabiting the various parts of the gastro-intestinal tract are shown in Figure 1.

Oral cavity
10⁵-10⁸ CFU/ml
saliva

Streptococcus
Actinomyces
Veillonella
Fusobacterium
Porphyromonas
Prevotella
Treponema
Neisseria
Haemophilus
Eubacteria
Lactobacillus
Capnocytophaga
Eikenella
Leptotrichia
Peptostreptococcus
Staphylococcus
Propionibacterium

Stomach
<100
CFU/ml
Helicobacter
Lactobacillus
Veillonella
Clostridium

Duodenum
10⁴ CFU/ml
Lactobacillus
Streptococcus
Veillonella

Distal ileum
10⁸ CFU/ml
Lactobacillus
Streptococcus
Enterobacteriaceae
Bacteroides
Clostridium



Esophagus
<10⁴ CFU/ml
Streptococcus
Prevotella

Colon
10¹⁰ – 10¹² CFU/ml
Bacteroides
Eubacterium
Bifidobacterium
Fusobacterium
Clostridium
Peptococcus
Peptostreptococcus
Ruminococcus
Actinomyces
Lactobacillus
Coprococcus
Faecalibacterium
Megasphaera
Veillonella
Collinsella
Eggertella
Enterobacteriaceae
Enterococcus

Figure 1. Numbers and species of the most common bacteria in the various parts of the alimentary tract

The oral cavity

More than 700 different bacterial species have been identified in the oral microbiota [5], the majority being anaerobes [6]. It is also clear that a number of species still remain to be identified [7]. Each individual usually harbours 100-200 species [5], the majority of which grow at a particular site, such as the back of the tongue, the hard palate, or the dental surfaces [8]. However, some bacterial groups, *e.g.* various streptococci, *Prevotella* and *Lactobacillus* species grow at most sites and are found in most individuals [5, 9]. The bacterial density varies widely between different oral niches, but the counts in saliva are approximately 10^5 - 10^8 / ml, and higher in dental plaques [10].

The stomach

The stomach harbours only a low number of bacteria due to the harsh conditions, where the low pH kills most bacteria within minutes. More acid-resistant bacteria, *e.g.* lactobacilli, *Veillonella* spp. and some clostridia can still survive, and some bacteria may even colonise niches where the pH is higher due to secretion of bicarbonate [11, 12]. In individuals colonised by *H. pylori*, the bacterial community is very much dominated by this species [13], whereas the same authors found evidence of a larger number of species in individuals who did not harbour *H. pylori* [13].

The small intestine

Moving from the ventricle towards the ileocaecal valve, the number of bacteria and the complexity of the microbiota gradually increase. The small intestine offers an aerobic environment and bacteria like lactobacilli and streptococci are common. Proximally, the bacterial numbers are low, only 10^{2-4} /ml, increasing to 10^{5-8} /ml in the distal ileum. Here the oxygen content decreases, and the microbiota also includes *Bacteroides*, clostridia and other anaerobes, along with facultative anaerobes such as enterococci and *E. coli* [12, 14].

The colon

The total number of bacteria in the colon amounts to 10^{14} , or 10^{11} / g faeces, which is equivalent to 60 % of the faecal mass [15]. The most common bacterial groups are presented in Figure 1. More than 99 % of the bacteria are strict anaerobes, as they are favoured by the lack of oxygen and low redox potential in this environment [16]. *Bacteroides*, *Bifidobacterium*, *Clostridium*, *Eubacterium*, *Ruminococcus*, *Coprococcus*, *Faecalibacterium*, *Megasphaera*, *Veillonella*, *Collinsella*, *Eggerthella* and *Fusobacterium* are most common [3, 17]. Species belonging to the genus *Lactobacillus*, which are defined as anaerobic or microaerophilic bacteria, are present in populations up to 10^{6-8} bacteria/g faeces.

Among the facultatively anaerobic bacteria, *E. coli* and enterococci are most common, reaching populations of 10^{7-8} bacteria/g faeces.

The microbial communities close to the epithelium in the crypts and in the mucus layer are likely to differ from the luminal microbiota. For instance, there is some oxygen diffusion from epithelial cells which may create a microaerophilic niche close to the epithelium, making this habitat less favourable for strict anaerobes than the lumen [16]. On the other hand, the mucosa-associated microbiota appears to be relatively similar all along colon [18, 19]. Luminal bacteria are likely to be a mixture of shed mucosal bacteria and a separate luminal nonadherent population [3]. A faecal sample will contain bacteria from all different intestinal niches and, thus, the bacterial species distribution in faeces has been found to be different from that of colonic epithelial biopsies [3, 19, 20]. However, not all studies have found these differences [21].

Lactobacilli

Lactobacilli are a diverse group of Gram-positive, non-sporulating, lactic acid producing anaerobic rods with varying oxygen tolerance. They are acid-tolerant and may grow at pH as low as 3.5 [22]. More than 150 species have been identified within the *Lactobacillus* genus (<http://www.bacterio.cict.fr/l/lactobacillus.html>), with substantial genetic and phenotypical differences between different groups [23]. Lactobacilli are ubiquitous where carbohydrate substrates are available, *i.e.* on mucous membranes of man and animals, on plant and plant materials, in manure, sewage and in fermented or spoiled food. *Lactobacillus*-containing food produced through fermentation includes *e.g.* sourdough, cheese, yoghurt, marinated fish and meat, fermented vegetables and wine. Lactobacilli are generally considered non-pathogenic or even health-promoting.

Lactobacilli are often found in carious lesions [9] and were preciously believed as being one of the main cariogenic bacterial groups. Today, however, they are mainly regarded as secondary invaders without a causative role in the caries process [24].

Speciation and grouping of lactobacilli

Lactobacilli ferment carbohydrates with lactic acid as end product [26]. Traditionally, they have been divided into three groups depending on their type of sugar fermentation: *obligately homofermentative*, which ferment hexose sugars by glycolysis and produce mainly lactic acid; *obligately heterofermentative*, which use the 6-phospho-gluconate/phosphoketolase (6PG/PK) pathway with the production of CO₂ and ethanol in addition to lactic acid; and facultative heterofermentative, that use both pathways.

Later genetic analyses have revealed that this division is not always in accordance with the genetic relationship between lactobacilli [26]. Certain species

of lactobacilli are impossible to distinguish with phenotypical methods. Therefore, the development of genetic typing methods is of great value for *Lactobacillus* identification and speciation. The 16S rRNA gene contains regions which are highly conserved among bacteria, but also regions which are highly variable between genera or species. Sequencing of 16S rRNA can be used both for determining genetic relatedness and for speciation of bacterial isolates. It can not, however, decide if two isolates from a certain species belong to the same strain. The grouping of lactobacilli according to their phylogenetic relatedness, as determined by 16S rRNA sequencing, is presented in Table 1.

Table 1. Phylogenetic groups of lactobacilli according to Felis and Dellaglio [27], with examples of *Lactobacillus* species within each group

Phylogenetic group	<i>Lactobacillus</i> species
<i>L. alimentarius</i> – <i>farciminis</i> group	<i>L. alimentarius</i> , <i>L. farciminis</i>
<i>L. brevis</i> group	<i>L. brevis</i>
<i>L. buchneri</i> group	<i>L. diolivorans</i> , <i>L. hilgardii</i> , <i>L. parabuchneri</i> , <i>L. parafarraginis</i>
<i>L. casei</i> group	<i>L. casei</i> , <i>L. paracasei</i> , <i>L. rhamnosus</i>
<i>L. coryniformis</i> group	<i>L. coryniformis</i>
<i>L. delbrueckii</i> group	<i>L. acidophilus</i> , <i>L. amylovorus</i> , <i>L. crispatus</i> , <i>L. delbrueckii</i> , <i>L. gasseri</i> , <i>L. jensenii</i> , <i>L. johnsonii</i>
<i>L. fructivorans</i> group	<i>L. fructivorans</i>
<i>L. perolens</i> group	<i>L. harbinensis</i>
<i>L. plantarum</i> group	<i>L. plantarum</i> , <i>L. pentosus</i>
<i>L. reuteri</i> group	<i>L. antri</i> , <i>L. fermentum</i> , <i>L. frumenti</i> , <i>L. gastricus</i> , <i>L. mucosae</i> , <i>L. oris</i> , <i>L. reuteri</i> , <i>L. vaginalis</i>
<i>L. sakei</i> group	<i>L. sakei</i>
<i>L. salivarius</i> group	<i>L. ruminis</i> , <i>L. salivarius</i>

However, 16S rRNA sequencing also has limitations in the recognition of *Lactobacillus* species, and it is, for instance, not possible to distinguish between *L. plantarum* and *L. pentosus* with this method [27]. Another bacterial group which often poses difficulties is the *L. casei*–*L. paracasei*–*L. rhamnosus* group [28]. Certain DNA sequences, e.g. the sequence of the 16S-23S rRNA intergenic spacer region and the flanking 23SrRNA may differ more between species. PCR using species- specific primers for these sequences has proved to be useful in the separation of *Lactobacillus* species in multiplex PCR assays [29]. Thus, it is often beneficial to combine different methods in the speciation of lactobacilli.

Lactobacillus colonisation rate and species distribution along the alimentary tract

Lactobacilli are found in the gastro-intestinal microbiota of almost all adult individuals [30], but, with the possible exception of the small intestine, they are not a dominating bacterial group in these habitats. An overview of lactobacilli commonly found in the alimentary microbiota is shown in Table 2.

Table 2. *Lactobacillus* species distribution in different parts of the gastrointestinal tract

Oral cavity	Stomach	Small intestine	Faeces	Colon epithelial biopsies
<i>L. paracasei</i>	<i>L. gasseri</i>	<i>L. gasseri</i>	<i>L. gasseri</i>	<i>L. plantarum</i>
<i>L. rhamnosus</i>	<i>L. reuteri</i>	<i>L. reuteri</i>	<i>L. paracasei</i>	<i>L. rhamnosus</i>
<i>L. fermentum</i>	<i>L. ruminis</i>	<i>L. rhamnosus</i>	<i>L. ruminis</i>	<i>L. paracasei</i>
<i>L. plantarum</i>			<i>L. reuteri</i>	
<i>L. gasseri</i>			<i>L. plantarum</i>	
			<i>L. salivarius</i>	
			<i>L. sakei</i>	

Lactobacilli commonly found in the oral cavity [9, 31-37, 42], stomach [38, 39], small intestine [38, 40] and large intestine [30, 31, 34, 38, 41,]

The lactobacilli of the oral cavity may differ between different biotopes in the mouth, and saliva contains a mixture of lactobacilli from the various niches. The species found in saliva vary between studies, but *L. rhamnosus*, *L. paracasei* and *L. fermentum* have been found to be common in several studies and *L. gasseri* is also frequently mentioned [33, 35-37]. The first three species are also found in most studies of caries lesions [9]. There are fewer studies of lactobacilli on oral mucosal surfaces, but *L. plantarum*, *L. rhamnosus* and *L. fermentum* may be common at the tongue surface [31, 32] and *L. rhamnosus* also at the gum mucosa [32]. The lactobacilli most commonly isolated from teeth and oral mucosal niches are shown in Table 3.

Lactobacilli may also be isolated from the gastric mucosa, including *L. gasseri*, *L. reuteri* and *L. ruminis* [38, 39]. Recently, four new *Lactobacillus* species were identified in gastric mucosal biopsies [43]. However, whether lactobacilli are only present there transiently, or colonise this habitat is not clear.

Table 3. Most common lactobacilli in different locations of the oral cavity

Saliva	Oral mucosa	Dental plaques	Cariious lesions
<i>L. fermentum</i>	<i>L. plantarum</i>	<i>L. fermentum</i>	<i>L. rhamnosus</i>
<i>L. casei/paracasei</i>	<i>L. rhamnosus</i>	<i>L. casei/paracasei</i>	<i>L. fermentum</i>
<i>L. rhamnosus</i>	<i>L. fermentum</i>		<i>L. casei/paracasei</i>
<i>L. gasseri</i>			
<i>L. salivarius</i>			
<i>L. plantarum</i>			
<i>L. acidophilus</i>			

Data were compiled from [9, 31, 34-37].

In the upper small intestine, lactobacilli are among the most dominant bacteria according to several authors [38, 44, 45]. However, not all studies have found lactobacilli to be common small intestinal colonisers [46], indicating individual variation. Which *Lactobacillus* species are most common in the small intestine is not well-known. According to one study *L. gasseri* and *L. reuteri* were most frequent, and another study found *L. Rhamnosus* to be most common [38, 40] (Table 2).

The *Lactobacillus* microbiota of the colon has been more studied. *L. gasseri*, *L. paracasei*, *Lactobacillus ruminis*, *Lactobacillus reuteri* and *L. plantarum* have been identified as predominant faecal *Lactobacillus* species using molecular typing methods. *Lactobacillus salivarius* and *Lactobacillus sakei* are also quite commonly found (Table 2) [30, 34, 37, 38, 41, 47, 48]. Some studies have identified lactobacilli in colonic biopsies, including *L. plantarum*, *L. rhamnosus* and *L. paracasei* [31].

Persistent colonisation by lactobacilli

The mere presence of lactobacilli in an oral or faecal sample does not necessarily imply colonisation. Since lactobacilli are ingested by food, they may also be transient passers-by. It is not clearly established which lactobacilli are transient, and which are resident members of the microbiota. This requires longitudinal studies, with typing of lactobacilli to the strain level. The isolation of the same strain over time could then imply persistent colonisation. Using this approach, oral persistence of *L. fermentum* and *L. vaginalis*, and gut persistence of *L. vaginalis*, *L. gasseri*, and *L. delbrueckii* was demonstrated in healthy individuals [34].

Lactobacilli in other human habitats

Lactobacilli are not only found in the alimentary tract canal, but also in the vagina of fertile women where they dominate the microbiota. The most commonly found vaginal lactobacilli in women of reproductive ages are *L. crispatus*, *L. iners*, *L. jensenii* and *L. gasseri* [49-51]. Lactobacilli are also isolated from breast milk, e.g. the species *L. gasseri*, *L. rhamnosus*, *L. plantarum* and *L. fermentum* [51, 52], but the origin of these bacteria is not clear.

Establishment of the microbiota of the alimentary tract

Colonisation of the alimentary tract starts at birth, when the baby leaves the sterile milieu of the uterus, and proceeds over several years.

Establishment of the oral microbiota

The oral microbiota is initially simple, but expands with teething, which provides new surfaces for adhering bacteria [53]. Among the earliest colonisers are streptococci, e.g. *S. mitis*, *S. oralis*, and *S. salivarius*, which usually appear in the infant within a few days [54]. *Actinomyces* species are other early colonisers [55] and also various anaerobes including *Bacteroides*, *Veillonella*, *Prevotella*, and *Fusobacterium* may be detected in the first two months of life [55, 56]. Colonisation by these and other anaerobes increases steadily over the first years of life. Colonisation by the caries pathogen *Streptococcus mutans* has previously been found to occur between 19 and 31 months of age [57]. However, more recent studies found *S. mutans* earlier [58, 59], including in 60 % of six month old preterm infants [58].

Establishment of the gut microbiota

The gut is initially colonised by facultatively anaerobic and oxygen-tolerant bacteria, since it is rich in oxygen. In older studies, *Enterobacteriaceae*, mainly *E. coli*, and enterococci dominated immediately after birth [60, 61]. However, in recent studies from Western countries, coagulase negative staphylococci, which are typical skin bacteria, have become the earliest colonisers, most likely because of a limited exposure of neonates to traditional faecal bacteria [62, 63]. *S. aureus* has also become a frequent early coloniser [63, 64].

The facultatively anaerobic bacteria soon consume the oxygen in the gut, and the anaerobic bacterial population starts to expand. Bifidobacteria are the most common anaerobes in the first weeks of life, followed by clostridia and *Bacteroides* [60, 61, 65, 66]. Most studies describing this early gut colonisation

pattern are based on stool cultures. The few and usually small culture-independent studies mostly agree with the results of the culture-based studies [67-69].

Over time, the number of species increases in the gut microbiota, and the dominance of anaerobes becomes more and more pronounced [69]. It takes several years until a full “adult-type” microbiota has developed [70]. Thereafter, it becomes more difficult for new species to colonise the intestine [71], and the species composition of the microbiota remains quite stable over time in healthy adults [72]. An adult is believed to harbour a few hundred different bacterial species in the gut [3] and the anaerobes outnumber the facultatives by a factor of 100 to 1000 [73].

Establishment of the *Lactobacillus* microbiota

Lactobacilli are rarely isolated from the oral cavity of infants in the first few months of life [55, 74], but are found in a majority of children aged two to five years [9, 59].

Regarding the presence of lactobacilli in the early gut microbiota, the results differ between studies. Most studies have reported low *Lactobacillus* colonisation rates in infants [60, 61, 75-77], and Stark and Lee, who followed infants over time, questioned that lactobacilli were able to form stable populations in the infant gut. Others claim that lactobacilli are present in substantial quantities (10^{7-9} CFU/g faeces) in most infant’s stools [78, 79]. Variations in methodology may, at least to some degree, account for the differences between older studies, since lactobacilli are hard to identify by traditional biochemical methods.

The *Lactobacillus* species most commonly isolated from infant faeces include *L. gasseri* [77, 80], *L. paracasei* and *L. crispatus* [77, 80]. However, there are substantial differences also in the *Lactobacillus* species distribution between different studies.

Bacterial pathogens causing gastroenteritis

Certain bacterial groups colonising the intestines have the capacity to cause gastroenteritis, the most common being diarrheagenic subgroups of *E. coli*, *Campylobacter*, *Salmonella*, and *Shigella*. *Clostridium difficile* is another enteric pathogen which may cause disease, especially during or after treatment with antibiotics.

Diarrhoea is the main symptom of gastroenteritis. Other common symptoms include vomiting, fever, nausea and abdominal cramps. Infectious diarrhoea can be divided into inflammatory and non-inflammatory. Inflammatory diarrhoea is characterised by signs of inflammation like blood and mucus in stools and fever. *Campylobacter*, *Salmonella*, and *Shigella* invade the intestinal mucosa and thereby induce acute inflammation. Non-invasive bacteria like

enteroaggregative *E. coli*, enterohemorrhagic *E. coli* and *C. difficile* also cause inflammatory diarrhoea by producing cytotoxins that stimulate the release of inflammatory mediators and damage the mucosa. The secretory diarrhoea caused by *Vibrio cholera* and enterotoxigenic *E. coli* is induced by enterotoxins which activate adenylate cyclase and cAMP leading to massive loss of fluid, but little inflammation and little damage to the mucosa [81].

Salmonella

Non-typhoid salmonellosis

Salmonella enterica is the type species of *Salmonella* including more than 2500 serotypes based on variation in O (LPS) and H (flagellar) antigens [82]. *Salmonella* Typhi and Paratyphi causes severe systemic disease, whereas other serotypes, often labelled as non-typhoid *Salmonella*, primarily cause intestinal disease. The most common non-typhoid serotypes worldwide include *S. Enteritidis*, *S. Typhimurium* and *S. Newport* [83]. Non-typhoid *Salmonella* is mainly acquired from contaminated foods. Attack rates are highest in infants, and lowest between 20 and 70 years of age [88]. Estimates of global incidence of non-typhoid *Salmonella* infection range from 200 million to 1.3 billion cases.

Diarrhoea is the cardinal symptom in non-typhoid *Salmonella* infection and is often accompanied by symptoms like abdominal cramps, myalgia, headaches, fever, and chills [85]. In some patients, septicaemia and focal infections occur. Most patients recover within a few weeks without treatment, but the acute symptoms may be quite severe and persistent gastro-intestinal disturbances are common [86].

Also, many individuals remain positive for *Salmonella* in faeces for various lengths of time after a symptomatic infection, median duration of excretion being approximately five weeks [87]. Less than one per cent continue to excrete *Salmonella* in faeces for more than a year [87], and they are defined as chronic or persistent carriers [88]. It is not clear if *Salmonella* actually colonises the gut for such prolonged periods, or if bacteria are only seeded to the intestine from other foci of colonisation, e.g. the biliary tract. Very low or very high age, biliary tract abnormalities, schistosomiasis, and diverticulitis are known risk factors for the carrier state [88]. *Salmonella* is cleared more rapidly in asymptomatic infection [89].

Pathogenesis in non-typhoid salmonellosis

Salmonella adhere [90] to epithelial cells and colonise the distal ileum and proximal colon [91]. They use type III secretion systems, a kind of molecular syringe consisting of more than 20 proteins, to inject so called effector proteins into the cells, which enables the invasion of the epithelium, induces fluid secretion and stimulates the production of inflammatory mediators [92]. Whereas *S. Typhi* is

spread systemically within macrophages and neutrophils, non-typhoid *Salmonella* normally remains in the intestinal tissues, and large numbers of neutrophils are attracted to the small intestinal wall [91,93]. Necrosis may be seen in the superficial mucosa layers in areas of the terminal ileum and colon [92].

Clostridium difficile

C. difficile is found in the normal gut microbiota of approximately ten per cent of adult individuals, but normally in low counts only [94, 95]. When permitted to reach high population numbers, *e.g.* during treatment with antibiotics, it may cause enteritis through its elaboration of toxins. Symptoms of *C. difficile* infection range from mild diarrhoea to life-threatening pseudomembranous colitis [96], a severe inflammation of the colon with production of fibrous membranes. *C. difficile* may produce two exotoxins, toxin A and B, which induce mucosal inflammation, fluid secretion, epithelial damage and in some cases necrosis of intestinal epithelial cells [97]. An aggressive *Clostridium difficile* clone has spread over several countries in the last decades, causing complicated and relapsing infections with a high mortality rate [98].

Defences of the alimentary tract

Barrier functions

Saliva

Saliva flushes microorganisms from teeth and oral mucosa and transports bacteria to the stomach through swallowing. The bacterial content of saliva is approximately 10^{5-8} CFU/ml and as we produce 750 to 1500 ml per day, 10^8 - 10^{11} bacteria from the oral cavity reach the acidic environment of the stomach daily. Saliva contains several protective factors and antimicrobial agents, *e.g.* secretory IgA, lactoferrin and lysozyme [99], which are described below.

The gastric acid barrier

The low pH of the stomach kills bacteria very efficiently. Most bacteria can pass only when the pH is higher, *e.g.* during meals, and reach the lower parts of the gastro-intestinal tract to possibly establish residency [11]. In neonates, gastric pH is relatively high, which might facilitate the establishment of the gut microbiota [100]. Some bacteria, *i.e.* lactobacilli, *Veillonella* and clostridia are, however, able to survive in acidic environments [12].

With a normal fasting pH below 3, gastric aspirate contains less than 10^{3-4} CFU/ml, whereas at a pH of 6-7.5 bacterial levels rise to 10^{6-8} CFU/ml [11].

Ageing, pernicious anaemia, malnutrition, medication such as proton pump inhibitors, and acid reducing gastric surgery result in a higher pH [11], and may disrupt the gastric barrier. With higher gastric pH, bacterial numbers increase both in the stomach and in the proximal small intestine [11, 101]. The bacterial species that proliferate mostly originate in the upper respiratory tract. As a higher gastric pH facilitates the survival of all bacteria reaching the stomach, it results in increased susceptibility to low doses of pathogens like *Salmonella* [102]. In accordance, acid-suppressing therapy was found to increase the risk of developing *Salmonella* infection in an outbreak situation [103].

Intestinal clearance

Intestinal clearance is the ability of the small bowel to clear its lumen of bacteria [11]. It is dependent on normal gastro-intestinal anatomy and motility. The flow rate is highest at the proximal end of the small intestine, where most bacteria are quickly removed. Under normal conditions bacteria need to be adherent to the mucosa to remain in the upper parts of the small intestine [104]. Animal experiments have shown that decreased intestinal peristalsis leads to colonisation of the small intestine by various types of gut bacteria [11]. Such small intestinal bacterial overgrowth may also occur in humans in conditions with disturbed peristalsis [105].

Multiplication of bacteria in the small intestine is also inhibited by bile [106] and pancreatic fluid [107]. Bacteria vary considerably in their sensitivity to bile acids and pancreatic enzymes, with Gram-negative bacteria in general being more resistant to bile than Gram-positive bacteria [108], although many Gram positives, *e.g.* lactobacilli, are bile resistant [109]. This influences the composition of the microbiota inhabiting the small intestine. *Salmonella* bacteria are highly bile resistant, and are even able to colonise the biliary tract [110].

In the colon, motility and transient times are much reduced as compared to the small intestine, and this is an important factor enabling the expansion of large bacterial populations at this site.

Mucus

The epithelium of the gastrointestinal tract is covered by a mucus layer where particles and bacteria are trapped, inhibiting direct contact between the epithelium and bacteria [21]. The layer is made up by water (>95 %) and mucins, highly glycosylated secretory glycoproteins [111]. Mucus also contains sloughed epithelial cells and transudated serum proteins and is rich in antibacterial peptides, lysozyme, lactoferrin and secretory IgA [112].

Mucins are produced at a high rate and are subject to constant degradation by human and bacterial proteases and glucosidases. Mucin oligosaccharides provide a source of carbohydrates, which are used as nutrients by the colonising bacteria [113]. Colonisation of the mucus layer may be beneficial for

the bacteria, as it protects them from being swept away by peristalsis [114]. The mucus layer provides binding sites for various bacteria, which is likely to facilitate their colonisation of this habitat [115].

Lactobacilli are among the bacteria which are likely to inhabit the mucus layer. This has been studied mainly *in vitro* [116, 117], but also *in vivo* [118].

The epithelium

Underneath the mucus layer, the epithelium covers the gastrointestinal canal from the mouth to the rectum. The continuous shedding of epithelial cells limits microbial colonisation of the mucosa. The area of the mucosa of the intestinal tract is 200 to 300 m² [119]. This large area is created by the organisation of the mucosa into villi and crypts in the small intestine, and crypts in the large intestine. Villi are about one mm long projections into the lumen, which increase the absorptive surface. In the large intestine there are no villi, but the crypts are generally deeper. The intestinal epithelial barrier is made up of a single columnar epithelium where the cells are tightly linked to each other with junctional complexes, providing a physical barrier that prevents bacteria from invading the body. There are several different types of epithelial cells in the small intestine: crypt cells, absorptive enterocytes, enteroendocrine cells, goblet cells and Paneth cells. Paneth cells are located at the base of the crypts from the duodenum to the ileum. Apically they are filled with granules which contain antibacterial substances produced within the cells [120]. In the large intestine, the epithelium consists of columnar epithelial cells, goblet cells, crypt cells and endocrine cells.

Antimicrobial compounds

Epithelial cells and Paneth cells are able to produce a large number of antibacterial compounds. Among these are antimicrobial peptides like defensins and cathelicidins with broad spectrum antimicrobial effect. Their mechanism of action is the formation of pores in bacterial membranes, resulting in bacteriolysis [122]. Other examples of antimicrobial compounds are the bacteriolytic enzymes lysozyme and group IIa phospholipase A2 (PLA2) [123], and lactoferrin which deprives microbes of nutrients through iron sequestration and induces cell lysis [124].

The immune system of the alimentary tract

Architecture

The alimentary tract is defended by organised lymphoid tissue, and a large number of immune cells scattered in the submucosal niches.

Around the oral cavity organised lymphoid tissues make up a ring consisting of the palatine tonsils, the lingual tonsils and the adenoid tissue in the roof of the nasopharynx. These tissues are rich in T and B lymphocytes, macrophages and dendritic cells, and are inductive sites for immune responses in the oral cavity. In the oral cavity, there are also many plasma cells, located in the salivary glands, producing dimeric IgA that is converted to secretory IgA during passage through the duct epithelium.

Organised lymphoid follicles are present along the entire small intestine, but become more abundant in the distal ileum. They are also frequent in the colon, especially in the caecum and the rectum. In the distal ileum they are grouped in large patches, the so called Peyer's patches, with B cells in the centre surrounded by T-lymphocytes. The Peyer's patches also contain macrophages and dendritic cells and are the primary sites for induction of immune responses towards bacteria that colonise or pass the gut. The epithelium overlaying lymphoid follicles differs from the villus epithelium. Instead of goblet cells and enteroendocrine cells it contains epithelial M cells which actively transport antigens and whole microbes across the epithelium.

The intestinal epithelium contains a large number of intraepithelial lymphocytes. Underneath, there is a thin layer of loose cell-rich connective tissue, the lamina propria containing fibroblasts and immune cells, including large numbers of activated T helper (T_H) lymphocytes, B cells, plasma cells, macrophages, and dendritic cells.

Innate defence

Bacteria that cross the epithelial barriers in the gastrointestinal tract will encounter the innate immune system which consists of cells and soluble proteins *e.g.* factors of the complement system. The innate immune system is activated within minutes and bacteria are eliminated by phagocytosis and complement activation, a response which produces inflammation.

Cells in the innate system, including *e.g.* macrophages, dendritic cells, mast cells, NK (natural killer) cells, monocytes and granulocytes are able to recognize microbial structures. Epithelial cells may also recognise and take part in the clearance of invading bacteria. Both commensal bacteria and pathogens are recognised by means of conserved structures that are specific for prokaryotes, *e.g.* LPS, peptidoglycan, lipoteichoic acid and flagellin [125]. These structures are defined as pathogen-associated molecular patterns, (PAMPs) and are recognised by specific receptors called pattern recognizing receptors (PRRs) [126] which are present on cells of the innate immune system and to some degree on epithelial cells. Receptor activation results in intracellular signalling via different pathways, and to expression of various genes, *e.g.* genes for production of inflammatory mediators and chemotactic compounds. The most well-known and well-characterised PRRs are the toll-like receptors expressed on the surface of *e.g.* macrophages, dendritic

cells and epithelial cells [127]. The intracellular NOD receptors are another important group.

The acquired immune system

When bacteria are taken up by M cells overlying lymphoid follicles, or cross the epithelial layer, the acquired immune system is also activated. This is a slower process, as lymphocytes produced in the bone marrow and thymus need time to mature into antibody producing plasma cells and effector T lymphocytes.

The cells of the acquired immune system are very specialised and can recognise an enormous array of structures. Each T- and B-lymphocyte has a unique receptor in its membrane, specific for one single structure, the antigen. T-lymphocytes recognise their specific antigen when presented by antigen-presenting cells (APCs), most commonly dendritic cells or macrophages. The T-lymphocytes are divided depending on which presentation-molecule, the so called MHC-molecule, they prefer. $CD4^+$ T cells recognise their specific antigen presented on MHC II molecules, whereas the cytotoxic $CD8^+$ T-cells recognise their antigen on MHC I molecules. $CD4^+$ T cells are further divided into T helper (T_H) cells or regulatory T cells.

The antigen-presenting cells are present in all tissues where they take up antigen or entire pathogens by endocytosis or phagocytosis. After degradation, pieces of antigens are presented on the MHC molecule to T-cells. This may occur in the Peyer's patches, where both APCs and T cells are present, or, if the APC has encountered bacteria in the lamina propria, they may migrate to the nearest lymph nodes, to present antigens to T cells. The $CD4^+$ T cell recognises its specific antigen through the T-cell receptor and starts to divide and mature into T_{H1} , T_{H2} or T_{H17} cells. T_{H1} cells activate macrophages, promote cytotoxic T lymphocyte activity, and mediate inflammation through the production of cytokines. T_{H2} cells are involved in the stimulation of antibody responses. T_{H17} cells are found mainly in the skin and intestinal epithelium and recruit neutrophils and induce a strong inflammatory response upon bacterial stimulation.

B cells recognise their antigens directly, with their surface bound antibodies. They can then proliferate and differentiate into plasma-cells, a process which usually requires activation by T-helper cells. B cells express MHC II and are able to present antigens to $CD4^+$ T cells. First, the antigen is presented by an APC to a T cell recognising the antigen. Then the T cell proliferates and differentiates into cytokine producing T_{H2} cells. The T_{H2} cell binds to the B cell presenting the same antigen as the APC did, and with the secretion of additional cytokines, the B cell differentiates into an antibody-producing plasma cell. Most naive B cells are originally expressing antibodies of the IgD or IgM class on their surfaces. Depending on the stimuli provided by the T cell, they may switch their production to another immunoglobulin class or subclass, *i.e.* IgG1, 2, 3 or 4, IgA1 or 2, or IgE.

Both the innate and the acquired immune system are regulated by cytokines. A cytokine is a soluble protein or glycoprotein released by cells, with an

effect on other cells, expressing receptors for this specific cytokine. Effects include for instance triggering or down-regulating inflammation.

Secretory IgA

Induction of secretory IgA responses

The production of secretory IgA (S-IgA) is induced by bacterial colonisation in the gut. Bacteria are taken up by the M cells overlaying the Peyer's patches and are transported across the epithelium. Dendritic cells process bacterial compounds and present them to T cells. The activated T cells stimulate the B cells which have recognised bacterial epitopes to proliferate and switch to IgA production. IgA positive cells thereafter leave the patches via the lymph, reach the blood stream, and "home" to mucosal surfaces where they mature to IgA producing plasma cells. The IgA is produced in a dimeric form, which binds to the poly Ig- receptor on the basolateral surface of epithelial cells and is transported through the epithelial cells and secreted into the lumen.

In addition to high affinity specific antibodies, a large amount of natural, or low affinity, IgA is produced in response to bacterial colonisation [128]. It is unclear where production of these natural IgA antibodies is induced, and to what extent T cells are involved in the induction.

Function of S-IgA

Saliva and gut secretions contain large quantities of S-IgA [129]. This antibody class is completely dominating at all mucosal surfaces that harbour a normal microbiota, where it is able to trap microorganisms and thus block or sterically hinder adherence of microorganisms to epithelial surfaces [130] and thereby prevent translocation, *i.e.* the passage of viable bacteria over the intestinal epithelium. S-IgA also neutralises toxins, inhibits virus replication and promotes clearance of microorganisms that have breached the epithelial barrier by binding and transporting microbes through the epithelium back to the lumen [131]. These barrier effects of SIgA are believed to reduce the intestinal inflammatory response and systemic antibody responses against the gastrointestinal commensal microbiota [132].

In addition to the specific antibody-antigen interaction, the carbohydrate chains of S-IgA may function as receptor sites for bacteria. For instance *E. coli* with mannose-binding type 1 fimbriae have been found to bind to carbohydrates on SIgA [133]. S-IgA, particularly of the IgA2 subclass, is rich in carbohydrate chains with terminal mannose [134]. Thus, S-IgA is particularly effective in binding microorganisms adhering to mannose-containing receptors [133]. *E. coli* and other *Enterobacteriaceae* species, including *Salmonella*, frequently express mannose-binding adhesins [135].

The influence of SIgA is probably most profound in the small intestine as the rapid peristalsis may remove bacteria which are not able to adhere to the mucosa. For instance, segmented filamentous bacteria, anaerobes closely related to clostridia, expand in the small intestine of mice in the absence, but not in the presence, of secretory IgA [136]. In the colon, however, S-IgA does not seem to prevent colonisation. *E. coli* colonise the gut regardless of the presence of specific S-IgA antibodies towards *E. coli* [137]. Indeed, a large share of faecal bacteria are coated with S-IgA under normal conditions [138]. However, it is likely that the presence of S-IgA may influence population numbers of certain bacteria in the large intestine. For instance, as S-IgA may prevent bacterial access to the epithelial surface, bacteria that prefer this specific niche could be disfavoured.

IgA deficiency

IgA deficiency is a lack of both IgA1 and IgA2 in serum and secretions and the most common primary immune deficiency. The prevalence of IgA deficiency is approximately one in 700 in Caucasian populations [139]. The background is not fully elucidated, but involves a failure of B lymphocytes to switch to IgA production [140]. The majority of IgA deficient individuals are healthy and may be discovered at *e.g.* blood donor screenings. Approximately one third have recurrent respiratory tract infections. Several *in vitro* studies have demonstrated that B cells from IgA deficient individuals become able to produce IgA when stimulated through CD40 together with IL-10 or IL-4 + IL-10 or [141, 142], especially B cells from healthy IgA deficient individuals [143]. Thus, defects in cytokine production may be involved in the pathogenesis of IgA deficiency.

Why some IgA deficient individuals suffer from recurrent infections could relate to differences in the ability to compensate for the absence of S-IgA, for instance by S-IgM production [144], but other studies show that healthy individuals with IgA deficiency do not have higher S-IgM concentrations at mucosal sites than patients with many infections [145]. Yet another hypothesis is that they compensate by production of antibacterial peptides, however, no effect of antimicrobial peptides on the expanding bacteria was found in small intestinal expansion of anaerobes due to lack of IgA [146].

IgA deficiency is associated with increased risk of certain autoimmune disorders [147], celiac disease and perhaps inflammatory bowel disease [148]. The latter condition could possibly relate to an increased inflammatory response towards gut bacteria in the absence of IgA.

Few studies have investigated the gastrointestinal microbiota of individuals with IgA deficiency. One study found increased counts of *Actinomyces* spp. in the oral cavity of IgA deficient individuals [149]. The same authors found increased counts of anaerobic bacteria and enterococci in faecal stool samples from IgA deficient as compared to healthy individuals [149].

Salmonella and the immune system

After invasion of the mucosa, non-typhoid *Salmonella* induces a massive inflammatory response, with production of inflammatory cytokines such as TNF, IL-1, IL-6, IL-12 and IL-18, and chemokines that recruit monocytes, macrophages, neutrophils and cells of the adaptive immune system to the site of infection [150].

Most studies of *Salmonella* pathogenesis and immune response are performed *in vitro* or in animals. The importance of S-IgA in *Salmonella* infections in humans is not clarified, but IgA deficient individuals do not seem to be at risk for more severe disease than individuals with normal IgA levels. Instead, individuals with deficiencies involving IFN- γ or IL-12 production have increased susceptibility to *Salmonella* infection [151], pointing towards the importance of cell-mediated immunity and macrophage activation.

Influence of gender on the immune system

Women seem to exert stronger cellular as well as humoral immune responses than men. For example, serum immunoglobulin levels and responses to a variety of antigens are higher in women [152]. Gender related differences have been observed in several infectious conditions, including parasitic infections, trauma-related bacterial sepsis and virus infections [153, 154].

Factors of importance for the composition of the microbiota of the alimentary tract

Host factors

A number of host factors, including the various mechanical and chemical barriers towards colonisation described above are important for if and where different bacteria are able to establish. Some additional host factors which possibly influence the microbiota are described below.

Hereditary factors

The predominant species of the gut microbiota are very stable over time in healthy adults and appear to be host-specific [155]. The highest levels of similarity is found in monozygotic twins [156], while the microbiota of the individuals of a married couple living together is not more similar than the microbiota of unrelated individuals [155]. Knowledge of how this genetic regulation is carried out is still scarce, but mechanisms could include *e.g.* genetic variations in carbohydrate-structures expressed in the mucosa, which could influence both adherence of

bacteria and the availability of nutrients. However, the high level of similarity between monozygotic twins could also indicate that the environmental conditions present when acquiring the microbiota in the first place have great influence on the final composition of the microbiota.

Various physiological and anatomical factors which are genetically determined could play a role for the composition of the oral microbiota. For instance tooth eruption, tooth fissure shape, and interdental space and amount of saliva play a role in deciding which bacteria will be favoured by their preferred niche in the oral cavity [157]. Also variations in host derived nutrients for bacteria available in *e.g.* saliva and gingival crevicular fluid are likely to be important [158].

Gender

Composition of the microbiota may to some extent be gender-dependent. Phylogenetic profiles of mice have been found to cluster together according to gender [159]. One group found that bacteria belonging to the *Bacteroides-Prevotella* group were more numerous in the male gut, without further distinctions [160] and another group that three *Clostridium* species, one species from the *Bacteroides* group and two from the phylum *Proteobacteria* are more common in males [161].

Environmental and lifestyle factors influencing the oral microbiota

The composition of the oral microbiota is influenced by several factors which are only briefly mentioned here. Lifestyle factors influencing the microbiota include *e.g.* diet, smoking, oral hygiene, and the presence of foreign materials [158]. Environmental factors, like variation in the level of exposure to different bacteria are also likely to influence the microbiota. In infants, acquisition of *S. mutans* was associated with habits that allowed saliva transfer from parents to infants [58].

Environmental and lifestyle factors of importance for the establishment and composition of the gut microbiota

The establishment of the gut microbiota early in life is influenced by several environmental and life style factors, including delivery mode, feeding pattern, and levels of bacterial exposure [162].

The origin of the bacteria colonising neonates may be the maternal faecal and vaginal microbiota, but also various environmental sources [62, 163]. Infants delivered by caesarean section show delayed acquisition of for instance *E. coli* and *Bacteroides*, and to some degree of bifidobacteria, indicating the importance of the maternal faecal microbiota as a source of these bacteria [62, 163]. Lactobacilli may sometimes be acquired from the maternal vaginal

microbiota during delivery, and some authors report somewhat delayed colonisation in sectio delivered infants [163, 164]. Clostridia and enterobacteria other than *E. coli*, e.g. *Klebsiella* and *Enterobacter* species are found equally early, or even earlier, in sectio delivered as compared to vaginally delivered infants [62] [63], indicating that these bacteria are easily acquired from the environment. By one year of age, sectio-delivered infants in Western countries have a lower ratio of anaerobic to facultatively anaerobic bacteria in the gut than vaginally delivered infants, possibly indicating a less “mature” microbiota [63]

Close contact with other individuals facilitates acquisition of bacteria. Infants with siblings have a higher ratio of anaerobic to aerobic bacteria by one year of age, which possibly indicates a more adult-like microbiota [63]. It is not clear if an early childhood with animals influence the colonisation pattern.

Breast milk may be a source of bacteria colonising infants. *Staphylococcus*, *Streptococcus*, *Bifidobacterium* and *Lactobacillus* are frequently isolated from breast milk [165]. Other foods given to neonates may also contain bacteria that colonise the infantile gut [166].

Other, less well defined, lifestyle factors also play a role. In a study of the microbiota of children aged five to 13 years from three European countries there were no differences between countries, but children attending anthroposophic schools had a more diverse dominant faecal microbiota than controls as well as farm children [167].

Influence of environmental bacterial exposure on the gut microbiota

It is clear that the level of microbial exposure influences the gut colonisation pattern. In neonatal intensive care units where great efforts are taken to reduce bacterial spread, infants acquire a microbiota where coagulase-negative staphylococci, enterococci and *Enterobacteriaceae* dominate, while anaerobes are almost absent [168, 169] Great differences are also observed when comparing the early microbiota between infants from different parts of the world, with different levels of environmental bacterial exposure. Infants in developing countries have a more rapid turnover of bacteria and a larger number of species in their early microbiota than infants in Western countries [166]. Also, in developing countries, infants delivered by caesarean section may be as rapidly colonised by *E. coli*, *Bacteroides*, and *bifidobacteria* as vaginally delivered infants, indicating pronounced spread of faecal bacteria in the hospital milieu [171]. Lactobacilli have been found to be more common in Ethiopian (two to six weeks of age) and also in Estonian infants (at one year) compared to Swedish infants of similar ages [172, 173].

It is not known if the level of environmental microbial exposure also influences the composition and complexity of the more stable adult gut microbiota.

Influence of diet on the gut microbiota

In young infants, the gut microbiota differs in several aspects between breastfed and bottlefed infants. Breastfed infants harbour less clostridia [66, 174], but tend to have more staphylococci [75]. Furthermore, *Bacteroides*, enterococci and *Enterobacteriaceae*, especially *Klebsiella* and *Enterobacter*, tend to be more common in bottle-fed infants [171, 175]. A majority of studies find no differences in *Lactobacillus* colonisation between breast and bottle-fed infants [79, 162, 176]. Also, Bifidobacteria are equally common in breast- and bottle-fed infants in most recent studies [177].

In adults, the gut microbiota differs between individuals in different geographical regions consuming different types of diets [178], but this may also relate to other factors differing between the populations.

Roseburia, *Eubacterium* and *Bifidobacterium* have been found to decrease as a result of a diet low in carbohydrate [179]. Vegetarians harbour less clostridia and *Faecalibacterium* than omnivores [180, 181].

Diet lipid content may also influence the gut microbiota, as fat stimulates bile flow, by which expansion of *e.g. Bacteroides* may be stimulated [182]. In a study of mice, dietary iron deprivation resulted in elevation of anaerobes including lactobacilli [183].

Bacterial factors of importance for establishment and composition of the gastrointestinal microbiota

Ability to utilise available nutrients

To establish in the gastrointestinal tract, bacteria must multiply at a rate exceeding their rate of elimination. Bacteria therefore compete for available nutrients which include both dietary and host derived components. Apart from indigestible carbohydrates and low levels of non-absorbed protein, not much of the food ingested by the host reaches the colon. However there is a broad range of other nutrient sources in this habitat, *e.g.* mucus and exfoliated epithelial cells.

Some bacteria are able to utilise a variety of different substrates, whereas others are much more specialised [184]. Many gut bacteria ferment indigestible carbohydrates into short chain fatty acids, which is discussed later.

Adhesins

Bacteria colonising the gastro-intestinal tract commonly express structures called adhesins mediating adherence to host cell receptors or mucus structures [135]. Most bacterial adhesins are proteinacious structures that recognise defined carbohydrate sequences in host tissues: glycoproteins, glycolipids or less often a defined protein

structure. Adhesins of Gram negative bacteria are often found on fimbriae, rigid protein rods reaching out from the bacteria, whereas adhesins of Gram-positive bacteria most commonly form part of the cell wall or of the cell coat [185]. The same bacteria may have more than one adhesin, and the expression of adhesins is commonly subject to phase variation and may be turned on and off by the bacteria depending on environmental stimuli, which has been shown for S layer protein adhesin in *L. acidophilus* and *L. brevis* [186].

Adherence is an important step in colonisation of mucosal sites. This has been shown most clearly for pathogenic bacteria, for instance enterotoxigenic *E. coli*, which colonise the small intestine of man and animals [187]. *Salmonella* adheres to enterocytes and M cells as a first step in the pathogenesis [90]. In the oral cavity and the small intestine, adherence is likely to be extra important for colonisation because of the salivary flow and peristalsis, respectively, which otherwise wash bacteria away. In the oral cavity, bacteria form complex biofilms, and in this process, they commonly also coaggregate, *i.e.* adhere to each other [158].

In the colon, the flow of gut contents and transient time are much lower, which could indicate that adherence is less important for colonisation of this habitat. However, adherence may promote colonisation of the epithelium or mucus layer, and provide advantages by increasing access to nutrients squamated from the tissues or present in mucus [188].

For *E. coli* colonising the large intestine, the possession of P-fimbriae and type-1-fimbriae mediating adherence to galactose- and mannose-containing receptors, respectively, on colonic epithelial cells, seem to promote long-term persistence in the gut [189, 190]. *E. coli* with mannose-binding type 1 fimbriae are also able to bind to mannose-containing oligosaccharides on S-IgA [133, 191]. *E. coli* retrieved from the commensal microbiota of IgA deficient individuals express less mannose-binding adhesins than *E. coli* from individuals with normal levels of IgA [191, 192]. Possibly, binding of *E. coli* to S-IgA, which is present in mucus, facilitates colonisation of the mucus layer, which may be the preferred niche for *E. coli* in the large intestine.

Adherence of lactobacilli

Lactobacilli have been shown to adhere to epithelial cells, mucus, and extracellular matrices. Several structures have been identified as target substances or receptors for lactobacilli, *e.g.* collagen, fibronectin, laminin, lectins, and oligosaccharide-chains of glycoproteins [193-195].

Most studies of *Lactobacillus* adhesion to epithelial cells have been performed using cell lines, but there are also studies of biopsy samples [196, 197]. A number of surface layer proteins of lactobacilli have been reported to mediate adhesion to intestinal epithelial cells [198, 199] but few specific receptors for lactobacilli on epithelial cells or in mucus have been identified. However, several *Lactobacillus* species are able to express adhesins mediating adherence to

mannose-containing receptors on colonic epithelial cells [31, 196]. Such mannose-specific (MS) adhesins have been demonstrated in *L. plantarum*, *L. salivarius*, *L. johnsonii*, *L. paracasei* and *L. fermentum*, all members of the oral and/or faecal microbiota [31, 196, 200-202]. At least in *L. plantarum*, the expression of these adhesins is enhanced by oxygen, since the mannose-specific adherence of lactobacilli to colonic epithelial cells is reduced after culture of bacteria under anaerobic conditions [196]. In *L. plantarum*, the adhesin was identified as a proteinaceous structure [196], more specifically as a multi-domain cell surface protein [201].

Adhesion to mucus structures by lactobacilli expressing MS adhesins have been described [201]. Also, in addition to the proteinaceous MS adhesin of *L. plantarum* [201], other mucus-binding *Lactobacillus* proteins have been identified: the extracellular mucus-binding protein (mub) of *L. reuteri* [203], and the mub of *L. acidophilus* [204].

Oral adhesion of lactobacilli is less well studied than adhesion to intestinal structures. Epithelial cells and teeth are covered with saliva, and several studies show *Lactobacillus* adherence to salivary structures [205-207]. Adherence to buccal epithelial cells of *L. rhamnosus* has also been found [205].

Adherence of Salmonella

As mentioned above, adherence to host cells is an important step in the induction of infection by mucosal pathogens, and may even be a prerequisite [90]. Several types of fimbriae have been found in *Salmonella*, including mannose-binding type 1 fimbriae, plasmid-encoded fimbriae, long polar fimbriae and thin aggregative fimbriae (curli) [208]. Variable expression of the many adhesin structures may enable adherence to different cell types and the colonisation of different hosts [209].

Bacteriocins and other antagonistic compounds produced by bacteria

Antibacterial substances are not only produced by the host but also by the bacteria themselves, and antimicrobial activity is thought to be an important way for members of the normal microbiota to competitively exclude or inhibit newcomers.

Most bacteria can make one or more antibacterial peptides, *i.e.* bacteriocins, with the function to suppress competing bacteria of the same or different species [210]. The best characterised bacteriocins are those from lactic acid bacteria, including lactobacilli. They are most often active towards closely related Gram-positive bacteria, while the producer cells are immune to their own bacteriocins [211]. However, activity against Gram-negatives, *e.g.* *Salmonella*, by *Lactobacillus* bacteriocins or bacteriocin-like substances has also been described [212]. Like for human antimicrobial peptides, the most common mode of action is the formation of pores in the bacterial membrane, but they can also act by

prevention of cell-wall synthesis, inhibition of RNA synthesis and inhibition of bacterial phospholipase [213].

Other antagonistic compounds produced by bacteria are also known. Lactobacilli produce a number of other substances with non-specific antimicrobial activity, *e.g.* SCFA, lactic acid, formic acid, and hydrogen peroxide [211]. For instance, the SCFA could possibly lower colonic pH, which may then limit the growth of certain bacteria [214]. However, little is actually known about the possible role of these compounds in the interaction between bacteria and for the composition of the microbiota in the gastro-intestinal tract.

Colonisation resistance

A full microbiota greatly hampers the implantation of new bacteria into the ecosystem, and, thus, protects its host from the colonisation by pathogens. This is called colonisation resistance [215], and is believed to be the result of competition for binding sites and nutrients, and from the production of bacteriocins and other substances harmful for the competitors. The same forces are likely to strictly regulate the population size of each bacterial strain in the microbiota.

Colonisation resistance is indeed an important defence mechanism against colonisation and proliferation of pathogenic or potentially pathogenic bacteria. For example, it has been shown that a dose of only ten *Salmonella* bacteria causes lethal infection in germfree animals, whereas animals with a normal gut microbiota can stand infection with up to 5×10^6 bacteria before lethal infection occurs [216]. Also, the colonisation resistance provided by the microbiota keeps down the population counts of the potential pathogen *C. difficile* in the gut. Other potential pathogens which are kept at relatively low numbers in the microbiota include *e.g.* *E. coli* and other enterobacteria.

Effects on the host of the gut microbiota

Gut maturation

Establishment of the gut microbiota contributes to the maturation of the intestines with thickening of the mucosa and deepening of crypts. The villi become broader and shorter and the mass of the small intestine increases [217]. The regulation of intestinal epithelial cell turnover and mucus biosynthesis increases as new genes are turned on [218]. Increased peristalsis reduces bacterial counts in the small intestine [219]. Germ-free animals have fewer goblet cells than conventional animals [114]. The intestinal microbiota may also contribute to the development of the capillary network in the small intestinal villi [220].

Maturation of the immune system

Both the innate and the adaptive immune systems need stimulation from bacteria to develop and function optimally. The commensal microbiota induces mediators of the innate defence. For example, Paneth cells in small intestinal crypts are stimulated through colonisation to secrete antimicrobial compounds. Macrophage chemotaxis and phagocytic activity are reduced in germ-free as compared to conventional animals [221].

There are even greater differences between germfree and conventional animals in the acquired immune system. The gut associated lymphoid tissue is poorly developed in germ free animals. There are fewer lymphocytes in the lamina propria and in the epithelium, and intestinal lymphoid aggregates, such as the Peyer's patches and mesenteric lymph nodes, are smaller [222]. Spleen and thymus are underdeveloped as compared to conventionally raised animals [223]. Also, concentrations of serum immunoglobulins are much lower in germfree than in conventional animals, and germ-free mice have very low levels of S-IgA at mucosal sites [223].

Energy and nutrients for the host

The gut microbiota has an important function in the processing of nutrients [25]. The presence of bacteria alters the metabolic apparatus of host cells, resulting in more efficient uptake and utilisation of nutrients [223, 224]. It contributes to the regulation of host fat storage, and increases the capacity to extract energy from the diet [225]. Bacteria are also able synthesise several vitamins, *e.g.* vitamin K which is taken up and utilised by the host [25].

Gut bacteria enable metabolism of otherwise indigestible dietary carbohydrates to host-absorbable compounds and thus contribute to energy production [25]. The major end products of the fermentation of indigestible carbohydrates by anaerobes in the colon are the short-chain fatty acids (SCFAs) acetate, propionate and butyrate [214].

Involvement of the gut microbiota in health and disease

The gut microbiota influences our wellbeing in several ways. For instance, the SCFA butyrate produced by gut bacteria, serves as energy for epithelial cells and may have a protective role against colon cancer and ulcerous colitis [226, 227], and propionate enhances colonic muscular contraction contributing to relief of constipation [228].

Stimulation of the immune system by the commensal microbiota may be beneficial for health. Such stimulation possibly contributes to the maturation of immunoregulatory mechanisms. It has been shown that so called regulatory T cells, which are important for the prevention of immune reactivity towards autoantigens

and harmless environmental antigens, *e.g.* allergens, have impaired function in germfree animals [229].

There is increasing evidence that the composition of the gastrointestinal microbiota could have implications for the development of a number of diseases, including allergy [230], inflammatory bowel disease [231], colon cancer colitis [226], and obesity [232].

Disturbances of the gut microbiota

Disturbances induced by antibiotics

Treatment with antibiotics is a common cause of disturbances of the normal gastrointestinal microbiota. Different agents have different effects, depending on antibiotic spectrum, dose, route of administration, pharmacokinetics and pharmacodynamics. In neonates and young infants, antibiotic treatment often has profound effect on the microbiota, and most anaerobic bacteria are strongly suppressed.

Suppression or elimination of large bacterial populations in the microbiota with loss of colonisation resistance may lead to the expansion and colonisation of potential pathogens in the gut microbiota, like *Clostridium difficile*, *S. aureus*, *C. perfringens*, and various *Enterobacteriaceae* or *Candida* species [233, 234]. Antibiotic-induced disturbances in the microbiota treatment also increases the risk of acquiring gastrointestinal pathogens. For instance, prior antibiotic treatment increases the risk of acquiring *Salmonella* [235].

Antibiotic associated diarrhoea

Antibiotic associated diarrhoea (AAD) is common during and after the administration of antibiotics. The incidence varies between five and 35 % in different studies [236]. Common risk factors include high age, hospitalisation and concomitant disease [236]. Clindamycin, extended-spectrum penicillins, cephalosporins and possibly fluoroquinolones are associated with a higher risk [236-238].

Toxin-producing *C. difficile* is reported to be the cause of 10 - 25% of AAD cases in most studies [239], but higher figures have been found [240]. Possibly, other pathogens like *S. aureus*, *C. perfringens*, various *Enterobacteriaceae* or *Candida* species may sometimes be responsible [233, 234]. The remainder of episodes of AAD may be due to several factors, including increased gastro-intestinal motility [233], osmotic diarrhoea secondary to decreased digestion and absorption of carbohydrates [233], and reduction of short chain fatty acids in faeces [241].

Clostridium difficile enteritis is treated with metronidazole or vancomycin, if discontinuation of antibiotics is not enough, but relapses are common. For other types of AAD there is no effective treatment available.

Disturbances in the microbiota induced by infectious gastroenteritis

Bacterial gastroenteritis may involve changes in the normal gut microbiota. There are several contributing mechanisms. Increased intestinal motility and fluid secretion change the intestinal environment and increase the oxygen content. Resident or introduced aerobic or facultatively anaerobic bacteria can expand, whereas several anaerobes decrease in numbers [242, 243]. In a recent study of children in India, significantly lower levels of *Bacteroides*, the *Prevotella-Porphyromonas* group, *Eubacterium rectale* and *Faecalibacterium prauznitzii* were found during acute diarrhoea [70]. There is also a decrease in bacterial fermentation products, particularly short-chain fatty acids, and an associated increase in luminal pH [243]. It is possible that this could then in turn allow further growth of bacteria that are usually inhibited by a lower pH.

Post infectious irritable bowel syndrome

Symptoms of irritable bowel syndrome (IBS), *i.e.* abdominal pain or discomfort, and altered bowel habits, commonly arise after gastroenteritis caused by *Campylobacter*, *Salmonella*, *Shigella* or diarrheagenic *E. coli* [86] but does not seem to be common after viral infection.

Proposed risk factors for developing IBS after bacterial gastroenteritis include infection by a virulent pathogen, long duration of diarrhoea, young age, female gender, and antibiotic treatment [244, 245].

The pathogenesis of IBS is unknown, but may include low grade inflammation of the gut mucosa [246]. Overgrowth in the small intestine by bacteria normally found in the colon has been described in IBS [247] and colonic gas-production (hydrogen or methane) is greater, possibly as a result of changes in the fermentation of carbohydrates by gut bacteria [248]. Changes found in the faecal microbiota include *e.g.* decreased numbers of *E. coli* and other *Enterobacteriaceae*, lactobacilli, *Collinsella* and to a lesser extent, bifidobacteria [249]. *Veillonella* was increased in IBS with constipation [250].

The prognosis of post infectious IBS is similar to that of IBS without preceding infection, with 50 % of the patients recovering within six years [251]. There is no widely accepted treatment for postinfectious IBS.

Probiotics

Probiotics are defined as “live microorganisms which, when consumed in adequate amounts as part of food, confer a health benefit on the host” [252]. Microorganisms commonly used as probiotics include the yeast *Saccharomyces boulardii*, and various bifidobacterial and *Lactobacillus* species. *Lactobacillus* species used as probiotics are e.g. *L. rhamnosus*, *L. reuteri*, *L. acidophilus*, *L. paracasei*, *L. johnsonii*, *L. salivarius*, and *L. plantarum*.

Why use probiotics?

There are a number of situations where probiotics could be an attractive treatment alternative. Conditions involving a disturbed microbiota could possibly benefit from the effects of probiotic bacteria on the microbiota. This includes e.g. patients treated with antibiotics.

Effective treatment is often lacking in bacterial gastroenteritis. For instance in *Salmonella*, antibiotics are indicated for severe and complicated infections, but have marginal effect against uncomplicated gastroenteritis. Antibiotics have also been reported to prolong the time *Salmonella* is excreted in stools [88], although this has been questioned [245]. Furthermore, there is an increasing problem with antibiotic resistance in enteric pathogens, including *Salmonella* [253].

Influence of probiotics on the gut microbiota

Intake of lactobacilli and other probiotics may alter the composition of the microbiota, but the changes are usually small and transient and there are large variations between individuals regarding the effects [47, 254]. Still, intake of probiotics may considerably alter levels of diverse bacterial metabolites, e.g. amino-acids, and SCFA [254]. Some studies have found no changes in the microbiota during or after the ingestion of a probiotic *Lactobacillus* strain (apart from an increase in *Lactobacillus* counts due to the strain administered) [255], whereas some find minor changes like increased numbers of *Enterococcus* [47]. A study of infants aged 12-24 months showed moderate changes in the faecal microbiota, with increased counts of several *Lactobacillus* species and decreased counts of clostridia with intake of the strain *Lactobacillus paracasei* A [256]. Clearly, the effects on the microbiota induced by probiotic bacteria are strain specific.

Other proposed effects of probiotics

Probiotics have been reported to influence the immune system through increased production of IFN- γ by lymphocytes [257] enhanced phagocytosis by polymorphonuclear leucocytes [258-260] and enhanced expression of complement

receptors on polymorphonuclear leucocytes [261, 262]. *L. rhamnosus GG* may increase production of nitric oxide (NO) in human T84 intestinal epithelial cells [263]. Anti-inflammatory effects have also been found, e.g. lowering of highly sensitive C-reactive protein and TNF [264]. Again, the effects appear to be strain specific.

In vitro studies indicate that probiotics may strengthen the mucosal barrier. Through competition for adhesion sites probiotics may diminish the attachment of pathogens [265]. Some *Lactobacillus* strains, including e.g. *L. plantarum 299v* and a probiotic mixture of lactobacilli, bifidobacteria and *S. thermophilus*, have been found to increase mucin production by epithelial cells *in vitro* [266, 267].

Clinical effects of probiotics

Health benefits of lactobacilli are claimed for a number of conditions (Table 4). Examples of conditions where certain probiotics may be of benefit are rotavirus diarrhoea in infants [268, 269], prevention of AAD [270-272], caries [273] and surgical infections [274]. However, evidence is often conflicting, and further studies are needed in most areas.

Probiotics for the prevention and treatment of antibiotics associated diarrhoea and *C. difficile* infection

Several studies have been performed to investigate if the incidence of antibiotic associated diarrhoea (AAD) can be reduced by administration of probiotic microorganisms. Positive results have been reported for the yeast *Saccharomyces boulardii*, which significantly reduced the incidence of AAD in several studies [270, 275, 276]. The widely used probiotic strain *L. rhamnosus GG* has also been tested in several trials [271, 277-279]. The effects seem quite good in children, but less certain in adults. Also, certain probiotic mixtures, for example *Lactobacillus casei* + *L. bulgaricus* + *Streptococcus thermophilus* have been shown to significantly reduce the development of AAD [272]. Probiotics have also been tried in the treatment of recurrent *C. difficile* associated diarrhoea where there is some evidence of effect of treatment with *S. boulardii* [282].

Table 4. Conditions where beneficial effects of probiotics have been claimed

Prevention of antibiotic-associated diarrhoea (by <i>C. difficile</i> or unexplained)	<i>Saccharomyces boulardii</i> [270, 275, 276] <i>L. rhamnosus</i> GG [271] [277-279] <i>L. bulgaricus</i> + <i>S. thermophilus</i> [272]
Treatment of recurrent <i>C. difficile</i> associated diarrhoea	<i>L. plantarum</i> 299v [280] ¹ <i>L. rhamnosus</i> GG [281] ² <i>S. boulardii</i> [282].
Rotavirus diarrhoea in children	<i>L. rhamnosus</i> GG [268] VSL#3 ³ [269] 3 <i>L. rhamnosus</i> strains [283]
Crohn disease	<i>Faecalibacterium prausnitzii</i> [284]
Prevention of pouchitis relapse	VSL#3 ³ [285]
Irritable bowel syndrome	<i>L. plantarum</i> 299v [286]
Post infectious irritable bowel syndrome	<i>B. animalis</i> [287]
Surgical infections	<i>L. plantarum</i> 299v + oat fibre [274]
Urinary tract infections	<i>L. crispatus</i> [288]
Necrotising enterocolitis	<i>L. acidophilus</i> + <i>B. infantis</i> [289] <i>B. infantis</i> + <i>B. bifidus</i> + <i>S. thermophilus</i> [290]
Caries	<i>L. rhamnosus</i> GG [273]
Atopic eczema	<i>L. rhamnosus</i> GG [291]

¹Tendency towards effect, but too few patients were included for significance

²Case report

³VSL#3 contains *L. acidophilus*, *L. paracasei*, *L. bulgaricus*, *L. plantarum*, *B. breve*, *B. infantis*, *B. longum*, and *S. thermophilus*

Antibiotic associated diarrhoea and probiotics, modes of action

The mechanisms by which probiotics might prevent AAD and *C. difficile* associated disease are not clarified, but may include *e.g.* an increased colonisation resistance and modulation of the host immune response.

As described elsewhere, suppression of the normal microbiota by antibiotics allows opportunists like *C. difficile* to multiply. By competing for space and nutrients probiotics may counteract the growth of this and other pathogens. Also, spore germination of *C. difficile* may be inhibited by the production of hydrogen peroxide and short chain fatty acids by probiotic *Lactobacillus* strains [293].

In a study by Gorbach the numbers of plasma cells in the intestinal mucosa increased with administration of *L. rhamnosus* GG, resulting in an enhanced immune response towards *C. difficile* or *C. difficile* toxins [294]. Certain *Lactobacillus* strains, including *L. plantarum* 299v, have been shown to increase the production of SCFAs in the gut [241]. A positive correlation between SCFA production, absorption of sodium and water in the colon, and decreased diarrhoeal symptoms has been observed in several studies [295].

Probiotics for the prevention or treatment of Salmonella infection

Several animal studies support an effect of probiotic lactobacilli against *Salmonella*. Good effects have been observed in chickens [296], pigs [297], and mice [298]. However, there are also studies showing no beneficial effects.

There are few studies involving human subjects. Alm *et al.* found in a non-blinded study that intake of *Lactobacillus acidophilus* shortened the duration of the *Salmonella* carrier state in patients that were asymptomatic at study start [300].

Salmonella and probiotics, modes of action

There are several possible mechanisms whereby probiotics could be effective against *Salmonella*: competition for binding sites in the gut, production of antibacterial substances, competition for nutrients, local reduction of luminal pH, and an influence on the host immune response. A number of studies, mostly *in vitro*, have been performed to test these possibilities. A short review of such studies is presented below, but to what extent these findings mirror what actually happens in the human gut is unknown.

Strains of several *Bifidobacterium* and *Lactobacillus* species, for instance *B. longum*, *L. acidophilus*, and *L. plantarum* are able to inhibit the adhesion of *Salmonella* to intestinal epithelial cells *in vitro* [301, 302]. It is not known if adhesion is blocked by competition for a specific receptor, or by steric hindrance.

Furthermore, several *Bifidobacterium* and *Lactobacillus* species, including *B. bifidum*, *B. animalis*, *L. acidophilus*, *L. johnsonii* and *L. plantarum*, have been shown to possess antagonistic action against *Salmonella* ssp. as a result of production of bacteriocins, organic acids like lactic acid, or other substances toxic to *Salmonella* [212, 303]. Antagonism by competition for growth limiting

amino acids between *S. Typhimurium* and a combination of five faecal species has been shown *in vitro* with a reduction of growth of the *Salmonella* strain [304].

There are also studies indicating that probiotic strains can down-regulate the inflammatory response induced by *Salmonella* [302], and increase the production of S-IgA in the gut against this pathogen [305]. On the other hand, certain probiotics may stimulate the inflammatory response, for example through activation of macrophages [306]. Probiotics were also found to stimulate proliferation of lymphocytes in response to *Salmonella* [307].

Lactobacillus plantarum 299v

Lactobacillus plantarum 299v (DSM 9843) is a probiotic strain which survives passage through the gastrointestinal tract and transiently colonises small intestinal and colonic mucosa [308, 309]. In animal models it has been found to reduce intestinal injury and inflammation after radiation, to reduce the severity of dextran sulfate sodium-induced colitis [310, 311] and to abolish *E. coli*-induced increase in intestinal permeability [312].

Two clinical studies have shown a reduction of abdominal pain in patients with irritable bowel disease receiving *L. plantarum* 299v, and trends towards less constipation and flatulence [286, 313]. However, another study found no clinical improvement in IBS using this probiotic strain [314].

Supply of *L. plantarum* 299v significantly reduced the incidence of post-operative infections in transplant recipients [315]. Furthermore, critically ill patients treated with this strain had decreased colonisation by *C. difficile* as compared to controls [316], and the same strain in combination with metronidazole tended to be more effective than metronidazole alone in the treatment of patients with recurrent *C. difficile* associated diarrhoea [280].

Safety of probiotics

There are several reports of sepsis associated with the probiotic yeast *S. boulardii* [317], which is widely used for the treatment of AAD and *C. difficile* infections. Infections have even occurred by aerosol transmission to patients with central venous catheters neighbouring a patient treated with the yeast [318]. Most infections occurred in severely debilitated patients.

Lactobacilli are generally regarded as safe [319] and it is believed that the risk of infection with a probiotic *Lactobacillus* strain is similar to the risk of becoming infected with a *Lactobacillus* strain from the commensal microbiota [320]. As mentioned earlier, endocarditis, bacteraemia and localised infections including abscesses caused by lactobacilli have been reported, mainly in individuals with severe underlying disease [321, 322].

There are some reports of infections caused by the widely used probiotic strain *L. rhamnosus GG* including bacteraemia in children with underlying disease and one case of endocarditis and one case of liver abscess in adults [317]. In a review from 2005 only one out of 241 reported *Lactobacillus* infections was caused by a probiotic *Lactobacillus* strain [322].

Plasmids carrying antibiotic resistance genes have been found in several *Lactobacillus* species, including *L. plantarum* [323]. Transfer of such plasmids to other bacteria has been considered to be rare, but is as yet not well studied [324]. Some studies indicate a higher risk of antibiotic resistance transfer than previously believed, especially during treatment with antibiotics [325, 326].

AIMS

The aims of this thesis were:

to study the establishment of the *Lactobacillus* microbiota at the species and strain level in Swedish infants followed over the first 18 months of life, and to identify factors influencing the colonisation pattern

to investigate how S-IgA influences the *Lactobacillus* microbiota, by studying the oral and faecal *Lactobacillus* microbiota of adults with and without IgA deficiency regarding species distribution and expression of mannose-specific adhesins

to determine if intake of *Lactobacillus plantarum* can prevent diarrhoea and other gastro-intestinal disturbances and decrease colonisation by toxin-producing *C. difficile* during treatment with antibiotics

to study if intake of *Lactobacillus plantarum* can reduce symptoms during and after *Salmonella* enteritis and/or reduce the time until clearance of *Salmonella* in faeces

MATERIALS AND METHODS USED IN THE STUDIES

A summary of the individuals studied and the methods used in study I-IV is presented here. Please refer to the individual papers for more detailed information.

Study populations and study design (I-IV)

Paper I

The establishment of the *Lactobacillus* microbiota was studied in Swedish infants followed over the first 18 months of life.

One hundred and twelve healthy Swedish infants born at the Sahlgrenska University Hospital were followed. The children were recruited to a prospective birth-cohort study aiming to investigate the relation between intestinal colonisation pattern and allergy development, the ALLERGYFLORA study [62] [63]. Parents-to-be were enrolled at the maternity clinic, and background data collected on atopic heredity, pets and siblings was collected.

During the infant's first 18 months of life, faecal samples were collected at regular intervals and analysed for lactobacilli. Parents recorded the child's health status and feeding habits during the first year. The influence of delivery mode, feeding mode and of siblings or pets in the household on *Lactobacillus* colonisation pattern was studied.

Paper II

The oral and faecal *Lactobacillus* microbiota of adult individuals with and without IgA deficiency was characterised regarding species distribution and expression of mannose-specific adhesins.

Thirty three individuals with selective IgA deficiency and thirty-four age-matched healthy individuals with normal serum immunoglobulin levels were included in the study. IgA deficiency was defined as a serum IgA level of less than 0.05 g/l in the presence of IgM, IgG and IgG1 - 4 in the normal range [327]. Ten of the individuals with IgA deficiency were healthy blood donors diagnosed at screening, while 23 at some time point had sought medical advice at the infectious diseases clinic for symptoms which could be related to their IgA deficiency. Symptoms included a history of frequent respiratory tract infections and/or of gastrointestinal complaints. Fourteen had at least one diagnosed auto-immune disease. All participants agreed not to consume any probiotic products for two weeks before sampling.

From each individual, one oral and one faecal sample were obtained and analysed for lactobacilli. The *Lactobacillus* species distribution and adhesin expression were compared between IgA deficient and control individuals, and between oral and faecal samples.

Paper III

The ability of the probiotic strain *L. plantarum* 299v to prevent diarrhoea and other gastro-intestinal disturbances during treatment with antibiotics was tested in a double blind placebo controlled study.

Inclusion criteria were age of at least 16 years, antibiotic treatment started within 48 hours before inclusion, an expected treatment period of 7 to 14 days, and ability to actively participate in the study by keeping a diary and providing stool samples. Patients were randomized to consume 200 ml/day of a test drink containing blueberries and 5% oats gruel with or without 5×10^7 colony forming units (CFU) of *L. plantarum*/ml once daily until a week after termination of antibiotics. Gastrointestinal symptoms, intake of antibiotics and intake of test drink were recorded daily by the patients. The patients were asked to avoid foods or products containing live bacteria during the study period and to continue the registration in the diary for at least one week after the last day of intake of test drink. On completion, the diary was mailed to the study centre. Faecal samples for the analysis of *C. difficile* toxins were obtained at study start before first intake of test drink and after discontinuation of antibiotics.

Primary outcomes were the proportion of patients in each group developing diarrhoea, defined as at least 3 loose or watery stools per day for at least 2 consecutive days, and the proportion of patients in each group positive for *C. difficile* toxin in faeces after treatment with antibiotics. Secondary outcomes were the risk of developing loose or watery stools, hard stools, abdominal pain, nausea, vomiting, flatulence, or blood in stools in each group.

Paper IV

The effect of intake of *L. plantarum* 299v on symptoms during and after *Salmonella* enteritis and on time until clearance of *Salmonella* in faeces was studied.

Patients were eligible for the study if they were at least one year of age, had sought medical advice for gastrointestinal symptoms and had a stool sample positive for *Salmonella*. Patients were randomised to consume 1 g of a skim milk powder with or without 5×10^{10} colony forming units (CFU)/g of freeze-dried *Lactobacillus plantarum* 299v. They were instructed not to consume other foods or products containing live bacteria during the study period, and to register symptoms, intake of study preparation and any use of antibiotics in the study diary. Faecal samples for the analysis of *Salmonella* were obtained at inclusion and then weekly during the study.

Primary outcomes were time until clearance of *Salmonella* and time until resolution of diarrhoea, *i.e.* time to the first week without a day with diarrhoea (defined as at least three loose stools in 24 hours). Secondary outcomes were time until cessation of other acute symptoms, *i.e.* loose stools, blood in stools, fever, nausea, vomiting, abdominal pain, or any of these symptoms. The occurrence of symptoms while still taking the test preparation after clearance of *Salmonella*, was also analysed and compared between the *L. plantarum* and the placebo group. Time to clearance of *Salmonella* symptoms, and effects of treatment, were also compared between female and male patients.

Permission from the Ethics Committee (I-IV)

Informed consent was obtained from included individuals or their parents, and the Ethics Committee of the Medical Faculty of Göteborg approved all four studies.

Sampling and culture for the isolation of lactobacilli (I, II)

From infants, faecal samples were obtained at 1, 2, 4 and 8 weeks, and at 6 and 12 months of age. In 65 of the 112 infants, faecal cultures were also performed at 18 months. Parents collected freshly voided faeces at home. The samples were kept refrigerated in a container without oxygen (AnaeroGen Compact, Oxoid Ltd., Basingstoke, UK) until transported to the laboratory, where they were processed within 24 h after collection.

In paper II, one oral and one faecal sample were obtained from each healthy and each IgA deficient individual. For oral lactobacilli, a cotton-tipped swab was pressed against the back of the tongue and transported in Stuart's transport medium [328] directly to the laboratory. For intestinal lactobacilli, freshly voided faeces was collected by the participants and kept refrigerated until transported to the laboratory within 5 h.

All faecal samples were serially diluted and plated on Rogosa agar for selective outgrowth of lactobacilli [329]. The limit of detection was 330 ($10^{2.52}$) CFU/g faeces. For oral lactobacilli, the cotton-tipped swab was streaked directly on Rogosa agar plates. All plates were incubated anaerobically at 37 °C for 3 days using the BBL GasPak anaerobic system (Becton Dickinson Microbiology Systems, Sparks, MD). From the plates of oral samples and from appropriate dilutions of the faecal samples, one representative colony of each morphotype (differing in *e.g.* size, shape, colour or texture from other colonies) was separately enumerated, Gram-stained, examined in the microscope and subcultured to purity. Unbranched Gram-positive rods were regarded as tentative lactobacilli and analysed further.

Identification of lactobacilli by PCR (I, II)

DNA extraction (I, II)

In paper I, putative *Lactobacillus* isolates were grown over night in *Lactobacillus* carrying medium (LCM) [330] and a crude cell extract was prepared by bead beating as previously described [331]. Briefly, cell extracts were prepared from overnight cultures at 28 °C. The cells were washed twice in 1 ml sterile Milli-Q® water (Millipore, Molsheim, France), and disrupted in an Eppendorf tube with glass beads.

In paper II, bacterial DNA was extracted as described by Song with some modifications [29]. Briefly, one to two bacterial colonies, which had been subcultured to purity on Rogosa agar, were suspended in 50 µl of 10 mM Tris HCl, 1 mM EDTA (pH 8.0) and 10 mM saline. The bacterial suspension was incubated for 10 min at 95 ° and centrifuged at 18 600 x g for 3 min. The supernatants were kept at -20 ° C until used.

Exclusion of bifidobacterial isolates using a Bifidobacterium-specific PCR (I, II)

As Rogosa plates are not entirely selective for lactobacilli, but also permit growth of bifidobacteria, all isolates were screened in a *Bifidobacterium*-specific PCR. The primers PbiF1 (5'CCG GAA TAG CTC C-3') and PbiR2 (5'-GAC CAT GCA CCA CCT GTG AA-3') [332] were used under previously described PCR conditions [332] (paper II) and as described in paper I, respectively. The amplicon specific for bifidobacteria, with a size of 919 bp, was visualised by agarose gel electrophoresis and ethidium bromide staining. Isolates identified as bifidobacteria were not analysed further.

RAPD for the distinction of different *Lactobacillus* strains combined with a Bifidobacterium-specific PCR (I)

In paper I, a Random Amplification of Polymorphic DNA analysis (RAPD) was performed to distinguish different *Lactobacillus* strains, as previously described [331]. In RAPD, primers of arbitrary sequences bind under low stringency conditions to various sites on both strands of template DNA. This results in a pattern of amplified DNA fragments which is unique for each strain [26].

The RAPD-primer 73 (5'-ACGCGCCCT-3') was used, and the RAPD analysis was performed in the PCR in which the *Bifidobacterium* specific primers were included. Gel electrophoresis for the separation of PCR products was performed on agarose gels that were stained with ethidium bromide and photographed under UV-light. Putative *Lactobacillus* isolates from a single child

showing the same band pattern on RAPD analysis were regarded as belonging to the same strain. Isolates identified as bifidobacteria were discarded.

Identification and speciation of lactobacilli by group and species-specific multiplex PCR assays (I, II)

At least one representative isolate of each different RAPD pattern from each child in paper I, and each putative *Lactobacillus* isolate in paper II, were subject to speciation by PCR. Lactobacilli were identified using a series of multiplex PCRs with group and species specific primers recognizing sequences of the 16S-23S rRNA and its flanking 23S rRNA gene [29]. The method was designed to cover *Lactobacillus* species commonly found in the human intestine [80]. In the first multiplex PCR, lactobacilli are differentiated into four groups (Fig.2). Group I contains only one species, *L. delbrueckii* which is identified directly, while isolates reacting with the primers specific for group II, III, or IV are analysed further in species-specific multiplex PCRs. The species identified in these analyses include *L. jensenii*, *L. acidophilus*, *L. crispatus*, and *L. gasseri* (group II); *L. paracasei* and *L. rhamnosus* (group III); and *L. salivarius*, *L. reuteri*, *L. plantarum* and *L. fermentum* (group IV).

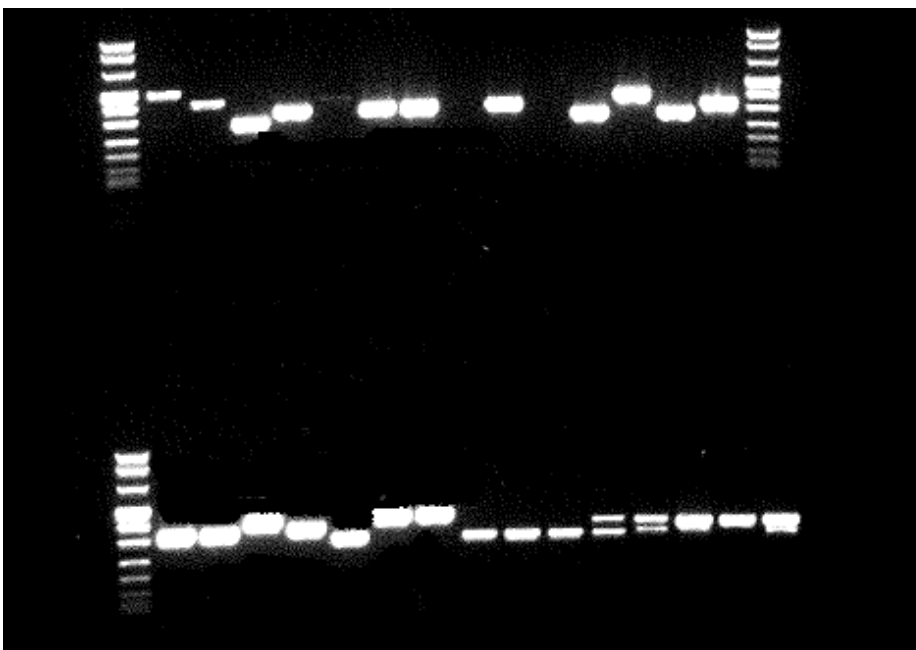


Figure 2. A photograph of PCR with group-specific primers

Speciation of lactobacilli by sequencing of the *Lactobacillus* 16S rRNA gene (I, II)

In paper I, one of the *Lactobacillus* strains that could not be typed to the species level using species-specific primers as described above was identified by partial sequencing of the *Lactobacillus* 16S rRNA gene. This analysis was performed at the Laboratory of Molecular Microbiology, Department of Clinical Bacteriology, Sahlgrenska University Hospital, using their standard protocol [333].

In paper II, a large number of isolates did not react in the group- or species-specific PCR assays. These isolates were speciated by partial sequencing of 16S rRNA genes, as described in the manuscript, at the Genomics Core Facility Platform at the Sahlgrenska Academy, University of Göteborg. The sequences were analysed using the Ribosomal Database Project (<http://rdp.cme.msu.edu/>) and FASTA (<http://www.ebi.ac.uk/Tools/fasta33/nucleotide.html>) databases. Only type strains were considered and a similarity of at least 98 % and a distance score of at least 0.5 % units to the next closest species required for the determination of species.

Adherence of lactobacilli to HT-29 cells (II)

The expression of mannose-specific (MS) adhesins by lactobacilli from IgA deficient and control individuals was tested by assessing adherence in a mannose-sensitive manner to cells of the colonic carcinoma cell line HT-29 as previously described [196]. Bacteria and cells were incubated in Hank's balanced salt solution with or without methyl- α -D-mannoside at 4 ° for 30 min. The cells were spun down, washed in PBS and fixed with neutral buffered formalin. The number of bacteria attached to each of 40 epithelial cells was determined using interference contrast microscopy. In each experiment, the *Lactobacillus* isolates from one IgA deficient and one control individual were analysed, the person examining adherence being blinded as to their identity. To calculate MS adherence, the number of bacteria adhering in the presence of methyl- α -D-mannoside were subtracted from the number of bacteria adhering in the absence of methyl- α -D-mannoside. Isolates adhering with at least three bacteria/cell and showing at least 30 % reduction of adherence in the presence of methyl- α -D-mannoside, were considered positive for MS adhesins [31].

Sampling for and detection of *C. difficile* toxin in faeces (III)

In paper III, faecal samples for the detection of *C. difficile* toxin were obtained before administration of the first dose of test drink and seven to ten days after discontinuation of antibiotics. Patients were instructed to deliver an extra faecal sample in case of diarrhoeal symptoms. Freshly voided faeces was collected by the patient and sent by mail if sampling was not performed at the hospital.

An ELISA was used to detect *C. difficile* toxin in faecal samples, either toxin A only (patients 1-154) or, as the standard method was changed at the clinical laboratory, toxin A and B (patient 155 and onwards) [334, 335]. The analyses were performed at the Clinical Bacteriology Laboratory at Sahlgrenska University Hospital.

Sampling and culture for the detection of faecal bacterial pathogens (IV, III)

In paper IV, rectal swab samples for culture and detection of *Salmonella* were performed at inclusion and once a week until four consecutive negative samples had been obtained. Thereafter, intake of test products was discontinued, but weekly samples were obtained and cultured for four additional weeks (Fig. 1, paper IV). The rectal swab samples were taken by the patients and sent in by mail in Stuart's transport medium [328].

In paper III, the faecal samples delivered during diarrhoeal symptoms were cultured and analysed for *Salmonella*, *Shigella*, *Yersinia*, and *Campylobacter*.

Culture and analysis for the identification of *Salmonella*, *Shigella*, *Yersinia* and *Campylobacter* were performed at the Clinical Bacteriology Laboratory at Sahlgrenska University Hospital according to their standard methods [336].

Salmonella serotyping (IV)

Salmonella isolates were serotyped at the Clinical Bacteriology Laboratory at Sahlgrenska University Hospital according to the Kauffman-White scheme [328].

Statistical methods

Fischer, Man-Whitney (I, II, III, IV)

Comparisons of characteristics between infants (paper I), study groups (paper II, III, IV), women and men (paper IV), and bacterial groups (paper II) were performed using Fisher exact test for proportions and the Mann-Whitney test for continuous characteristics.

McNemar's test (II)

McNemar's chi-squared test was used for comparing paired proportions, *i.e.* oral and faecal isolates.

Exact logistic regression (III)

The probability of antibiotic associated diarrhoea was compared between the treatment and placebo groups using exact logistic regression.

Generalised estimating equations (III, IV)

To analyze the effect of treatment on the risk of experiencing loose or watery stools or other gastrointestinal symptoms in paper III, we used generalized estimating equations [337, 338], modelling the correlation structure between the responses within an individual by the autoregressive model, where the correlation between responses gets smaller with time.

In paper IV, we used the empirical model of generalized estimating equations to compare the treatment groups, as well as women and men, regarding symptoms after clearance of *Salmonella* while still taking the test preparation.

Survival analysis (IV)

Kaplan Meier (IV)

Kaplan Meier curves with the log rank test were used to detect the effects of probiotic treatment and gender on the primary outcomes time to clearance of *Salmonella* and resolution of diarrhoea, and on time to resolution of other acute symptoms. Finally, they were used to study the effect of certain other factors and patient characteristics on time to *Salmonella* clearance and resolution of diarrhoea, respectively.

Cox regression (IV)

Cox regression was used to perform a multivariable analysis of the factors that were found to influence time to clearance of *Salmonella* and resolution of diarrhoea in the univariable analyses, to determine the possible independent contribution of these factors to the outcomes.

RESULTS

Studies on the *Lactobacillus* microbiota of infants and adults (I, II)

A summary of the results from study I-IV is presented here. Please refer to the individual papers for more detailed information.

Characteristics of infants (I)

Gut colonisation by lactobacilli was studied in 112 Swedish infants followed from birth to 12 or 18 months of age. Eighty-five per cent of the infants were delivered vaginally. The majority (70 %) were fully breast-fed for at least four months, and 78 % still received some breast-milk at six months of age. Forty-seven per cent of the infants had one or more siblings, and 23 % grew up in homes with pets. In most cases (89 %) at least one parent had some kind of allergy.

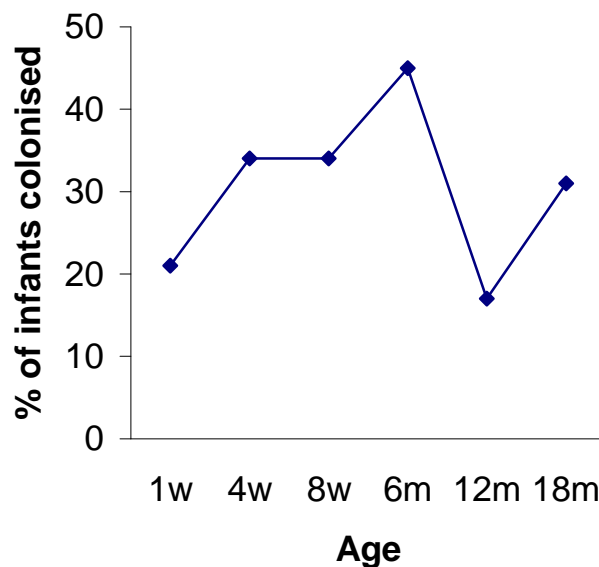


Figure 3. Lactobacilli were cultured from faeces of 112 infants at regular intervals during the first year. Sixty-five of the infants were also cultured at 18 months of age.

Lactobacillus colonisation rate and population counts in the infant gut (I)

Lactobacilli were isolated from 21 % of 1-week-old infants. The *Lactobacillus* colonisation frequency increased to a maximum of 45 % by 6 months of age, then dropped to 17 % by 12 months ($p < 0.0001$), to increase again to 31 % at 18 months ($p < 0.05$) (Fig.1, paper I)(Fig. 3 above). During the first months, population counts in colonised infants rose from $10^{6.8}$ CFU/g at one week to a maximum of $10^{8.8}$ CFU/g stools by six months. The counts then dropped significantly to $10^{5.4}$ CFU/g by 12 months of age ($p < 0.0001$) and increased only slightly to 10^6 CFU/g by 18 months (Fig. 2 paper I).

Lactobacillus species distribution in the infant gut (I)

Lactobacilli were speciated using multiplex PCR [29], and *Lactobacillus* species distribution in infant faecal samples are shown in Figure 3, paper I and in Table 5 below. *L. rhamnosus* and *L. gasseri* were the most frequently isolated species during the first two months of life. *L. rhamnosus* remained dominant between two and six months, followed by *L. paracasei*. At twelve months, the most frequently isolated species, *L. paracasei*, was found in only 7 % of the infants, but the isolation frequency of this species increased to 22 % at 18 months. By that time, *L. plantarum* and *L. delbrueckii* were isolated from 6 % and 5 % of infants, respectively, and *L. rhamnosus* was almost absent. No infant harboured *L. gasseri* in the 12 or 18 months samples.

The counts of *L. rhamnosus* in colonised infants were quite stable, approximately 10^8 CFU/g faeces over the first six months of life, whereas the counts of *L. gasseri*, *L. paracasei* and *L. fermentum* increased over the same period (Fig. 4, paper I). The counts of all early colonising species decreased after six months of age (Fig. 4, paper 1)

Persistence of individual Lactobacillus strains in the gut microbiota of infants (I)

Individual *Lactobacillus* strains within a child were distinguished using RAPD. During the first six months, 17 % of the infants harboured *Lactobacillus* strains that persisted for at least three weeks in the microbiota, most commonly *L. rhamnosus*, *L. gasseri*, or *L. paracasei* (Fig. 5, paper I). As samples were collected at six months intervals after six months of age, persistence of the later colonising strains could mostly not be determined. However, one infant harboured the same *L. rhamnosus* strain at six and twelve months, and one harboured the same *L. paracasei* strain at six, twelve and 18 months of age (Fig 5, paper I).

Table 5. Per cent of infants colonised by various *Lactobacillus* species at different time points over their first 18 months of life

	Isolation frequency (%)						
	1 w	2 w	1 m	2 m	6 m	12 m	18 m
<i>L. rhamnosus</i>	7.1	8.9	14	21	21	1.8	1.5
<i>L. gasseri</i>	4.5	8.9	11	8.9	3.6	-	-
<i>L. fermentum</i>	3.6	3.6	2.7	0.9	3.6	3.6	-
<i>L. paracasei</i>	0.9	0.9	2.7	5.4	15	7.1	22
<i>L. plantarum</i>	1.8	-	1.8	-	4.5	2.7	6.2
<i>L. delbrueckii</i>	-	-	-	-	0.9	0.9	4.6
<i>L. crispatus</i>	1.8	-	1.8	-	-	-	-
<i>L. reuteri</i>	-	-	-	-	1.8	0.9	-
<i>L. salivarius</i>	-	-	-	-	-	-	1.5
<i>L. mucosae</i> *	0.9	0.9	0.9	0.9	-	-	-

*One strain identified by partial sequencing of 16S rDNA

The *Lactobacillus* microbiota in infants in relation to lifestyle factors (I)

There were no significant differences in *Lactobacillus* colonisation rate or species distribution depending on delivery mode, nor did presence of siblings or pets in the household significantly influence colonisation. Because of the high breast-feeding rates in the first months of life, it was not possible to compare the *Lactobacillus* colonisation pattern between breast- and bottlefed infants during this period. At six months 22 % of the infants were weaned, and they harboured lactobacilli significantly less often (20 % vs. 52 %, $p < 0.01$) and had a tendency towards lower population counts ($10^{6.8}$ vs. $10^{8.8}$ CFU/g, $p = 0.18$) than did breastfed infants of the same age.

L. rhamnosus tended to be more common in vaginally than in sectio-delivered infants, but the difference was not significant. Colonisation by *L. gasseri*, which is a common species in the maternal vaginal microbiota [339], was not related to delivery mode. In infants still receiving breast milk at six months, *L. rhamnosus* was more commonly isolated (27 % vs. 4 %, $p < 0.05$) and tended to

reach higher population counts ($10^{8.9}$ vs. $10^{6.8}$ CFU/g, $p=0.19$) than in infants weaned by that age.

Most of the infants studied (89 %) had at least one allergic parent. However, the *Lactobacillus* colonisation pattern did not differ between children of atopic and children of non-atopic parents.

Lactobacillus colonisation frequency and species distribution in the oral microbiota of adult individuals with and without IgA deficiency (II)

To investigate the effect of secretory IgA on the *Lactobacillus* microbiota, we studied and compared the *Lactobacillus* microbiota of healthy and IgA deficient individuals.

Lactobacilli were isolated from the tongue of more than 80 % of control individuals, and the isolation frequency was only slightly lower in IgA deficient individuals. The various species isolated are shown in Table 1, paper II. *L. paracasei* and *L. gasseri* were the most common species in the oral microbiota of both IgA deficient and control individuals. The third most common species was *L. vaginalis* in IgA deficient persons and *L. fermentum* in controls. The latter species was significantly more common in the oral microbiota of controls as compared to IgA deficient individuals, while *L. vaginalis* tended to be more common in IgA deficiency. A large number of other species were also isolated, many of which were found in one or two individuals only (Table 1, paper II).

When comparing the *Lactobacillus* species distribution between symptomatic and asymptomatic IgA deficient individuals, *L. salivarius* was significantly more common in the oral cavity of the asymptomatic group. None of the individuals with a history of frequent respiratory infections carried this species.

Lactobacillus colonization frequency, population counts and species distribution in the gut microbiota of adult individuals with and without IgA deficiency (II)

Lactobacilli were isolated from faeces of more than 90 % of both IgA deficient and control individuals. The mean faecal *Lactobacillus* population counts were approximately 10^6 CFU/g in both groups, slightly lower in IgA deficient individuals than in controls. *L. paracasei* dominated by far in the faecal microbiota of both study groups, followed by *L. gasseri*, *L. plantarum* and *L. rhamnosus*. There were no significant differences between IgA deficient and control individuals regarding *Lactobacillus* species distribution in faecal samples. We also compared the faecal *Lactobacillus* species distribution between symptomatic ($n=23$) and asymptomatic ($n=10$) IgA deficient individuals, but no significant differences were observed.

Similarities and differences between the oral and faecal *Lactobacillus* microbiota of IgA deficient and control individuals (II)

A majority of individuals harboured lactobacilli in both oral and faecal samples. More than two thirds of these individuals had at least one species in common between the two locations and approximately one third had two or more species in common.

L. gasseri, *L. vaginalis* and *L. fermentum* were significantly more common in the oral cavity than in faeces. No individual was positive for *L. vaginalis* or *L. fermentum* in faeces without being positive also in the oral sample. The diversity of species also seemed to be somewhat higher in oral than in faecal samples (Table 1, paper 2). When analysing IgA deficient and control individuals together, oral samples yielded on average 2.4 different species, and the corresponding figure for faecal samples was 2.0.

Expression of mannose-specific adhesins by oral and faecal *Lactobacillus* from IgA deficient and control individuals (paper II)

The carbohydrate chains of SIgA are rich in mannose, and could possibly function as receptors for MS adhesins of lactobacilli. Such interactions could then in turn influence the *Lactobacillus* microbiota by favouring, or disfavouring, lactobacilli expressing MS adhesins at mucosal surfaces. To explore this, we examined the expression of MS adhesins by *Lactobacillus* isolates from IgA deficient and control individuals by testing their ability to adhere in a mannose-sensitive manner to HT-29 colonic epithelial cells. MS adhesins were more commonly expressed by faecal isolates from IgA deficient individuals than by faecal isolates from controls, but a corresponding difference between IgA deficient and control individuals was not observed in oral isolates (Fig. 2a, paper 2). There were no significant differences between IgA deficient and control individuals in the level of MS adherence (bacteria/cell) in adhesin positive isolates.

Among both IgA deficient and control individuals, MS adhesins were more common in oral than in faecal *Lactobacillus* isolates (Fig. 2a, paper II) and adhesin-positive isolates also displayed a higher average adherence (bacteria/cell) when isolated from the oral cavity (Fig. 2b, paper II).

Isolates of a large number of species expressed MS adhesins, including *L. fermentum*, *L. parabuchneri*, *L. vaginalis*, *L. plantarum*, *L. brevis*, *L. acidophilus*, *L. paracasei*, *L. salivarius*, *L. reuteri*, *L. gasseri*, *Lactobacillus hilgardii* and *Lactobacillus parafarraginis* (Table 2, paper 2). In total more than 80 % of isolates from both oral and faecal samples and in both study groups belonged to species with the capacity to express MS adhesins. Among the more commonly isolated species, MS adherence was absent in *L. rhamnosus* and *L. oris*.

Studies on probiotic effects of *L. plantarum* 299v (III, IV)

Characteristics of patients receiving *L. plantarum* 299v or placebo during treatment with antibiotics (III)

In this double blind placebo controlled trial, we investigated if intake of *L. plantarum* 299 could reduce the risk of diarrhoea or other gastrointestinal symptoms during treatment with antibiotics. Two hundred and thirty-nine patients, 93 men and 146 women, were initially included in the study, and randomised to daily intake of either a blueberry/oat drink with *L. plantarum* 299v (10^{10} CFU/day) or a blueberry/oat placebo drink. Seventy-three of the patients left the study without returning the study diary where symptoms and intake of test drink should be recorded, and were therefore excluded. In addition one patient was excluded because of cultivation of *Campylobacter jejunii* in blood, one because the diary was impossible to interpret, and one patient did not take the test drink. The excluded patients were equally distributed between the *L. plantarum* and placebo groups, and reasons for withdrawal did not differ between the study groups. Characteristics of patients who remained in the study and those who did not are presented in Table 1, paper III. The median age was significantly higher in patients who completed the study ($p=0.0015$).

Thus, 163 patients could be analysed and they were equally distributed between the *L. plantarum* and the placebo group (Table 2, paper III). The proportion of women tended to be higher in the *L. plantarum* group ($p=0.081$) and somewhat more patients in this group were positive for *C. difficile* on inclusion ($p=0.20$), but no clear differences between the study groups were observed regarding diagnoses, hospitalisation or antibiotics given (Table 2, paper III).

Side effects of the *L. plantarum* and placebo blueberry drinks (III)

Three patients in the *L. plantarum* group and three in the placebo group reported constipation during the study, all during intake of test drink. No serious side effects were recorded.

Effects of *L. plantarum* 299v on the incidence of diarrhoea in antibiotic treated patients (III)

The patients recorded symptoms, stool frequencies and intake of test preparation in a diary during the study. Diarrhoea, defined as at least three loose or watery stools per 24 hours for at least two consecutive days, was only diagnosed in eleven

patients during the entire study period, in six patients in the *L. plantarum* group and five in the placebo group.

When comparing the risk of diarrhoea and other symptoms between the study groups, we also divided the study period into three separate periods: the period of antibiotic treatment (A), the period of continued intake of test drink after discontinuation of antibiotics (B) and a follow-up period after intake of test drink (C) (Fig. 1, paper III). Four of 80 patients in the *L. plantarum* group and five of 83 in the placebo group had diarrhoea during treatment with antibiotics (period A) (OR 0.96; 95% confidence interval (CI) 0.18-4.7; $p=1.0$). During period B, with continued intake of test drink after antibiotic treatment, 3/71 vs. 0/69 patients had diarrhoea (OR not estimable, $p=0.17$), and during the follow-up period (C) 3/45 vs. 0/42 (OR not estimable, $p=0.25$).

Patients developing and those not developing diarrhoea are compared in Table 3, paper III. Treatment with more than one antibiotic and treatment with clindamycin, cephalosporines or ampicillin was more common among patients developing diarrhoea than among other patients.

Effects of *L. plantarum* 299v on gastro-intestinal symptoms not defined as diarrhoea in antibiotic-treated patients (III)

The risk of gastrointestinal symptoms not defined as diarrhoea was also compared between the study groups. The risk of developing loose or watery stools during the study was significantly lower in the *L. plantarum* group (OR 0.69; 95 % CI 0.52-0.92; $p=0.012$) (Table 4, paper III). The effect was significant during antibiotic treatment (period A) (OR 0.65; 95 % CI 0.45-0.94; $p=0.020$). There was a tendency towards an effect also after discontinuation of antibiotics, during continued intake of test drink (period B), but not thereafter (period C) (Table 4, paper III).

Patients in the *L. plantarum* group also had a lower risk of nausea than patients on placebo. When analysing the three study periods separately, the effect was significant during the period of antibiotic treatment, but not during the following periods (Table 5, paper III), possibly due to the lower number of patients included in the analysis of the two later periods.

Intake of *L. plantarum* did not alter the risk of abdominal pain, vomiting, hard stools, flatulence, or presence of blood in faeces (data not shown).

Women had significantly more nausea (OR 3.2; 95 % CI 1.6-6.2; $p=0.0006$) and abdominal pain (OR 2.9; 95 % CI 1.5-5.5; $p=0.0017$) than men during treatment with antibiotics (period A). There were no gender-specific differences for other symptoms (data not shown). All statistical analyses performed to compare the *L. plantarum* and the placebo groups were adjusted for age and gender.

C. difficile toxin in faeces of antibiotic-treated patients receiving *L. plantarum* 299v or placebo (III)

C. difficile toxin was found in four of 69 patients in the *L. plantarum* group and in one of 74 patient in the placebo group at baseline ($p=0.20$). Three of 74 patients in the *L. plantarum* group and three of 76 in the placebo group were positive for *C. difficile* toxin after antibiotic treatment. One of the eleven patients who developed diarrhoea was positive for *C. difficile* toxin on inclusion. The same patient was also the only one positive for *C. difficile* during diarrhoea. However, two of the patients developing diarrhoea did not provide samples for analysis at baseline, and only four delivered samples during diarrhoeal symptoms.

Characteristics of patients with non-typhoid salmonellosis receiving *L. plantarum* 299v or placebo (IV)

In this double-blind, placebo controlled trial we investigated if intake of *L. plantarum* 299v could accelerate clearance of *Salmonella* in faeces, and reduce infection-related symptoms in patients with non-typhoid salmonellosis. The patients were randomised to daily intake of 5×10^{10} CFU of freeze dried *L. plantarum* 299v or placebo. One hundred and fifty-four patients were initially enrolled in the study, five of whom were excluded as they had not had any symptoms from their *Salmonella* infection. Of the remaining patients, 46 % were male, and the median age was 36 years (range 5 – 68 years). Patient characteristics are shown in Table 1, paper IV. No significant differences between the *L. plantarum* and placebo groups were observed regarding concomitant diseases, medication at study start or recent intake of antibiotics or probiotics. Initial symptoms are shown in Table 2, paper IV. All but one patient reported diarrhoea, and a majority reported fever and abdominal pain at their first hospital visit, which occurred on average five days before inclusion in the study, and seven days after first onset of symptoms. Vomiting was more common as reported on inclusion among patients later randomised to receive placebo (Table 2, paper IV). Eighteen per cent of the patients were initially hospitalised.

Patients were asked if they wanted to participate in the study once it was clear that they had a stool culture positive for *Salmonella*. At inclusion, a new stool sample was collected. Eighty-five per cent of the patients, 83 % in the placebo and 87 % in the *L. plantarum* group, still had *Salmonella* in stools when entering the study.

Occurrence of symptoms at study start was defined as occurrence of the symptom on at least one day during the first week after inclusion in the study (Table 2, paper IV). All symptoms were less common at study start than at the first hospital visit. Loose stools were more common in the *L. plantarum* group at study start and the same tendency was observed for fever (Table 2, paper IV). Median

time from onset of symptoms to study start was eleven days in the group receiving *L. plantarum* and ten days in the placebo group. The most common *Salmonella* serotype was *S. Enteritidis* in both study groups (Table 3, paper IV).

Differences at study start between female and male patients with non-typhoid salmonellosis (IV)

We also analysed whether women and men differed in various patient characteristics and initial symptoms.

Median age was 37 years in female and 34 years in male patients. Women tended to have a history of gall bladder disease more often than men ($p=0.13$). Reported symptoms at the first hospital visit and symptoms at study start in female and male patients are shown in Table 4, paper III. Vomiting and abdominal pain were somewhat more common in women than in men before inclusion, but the differences were not significant. During the first week of the study, all symptoms were somewhat more common in female than in male patients, and significant differences were observed regarding nausea and abdominal pain. The differences remained when leaving out women reporting menstruation during the same time period.

Women in the *L. plantarum* group significantly more often had fever during the first study week than women in the placebo group.

Side effects of the freeze-dried *L. plantarum* 299v and placebo preparations (IV)

Urticaria was reported by one patient in the placebo group. Symptoms resolved quickly after discontinuation of intake of the placebo skim milk powder. Treatment with *L. plantarum* tended to increase some gastrointestinal symptoms, and these effects are presented below under treatment effects.

Effects of treatment with *L. plantarum* and influence of gender on clearance of *Salmonella* (IV)

Faecal samples were cultured for *Salmonella* at inclusion and once a week thereafter until four weeks after discontinuation of intake of the study preparation. Among patients positive for *Salmonella* at inclusion (85 %), time to clearance of *Salmonella* was studied with survival analysis. There was no significant difference in time to clearance of *Salmonella* between the *L. plantarum* and the placebo groups as analysed using the log rank test (Fig. 2a, paper IV).

Time to clearance was also compared between women and men. Women tended to clear *Salmonella* more quickly than men according to the survival analysis (Figure 2b, paper IV). The same tendencies regarding gender were seen when analysing separately patients receiving *L. plantarum* and patients on

placebo. Intake of *L. plantarum* did not affect time to clearance of *Salmonella* when analysing female and male patients separately (table 9, paper IV).

Effects of treatment with *L. plantarum* 299v and influence of gender on time to resolution of diarrhoea and other symptoms (IV)

Among patients who had diarrhoea at study start, time to resolution of diarrhoea was analysed by survival analysis and compared between groups using the log rank test. There was no significant difference between patients receiving *L. plantarum* and patients receiving placebo in time to resolution of diarrhoea (Fig. 3a, paper IV). However, men cleared diarrhoea within significantly shorter time than women (Fig. 3b, paper IV). This difference was observed also when analysing only patients on placebo. There was no effect of *L. plantarum* on time to resolution of diarrhoea when analysing female and male patients separately.

Time to cessation of various acute symptoms in patients receiving *L. plantarum* or placebo is shown in table 5, paper IV. There were no significant differences in time from study start to cessation of loose stools, nausea or abdominal pain between patients receiving *L. plantarum* or placebo as compared using the log rank test. In addition, there were no differences in time from study start to resolution of blood in stools, fever, or vomiting, but patients still having these symptoms at study start were too few for meaningful comparisons (Table 2, paper IV).

There were no significant differences between female and male patients in time to resolution of these symptoms (Table 6, paper IV), and no differences between the *L. plantarum* and the placebo group was observed when women and men were analysed separately.

Additional factors affecting time to clearance of *Salmonella* and resolution of diarrhoea (IV)

To identify factors influencing time to clearance of *Salmonella* and resolution of diarrhoea we used survival analysis and log rank tests to compare patients positive or negative for a specific factor of possible importance. Higher age, history of diverticulitis, and vomiting at study start were associated with *Salmonella* clearance, and diarrhoea, fever, and abdominal pain were associated with prolonged carriage in univariate analyses. In the multivariate analysis (Cox regression), diarrhoea at study start, associated with longer *Salmonella* carriage, was the only factor which remained significant. There was a non-significant tendency for vomiting to shorten *Salmonella* carriage.

Male sex and higher age were associated with shorter time to resolution of diarrhoea in univariate analyses. Only gender remained significant in the multivariate analysis.

Effects of treatment with *L. plantarum* and influence of gender on symptoms after clearance of *Salmonella* (IV)

We also analysed the effect of treatment with *L. plantarum* 299v on symptoms occurring after clearance of *Salmonella* from stools, as gastrointestinal symptoms commonly persist for some time after the initial infection. In the *L. plantarum* group, the proportion of days with hard stools was lower than in the placebo group, but the proportion of days with fever was increased. Also, the risk of abdominal pain tended to be higher in the *L. plantarum* group (Table 7, paper IV).

The risk of experiencing symptoms after clearance of *Salmonella* was also compared between women and men, revealing that women had a larger proportion of days with loose stools, nausea, abdominal pain and flatulence (Table 8, paper IV). The difference in abdominal pain was primarily observed among women receiving *L. plantarum*. Comparing the effect of *L. plantarum* in women and men separately, women in the treatment group reported more days with fever and abdominal pain than women in the placebo group. Men in the *L. plantarum* group had a higher proportion of days with diarrhoea, but fewer days with hard stools than men in the placebo group (Table 8, paper IV).

DISCUSSION

The microbiota of the alimentary tract is a complex system with interactions microbe to microbe as well as between microbes and host. As the bacteria residing within us affect our health and wellbeing, ways of influencing the microbiota are being increasingly explored. Lactobacilli are believed to have health-promoting effects and are commonly used as probiotics. There is still much to be learned about the normal *Lactobacillus* microbiota and factors of importance for its composition.

In the present thesis, the *Lactobacillus* microbiota in infants and adults was studied. The effects of the probiotic strain *Lactobacillus plantarum* 299v on the prevention of gastrointestinal symptoms caused by antibiotic treatment and on the course of non-typhoid salmonellosis were also examined.

Lactobacillus colonisation frequency, population counts and species distribution in healthy infants

In study I faecal *Lactobacillus* populations were studied in healthy infants from the age of one week to 18 months. Lactobacilli never dominated in the gut microbiota, and the frequency of infants colonised by lactobacilli did not exceed 45 % at any time. The low colonisation rates are in line with other culture-based studies, where the majority found similar or lower rates [60, 61, 77, 340]. Higher colonisation rates have been reported occasionally [78, 79]. The differences could relate to the different methods used for culture and detection of lactobacilli in different studies. We found bifidobacteria to be very common, which is confirmed by other studies where bifidobacteria dominate over lactobacilli in this age group [61]. Bifidobacteria also grow well on the supposedly *Lactobacillus*-specific medium Rogosa agar. This could contribute to difficulties in the isolation of lactobacilli from this medium, since they may be overgrown by bifidobacteria. However, most lactobacilli form large colonies on Rogosa agar, which distinguish them from bifidobacteria.

A recent study from Sweden, which used non-culture-based methods for the identification of lactobacilli in faecal samples reported higher colonisation rates in infants than those reported here [341]. Some lactobacilli are difficult to culture, and may therefore be more easily found with non-culture based methods. However, it is also possible that there are true differences in *Lactobacillus* colonisation between different infant populations. For instance lactobacilli are more common in Ethiopian and in Estonian children than in Swedish infants [172, 173, 342], possibly reflecting different exposure to these bacteria.

There were no significant differences in *Lactobacillus* colonisation rates between the vaginally and sectio delivered infants studied here. Several other studies have found that vaginally delivered infants are more likely to harbour lactobacilli in the early gut microbiota [78, 163, 164], whereas some find no difference [66, 343]. In most studies the difference in colonisation rate between sectio and vaginally delivered infants disappear within one or two weeks, and the studies finding equal *Lactobacillus* colonisation rates regardless of delivery mode have mostly studied infants at later time points only. The infants followed here were first sampled at one week of age, and it is possible that a significant difference would have been revealed, had we sampled the children a few days earlier. It seems likely that some vaginally delivered infants do pick up lactobacilli from the maternal vagina. This has been documented in Japanese infants, where approximately 20 % of vaginally delivered infants acquired maternal vaginal *Lactobacillus* strains [77]. However, only a few infants retained these strains until one month of age. Thus, vaginal lactobacilli are not likely to influence colonisation rates at later time points. Also, the fact that sectio delivered infants catch up so quickly regarding *Lactobacillus* colonisation rate indicates that other sources are more important for the acquisition of these bacteria.

Differences between sectio and vaginally delivered infants in *Lactobacillus* colonisation could also relate to antibiotic treatment of mothers giving birth by caesarean section, or different practices in postpartum routines for sectio delivered children. For instance, in some countries all mothers receive antibiotics before sectio [163], whereas in other countries, *e.g.* Sweden, it is mainly used in emergency sections. Antibiotics administered to the mother during delivery may pass to the infant and influence the early colonisation pattern [344]. There may also be differences between countries and hospitals in *i.e.* how quickly a sectio delivered infant is handed over to the mother, which could influence the colonisation rate. Since lactobacilli are common in the oral flora of adults, they could easily be transferred to the neonates through close contact. Furthermore, differences in which other bacteria first colonise the gut may influence the establishment of lactobacilli.

Close contact with other individuals, as well as contact with animals could influence the acquisition of lactobacilli. In the infants studied here, there were no significant differences in *Lactobacillus* colonisation pattern in relation to presence of siblings or pets, although there were tendencies towards higher colonisation frequencies in infants in households with older brothers or sisters an/or household animals.

Some studies have found higher counts and/or colonisation rates of lactobacilli in the faeces of breastfed infants [345-347], but most studies report no difference or even more lactobacilli in bottle-fed infants [75, 79, 176, 348, 349]. The Swedish infants examined here were mostly breastfed exclusively until at least four months of age. At six months, when 25 infants were completely weaned, lactobacilli were

significantly more often isolated from infants still receiving breast milk. At the same age, population counts reached a maximum of $10^{8.8}$ CFU/g faeces in breast-fed as compared to $10^{6.8}$ CFU/g faeces in bottle-fed infants. These differences between breast- and bottlefed infants could indicate that infants are supplied with lactobacilli from maternal milk, as further discussed below. It could also be that lactobacilli are favoured in the gut milieu of breastfed infants. It is unclear why results regarding infants studied here differ from most other studies comparing breast- and bottlefed infants. It could relate to the fact that the Swedish infants here were compared at six months of age whereas the majority of other studies compared breast- and bottlefed infants at younger ages. Also, the influence of breast milk on the gut milieu and colonisation by lactobacilli may depend on other bacteria in the microbiota, which may differ between different infant populations studied.

Regarding *Lactobacillus* species distribution, we found *L. gasseri* and *L. rhamnosus* to dominate in the early phase of infancy. The finding of early colonisation by *L. gasseri* is in agreement with several other studies [77, 80]. *L. rhamnosus* was not found to dominate in the majority of earlier studies of infants from other countries, e.g. the Netherlands, Japan or Scotland [77, 80, 176, 346], but was common in a recent study of Greek infants [164]. Also another recent study of Swedish infants found *L. rhamnosus/L. paracasei* in faeces of around 50 % of infants during the first two months of life [341]. *L. casei/paracasei* and *L. rhamnosus* are sometimes difficult to distinguish, which may lead to different results in different studies. The studies mentioned above, which found *L. rhamnosus* in very few infants, did find *L. casei/paracasei* to a somewhat higher extent [77, 80, 176, 346]. It is also possible that colonisation by *L. rhamnosus* has become more common in mothers in some countries in recent years, and therefore in infants, due to that *L. rhamnosus* has become a commonly used probiotic species in e.g. dairy products. True differences can also relate to that the early microbiota exhibits large interindividual variation [350], and the composition is likely to influence which bacteria are able to establish.

In the Swedish infants studied here, infants still breastfeeding at six months were more often colonised with *L. rhamnosus* than weaned infants, indicating that breastfeeding favoured colonisation by this species. Thus, the differences found between countries in colonisation rates of *L. rhamnosus* could also be related to different feeding patterns in early infancy.

L. gasseri and *L. rhamnosus*, as well as *L. fermentum*, which was also sometimes present in faeces of the infants studied here, may all be found in breastmilk [52, 351]. Thus, if breastmilk is a source of lactobacilli colonising infants, this could explain the dominance by this species in breastfed infants. Colonisation by *L. gasseri* was not more prevalent in breastfed than in weaned infants at six months in the present study, but strains of *L. gasseri* with identical RAPD patterns have been

isolated from breast-milk and infant faeces [51, 351], which indicates that breast milk could be the source also for at least some of the *L. gasseri* bacteria found in infants. The origin of bacteria isolated from human milk is not clear. Some authors claim that lactobacilli are transported by macrophages from the gut of the mother to the breast tissue [352], but this has not been proven. Another possibility is that lactobacilli present in breast milk are simply transferred from the mouth of the infant. Although lactobacilli are normally not part of the early oral microbiota [74] [55], they may well be transiently present within infants' mouths.

L. gasseri is common in the vaginal flora of fertile women [49-51, 353] and, as mentioned, vaginal lactobacilli have been found to transiently colonise newborn infants [77]. However, another study found that few lactobacilli from the vagina of the mother were present in infant faeces [51] which is in line with our study where *L. gasseri* was no more common in vaginally delivered than in infants delivered by caesarean section. In addition, *L. gasseri*, *L. rhamnosus* and *L. fermentum* are common colonisers of the mouth and gastrointestinal tract of adults [30, 36, 37] and could easily be transferred from the parents through handling and kisses.

The majority of the infants studied here had at least one atopic parent. Parental allergy did not significantly influence the colonisation pattern. Atopy in the parents does not seem to play an important role in infant intestinal colonisation pattern in general [62].

The *Lactobacillus* colonisation pattern was very different at six months as compared to at one year of age, with colonisation rates and population counts being highest at six months and reaching its lowest points at 12 months of age. It is likely that the shift is related to changes in the gut milieu occurring with weaning, making it less favourable for lactobacilli. It is also possible that less lactobacilli are consumed in the first period after weaning than during breastfeeding, if lactobacilli are ingested with the breastmilk.

From 12 months, *L. paracasei* started to be the most prevalent *Lactobacillus* species, and it became even more common at 18 months, followed by *L. delbrueckii*, *L. plantarum* and *L. acidophilus*. These species are found in foods, e.g. cheese (*L. paracasei*), fermented vegetables (*L. plantarum*) and milk products (*L. delbrueckii* and *L. acidophilus*). *L. paracasei* has previously been reported to dominate in Swedish 18-month old children [342] and is a common member of the adult gut microbiota [30, 37], as is *L. plantarum* [30, 38].

The faecal Lactobacillus microbiota of healthy adults

In study II, adult individuals with and without IgA deficiency were cultured for oral and faecal lactobacilli. In this section, the findings of faecal lactobacilli in healthy adult individuals with normal IgA levels are discussed.

Lactobacilli were detected in more than 90 % of the individuals. It is possible that the true *Lactobacillus* colonisation rate is almost 100 % in the gut microbiota of adults. Indeed, a recent non-culture based study using real time quantitative PCR with a sensitivity of 10^2 to 10^4 cells / g faeces for the detection of lactobacilli, found lactobacilli in 98 % of faecal samples from healthy adults [30]. We found a mean population count in adults of around 10^6 CFU/g faeces. Numbers similar to ours are commonly reported in recent both culture based and non-culture based studies [30, 37].

In the faecal *Lactobacillus* microbiota of adults, *L. paracasei* was the most commonly isolated species, being present in more than 40 % of the individuals studied, and *L. gasseri* and *L. plantarum* were also quite common. These findings are in agreement with another recent culture-based study [37]. The non-culture-based study by Matsuda *et al.* found *L. fermentum* in 60 % [30], which is a considerably higher isolation frequency than we found. Since *L. fermentum* grows readily on Rogosa plates we do not believe that methodological differences explain our comparatively lower isolation rates, but rather that this reflects a difference between the populations studied. Japanese and Swedes differ in *e.g.* eating habits, which could possibly affect the *Lactobacillus* microbiota.

Several early studies identified *L. acidophilus* as the predominant faecal *Lactobacillus* species in adults [73, 178]. Many of these bacteria probably belonged to the species *L. gasseri*, as these two species are not distinguished by the classical phenotypic identification methods used in early studies [354].

Comparison between infants and adults regarding faecal lactobacilli

Since we used much the same methodology to isolate and identify lactobacilli in the studies involving infants and adults, the results may be directly compared. The faecal *Lactobacillus* microbiota differs between infants and adults in colonisation frequency, population counts and species distribution.

In our studies, adults were much more frequently colonised with lactobacilli in faeces than were infants in their first 18 months of life. Thus, the acquisition of lactobacilli as a component of the gut microbiota may occur after the first 18 months in many infants.

In adults, a large number of species were isolated, many of which were found in one or two individuals only. Possibly, the larger species diversity in adults is related to a more varied food intake than in infants, as some of the species isolated from adults originate in food not consumed early in life, like marinated fish and meat products (*L. alimentarius*) [355] and wine (*L. hilgardii*) [356]. Furthermore, commercial baby foods are often completely devoid of bacteria, including lactobacilli (Annika Ljung, personal communication). However, minor methodological differences between our two studies probably also have a role. Seven infants had lactobacilli in their stools reacting with primers specific for *Lactobacillus* group III or IV, but not with any of the primers specific for these groups included in the multiplex PCR. These isolates were only sequenced in one case, and were identified as *L. mucosae*. The rest were not speciated. In adults, there were several isolates especially in group IV not reacting with primers in the multiplex PCR. Most of these were identified as species which were isolated from very few individuals, but *L. parabuchneri*, *L. ruminis*, and *L. vaginalis* were each found in the faeces of around 10% of individuals. Of these, at least *L. ruminis* [256] and *L. vaginalis* [77] have been found in infant faeces. It is possible that infant *Lactobacillus* isolates which were not speciated belonged to these species.

It is also possible that the large number of bifidobacteria growing on Rogosa plates inoculated with infant faecal samples hid some lactobacilli. Certain *Lactobacillus* species, e.g. *L. ruminis*, and *L. parabuchneri*, are difficult to culture on Rogosa agar [34], and an appearance as very small colonies or a presence of very few colonies may have contributed to that they were overlooked in the infant faecal cultures. Thus, it is possible that also infants harbour a larger variety of species than revealed in our studies, especially at the older ages when starting to consume a more varied diet.

The *Lactobacillus* species distribution differed over time in infants, and *L. rhamnosus* was clearly more prevalent in early infant faecal samples than in the adult faecal microbiota. As discussed above, we believe that this dominance is related to breastfeeding. The dominance of *L. paracasei* from twelve months of age and also in adults could possibly indicate that the *Lactobacillus* microbiota becomes more adult-like by one year of age.

While a higher number of adults harboured lactobacilli, colonised infants had higher *Lactobacillus* count in their first six months of life. A probable reason for the higher counts in young infants is that they have a less complex microbiota which allows bacteria that reach the intestine to expand in a way that the more complex microbiota of adults does not. Also, the gut milieu of breast fed infants could possibly favour the proliferation of certain lactobacilli, as discussed above, and as previously described [52, 351]. The sharp decline in *Lactobacillus* counts in infants after weaning could support this.

Persistence of lactobacilli in the gut

In our longitudinally followed infants, we typed lactobacilli to the strain level and could thus follow individual strains in the microbiota over time. A single *Lactobacillus* strain persisted for at least three weeks in 17 % of the infants. In a study of Japanese infants only 2/86 infants harboured the same strain of lactobacilli at five days and one month of age [77]. Thus, persistent gut colonisation by *Lactobacillus* strains appears not to be common in infancy. In our study, strains that persisted in the infant gut belonged to the species that were most common in the microbiota: *L. rhamnosus*, followed by *L. gasseri*, *L. paracasei* and *L. fermentum*. One infant harboured *L. mucosae* for seven weeks. These persistent strains are likely to be true colonisers of the infant gut.

There are few studies of intestinal persistence of lactobacilli also in adults. In a study of three healthy adults, gut persistence of *L. gasseri*, *L. delbrueckii* and *L. vaginalis* was found [34]. Faecal samples in our study of adults were only obtained once, and persistence of lactobacilli could therefore not be tested. It is likely, however, that adults harbour a higher number of persistent strains, as the adult microbiota is more stable, and it seems as if several *Lactobacillus* species are able to persist for some time in the gut.

Are the findings representative?

In our studies of the intestinal *Lactobacillus* microbiota of infants and adults we analysed faecal samples only. It is possible that lactobacilli isolated from biopsies, representing the mucosa-associated *Lactobacillus* microbiota, are the lactobacilli with the most influence on the host. A number of studies have found considerable differences between the microbiota in biopsies as compared to faeces [3, 20], whereas others have not [21]. In a study of rectal biopsies *L. plantarum* found in 29% of healthy adult volunteers was most common, whereas *L. paracasei* and *L. gasseri* were found much less frequently than in our study of adults [31]. This could of course also relate to differences between the populations studied. However, it is likely that all lactobacilli reaching the intestine in significant amounts are able to influence its host [241, 254]

Our studies of the *Lactobacillus* microbiota in infants and adults, respectively, were both culture based. With initial culturing, bacteria which are difficult to culture may be missed. Many non-culture based studies are based on a very low number of individuals, which do not readily allow comparisons. The previously mentioned study by Matsuda *et al.* [30] reported a somewhat higher *Lactobacillus* isolation rate in faecal samples than what we found in healthy adult individuals, and especially higher colonisation frequencies of individual species, including *e.g.* *L.*

plantarum/pentosus identified in 80 % and *L. fermentum* in 60% of the 40 healthy adults studied [30]. However, much the same species were found as in our study.

The oral microbiota of healthy adults

In our study of adult individuals, we also investigated the oral *Lactobacillus* microbiota.

Lactobacilli were isolated from the tongue of more than 80 % of healthy adults. There are few studies of *Lactobacillus* colonisation rate from this location, but a study of healthy individuals aged 9- 28 years found a *Lactobacillus* colonisation frequency of 42 % in tongue samples [32]. The same study found lactobacilli in the saliva of only 28 %, as compared to 100 % in a study of adults [357]. Thus oral *Lactobacillus* colonisation frequencies differ considerably between studies as well as between different oral niches. There are several factors associated with higher numbers of oral lactobacilli, e.g. increasing age [9], smoking [358], presence of foreign materials in the mouth [359], increased carbohydrate intake [360], intake of *Lactobacillus* containing food, and the presence of caries [361]. The impact of these factors was not analysed in our study.

L. paracasei and *L. gasseri* were most common in tongue samples of adults, present in about 40 % each. This differs from another study of lactobacilli isolated from the same location where *L. plantarum* was most common, found in 45 %, and *L. casei/paracasei* was found in only 14 %. In the same study, *L. gasseri* was not identified, but could have been present in the group “acidophilus-like” isolated from 7 % [31]. However, others have found *L. paracasei* and *L. gasseri* to be common in other niches of the oral cavity [36, 37].

Which lactobacilli are found in the oral microbiota may be influenced by recent food intake, since lactobacilli ingested by foods are likely to reside in the mouth for some time, even if not colonising. Like *Lactobacillus* colonisation frequencies and numbers, species distribution is also likely to be influenced by a number of factors as described in the previous paragraph.

Since we only sampled individuals once, we do not know which lactobacilli could have been persistent oral colonisers. However, oral persistence of strains of at least *L. fermentum* and *L. vaginalis* has been reported previously [34].

Differences and similarities between the faecal and oral Lactobacillus microbiota of healthy individuals

L. gasseri and *L. fermentum* were significantly more common in the oral cavity than in faeces of adult individuals. No individual was positive for *L. fermentum* or

L. vaginalis in faecal samples while negative in the oral sample. This could indicate that the presence of these species in the gut is dependent on their presence in the oral cavity, and in saliva, which is a constant source of lactobacilli reaching the intestine. However, persistence of a *L. gasseri* strain in the gut, but not in the oral cavity, was described in two of three individuals studied by Dal Bello and co-workers [34], indicating that the gut microbiota may harbour at least this species independent of its presence in the oral microbiota.

There were great similarities between the oral and faecal microbiota at the species level. More than two thirds of individuals positive for lactobacilli in both the oral and faecal microbiota had at least one *Lactobacillus* species in common in these two locations. However, the variety of species was somewhat broader in the mouth, both collectively and individually, which could reflect a higher isolation rate of food-derived lactobacilli in the mouth. Others have also found that the same species often inhabit both the oral cavity and the gut [34, 37]. In Dal Bello's study of three healthy adults all three harboured several *Lactobacillus* strains in both saliva and faeces. These strains belonged to the species *L. gasseri*, *L. paracasei*, *L. rhamnosus* and *L. vaginalis* [34], *i.e.* similar species as identified in both faecal and oral samples in the present study. *L. fermentum* was found in two of the three individuals and only in the oral cavity [34].

Influence of IgA deficiency on the Lactobacillus microbiota

IgA deficiency is the most common primary immune deficiency in humans. IgA is normally abundant in the gut and in the oral cavity in the form of secretory IgA, and therefore it is possible that a lack of S-IgA could influence the composition of the *Lactobacillus* microbiota. However, the rate of colonisation by lactobacilli, as well as the species distribution in oral and faecal samples, was very similar in persons with and without IgA deficiency. The only significant difference was that *L. fermentum* was more common in the oral cavity of controls as compared to IgA deficient individuals. *L. fermentum* is a major species in the oral microbiota of healthy individuals, but is also common in carious lesions [9]. Lactobacilli are today mainly regarded as secondary invaders in the caries process [24], although some still consider them to be important cariogens [362]. Some *Lactobacillus* species have been found to be associated with a lower risk of caries. Even certain *L. fermentum* strains may be beneficial, as for instance a *L. fermentum* strain isolated from children without dental caries inhibited the formation of biofilm by the cariogenic bacterium *Streptococcus mutans* [363]. We found no difference in *L. fermentum* colonisation rates in the faecal flora between IgA deficient and control individuals, which was quite low in both groups.

Lactobacillus population counts in faeces did not differ significantly between healthy and IgA deficient individuals, although a weak trend towards lower counts

in the latter group was observed. According to animal studies, S-IgA has a greater influence on the small intestinal than on the colonic microbiota [364]. Therefore it is possible that differences in *Lactobacillus* population counts between IgA deficient and control individuals would have been found had we examined small intestinal fluid from these individuals.

L. salivarius was isolated less often from the oral cavity of IgA deficient individuals with a history of frequent respiratory tract infections than from oral samples of IgA deficient individuals without this history. Several studies have shown that *L. salivarius* has antibacterial activities against bacterial pathogens, e.g. *Streptococcus pneumoniae* [35].

The expression of mannose-specific adhesins by lactobacilli from IgA deficient and control individuals

In addition to the classical antigen-antibody interactions between S-IgA and bacteria, the carbohydrate chains of S-IgA may also function as receptors for certain bacterial adhesins. The carbohydrate chains of S-IgA are rich in mannose, and *E. coli* with mannose-binding type 1 fimbriae have been found to bind to S-IgA [133, 191, 192]. Furthermore, intestinal *E. coli* isolates from healthy individuals express more type 1 fimbriae than isolates from IgA-deficient individuals. This suggests that the interaction between type 1 fimbriae and S-IgA may be of advantage for the *E. coli* in the gut [191, 192].

Many *Lactobacillus* species are also able to express mannose-specific (MS) adhesins [196, 200, 202]. In our study of IgA deficient and healthy individuals, we examined the ability of each *Lactobacillus* isolate to express MS adhesins by testing their ability to adhere to HT-29 colonic epithelial cells in a mannose-sensitive manner. Lactobacilli are known to adhere to several structures of epithelial cells, mucus, and extracellular matrices in the gut [365]. It is possible that MS adhesins of lactobacilli are able to interact with mannose-containing oligosaccharides on S-IgA. Our hypothesis was that lectin-carbohydrate interactions between lactobacilli expressing MS adhesins and S-IgA could be of advantage for lactobacilli, for instance, binding to S-IgA in the mucus layer could facilitate colonisation of this habitat. However, it is also possible that binding to S-IgA could be disadvantageous for lactobacilli, since it would prevent direct contact with epithelial cells [130].

The expression of MS adhesins by intestinal lactobacilli was more common in IgA deficient persons than in controls. Thus, in the presence of IgA, expression of MS adhesins seems to be disadvantageous for lactobacilli in the gut, possibly by S-IgA trapping lactobacilli in the mucus layer and preventing their association with the epithelium, which may be most advantageous for the bacteria. However, we did not

find a difference in expression of MS adhesins between oral *Lactobacillus* isolates from IgA deficient and healthy individuals, respectively. IgA, including secretory IgA, exists in two subclasses, IgA1 and IgA2. Secretory IgA2 is somewhat more common in colonic secretions, while relatively more S-IgA1 is found in saliva [366]. The IgA2 subclass expresses much more mannose than IgA1 [134], and this could possibly influence the ability to interact with MS adhesins of lactobacilli. The lack of differences between IgA deficient and healthy individuals regarding MS adhesion of oral lactobacilli could indicate that the MS adhesins of lactobacilli interacted with S-IgA2 but not with S-IgA1.

We also found that MS adhesins were significantly more common among oral than faecal *Lactobacillus* isolates, especially in healthy individuals. Isolates expressing MS adhesins also adhered in higher numbers when originating in the mouth, possibly supporting that this adherence specificity is beneficial for lactobacilli in the oral cavity. Adhesion of lactobacilli in this location is less well studied than intestinal adhesion, but lactobacilli have been found to bind to structures in saliva [205-207] as well as to buccal epithelial cells [205].

MS adhesins were expressed by isolates from a large number of *Lactobacillus* species, including *L. plantarum*, *L. salivarius*, *L. johnsonii*, *L. paracasei* and *L. fermentum*, in which the expression has been demonstrated previously [31, 196, 200, 202]. It was also found in several additional species in our study, and more than 80 % of all isolates from IgA deficient and healthy individuals belonged to species with the capacity to express MS adhesins. Thus, the ability to express MS adhesins is a common trait in lactobacilli.

Are lactobacilli in the gut microbiota important for health?

The fact that lactobacilli are not numerous in stools does not preclude that they are an important part of the intestinal microbiota. Lactobacilli may preferentially colonise the small intestine, and adhere to the mucosa there [367]. It is clear that suppression of the commensal microbiota may lead to disease such as *C. difficile* enteritis, that lactobacilli are common in the gut microbiota of healthy individuals and that they are able to influence *e.g.* the immune system, the epithelium as well as other gut bacteria. Lactobacilli produce short chain fatty acids (SCFA), which are believed to be beneficial for the host in several ways [226, 227, 295]. Certain *Lactobacillus* species have been associated with a lower risk of colon cancer [178].

As previously described, lactobacilli are commonly used as probiotics, with a large number of claimed effects. In some cases there is good evidence, but in many others further studied are needed to confirm the effects.

L. plantarum 299v for the prevention of diarrhoea and other gastrointestinal symptoms associated with antibiotic treatment

We performed a randomised placebo controlled trial where we examined the ability of the probiotic strain *L. plantarum 299v* to prevent diarrhoea and other gastrointestinal side effects during and after treatment with antibiotics.

We found a low incidence (6.7 %) of antibiotics associated diarrhoea (AAD), defined as at least three loose or watery stools a day for at least two consecutive days. The incidence was still within the limits of previously reported figures of 5-35 % [236] and slightly higher than in a large prospective Swedish study which found AAD in 4.9 % [240]. One explanation for the lower incidence in Sweden as compared to some other countries could be the preferred use of narrow-spectrum antibiotics in Sweden. Patients treated with clindamycin, a cephalosporin or an ampicillin derivate tended to have diarrhoea more often than other patients, which is in line with previous studies [237, 368]. We also found an increased risk of AAD with treatment with more than one antibiotic, which is also known before [240, 369]. Another contributing factor to the low incidence of AAD in our study is probably that the prevalence of known risk factors such as enteral feeding or comorbidities was low among the patients studied.

We did not find any protective effect of *L. plantarum 299v* against diarrhoea, as strictly defined. However, we did find a significantly reduced risk of loose stools during the study in patients receiving *L. plantarum 299v*. This effect was most obvious during antibiotic treatment and lost after the discontinuation of intake of test drink. Thus, *L. plantarum* seems to have some preventive effects on milder antibiotic associated intestinal disturbances. The fact that the effect on loose stools was observed only as long as the probiotic was administered indicates that continuous intake of the probiotic is required for beneficial effects. Why there was an effect against loose stools, but not against diarrhoea is not clear. It is likely that the reasons for diarrhoea, as strictly defined, differs from the aetiology of the milder symptom loose stools. A significant part of the diarrhoeal cases may have been caused by overgrowth by potential pathogens in the microbiota, whereas loose stools could result *e.g.* from minor disturbances in the microbiota, or other disturbances induced by the antibiotic. For instance, *L. plantarum* has been shown to reduce negative effects of antibiotics on colonic fermentation [241].

The risk of experiencing nausea was also reduced among patients receiving *L. plantarum*. The mechanisms behind this effect are not clear. A recent study showed that treatment with a mixture of *L. acidophilus* and *B. longum* reduced nausea in individuals with stress-induced gastrointestinal symptoms [370].

Effects of L. plantarum 299v on Clostridium difficile toxin in faeces after antibiotic treatment

Few patients harboured toxin producing *C. difficile* after having been treated with antibiotics. Several studies have shown a much higher prevalence of toxin producing *C. difficile* with antibiotic treatment, including the large Swedish study by Wistrom and co-workers [239, 240]. *C. difficile* is a spore forming bacterium, and the spores are able to persist on surfaces like the floor for several months [371], and may spread with aerial dissemination [372]. They are easily transmitted between patients and have been isolated from the hands of hospital staff [373]. One reason for the lower isolation rate of toxin-producing *C. difficile* in our study may thus be that almost 50 % were outpatients. Furthermore, all the patients in our study were recruited from an infectious diseases clinic, while the patients in the study by Wistrom *et al.* were admitted to wards within several medical specialities. It is possible that different hygiene routines, and/or fewer patients per room at the infectious diseases clinic may have contributed to less spread of *C. difficile*. Furthermore, the patients in our study were younger than in the study by Wistrom and co-workers, and they had fewer concomitant diseases, and interventions like endoscopy and abdominal surgery were less common. These factors may influence colonisation by *C. difficile*

There was no difference between patients receiving *L. plantarum* and those receiving placebo in the colonisation by toxin-producing *C. difficile*. As the total number of individuals positive for *C. difficile* toxin was low, it is not possible to draw any far-reaching conclusions from this. There are previous studies which found no influence on the number of clostridia after the administration of *L. plantarum 299v* [309, 374], and in one study there was even an increase in total *Clostridium* counts after the administration of this strain [375]. However, *C. difficile* was not studied specifically in these studies, and *Clostridium* is a genus containing a variety of highly differing species which are not likely to be influenced by probiotics in a similar way. Another previous study has shown reduced colonisation by *C. difficile* in critically ill patients receiving *L. plantarum 299v* [316]. In a small study of recurrent *C. difficile* enteritis, *L. plantarum 299v* combined with metronidazole tended to be more effective than only metronidazole in clearing the infection [280].

Lactobacillus strains have so far mainly been tried in very small studies or case studies for the treatment of infections caused by *C. difficile* [280, 281, 376]. The probiotic yeast *Saccharomyces boulardii* has been found to have a significant effect against recurrent, but not initial *C. difficile* associated diarrhoea [377] and also to prevent recurrences of *C. difficile* enteritis in patients with more severe disease only [282]. Whether also certain lactobacilli could have better effects against more severe *C. difficile* infections is not clear, but it is possible that probiotics in general

are more efficient in conditions where larger derangements of the gut microbiota could allow more efficient colonisation by the probiotic strain.

L. plantarum 299v for the treatment of non-typhoid salmonellosis

In a second randomised placebo controlled study, we tested if intake of *L. plantarum 299v* could shorten the time of *Salmonella* carriage and ameliorate symptoms in non-typhoid *Salmonella* infection. There was no positive effect at all of *L. plantarum 299v* administered to patients diagnosed with non-typhoid *Salmonella* gastroenteritis neither on time to clearance of *Salmonella* from stools, nor on resolution of acute symptoms. The lack of effect was not due to under-powering and after *Salmonella* clearance, loose stools and even diarrhoea seemed to be more frequent in patients receiving *L. plantarum*.

Patients had had symptoms of their *Salmonella* infection for a median of eleven days when entering the study. One possible way by which *L. plantarum* could counteract *Salmonella* infection could be through inhibition of adherence of *Salmonella* to epithelial cells. Both *L. plantarum* and *Salmonella* may adhere by binding to mannose-containing receptors. However, it is not clear if they can adhere to the same receptors. Adherence of *Salmonella* is important early in the infection [90], and thus it is possible that very early administration of *L. plantarum 299v* could have had some positive effects. *L. acidophilus* has been found *in vitro* to prevent attachment of *Salmonella Pullorum*, and *L. fermentum* has been shown to inhibit attachment to some extent of *Salmonella Typhimurium* to epithelial cells, but both lactobacilli were unable to displace already attached *Salmonella* bacteria [301]. *Salmonella* is likely to be the causative agent in a significant part of cases of travellers' diarrhoea, but studies using probiotics to prevent this condition have not been conclusive [378].

Why *L. plantarum* seemed to somewhat increase the risk of loose stools and diarrhoea after clearance of *Salmonella* is not clear. However, *L. plantarum* also seemed to decrease the risk of experiencing hard stools, and the same mechanism may be responsible for these effects. *L. plantarum* has previously been shown to be effective in treatment of constipation [379], and a trend towards reduction of constipation in IBS patients through intake of *L. plantarum 299v* has also been found [286]. In a study in pigs, *L. plantarum 299v* decreased jejunal net fluid absorption [380]. Further, propionate may play a role. This short chain fatty acid (SCFA) which was found to increase after intake of *L. plantarum 299v* [308] can induce colonic muscular contraction in rats, which may counteract constipation [228].

While it seems as if some probiotics, *i.e.* *S. boulardii* and *L. rhamnosus GG*, may be more effective in the prevention of AAD than *L. plantarum* [270, 277], it is not

known whether other probiotics would be more effective against *Salmonella* carriage and symptoms, since very few studies involving humans have been performed. *Lactobacillus acidophilus* was used in one study and was shown to shorten the duration of the *Salmonella* carrier state in asymptomatic patients [300]. This lack of studies of probiotic treatment against *Salmonella* infection in humans could be a result of publication bias, where studies of probiotic treatment in *Salmonella* infections have been performed but not published due to lack of effect.

It could be worth trying a *Lactobacillus* strain which has been shown to have effect against *Salmonella* in animals, but it is not at all certain that such a strain would have positive effects against salmonellosis in humans.

Patients in our study were infected by a number of different *Salmonella* subtypes, *Salmonella* Enteritidis being most common. It is possible that different probiotics are to prefer against different serotypes of *Salmonella*. *In vitro*, *L. acidophilus* was found to prevent the attachment of *S. Pullorum* to ileal epithelial cells, but not the attachment of *S. Typhimurium* [301].

Why did L. plantarum decrease the number of loose stools during antibiotic treatment, but not during Salmonellosis?

There was no effect of *L. plantarum* 299v against diarrhoea in either of our two studies discussed here. However, the probiotic did reduce the risk of loose stools in the study of antibiotic-treated patients, whereas there was no such effect in the study of patients with non-typhoid salmonellosis, where the number of days with loose stool and also with diarrhoea after clearance of *Salmonella* instead tended to be higher in patients receiving *L. plantarum* than in patients on placebo.

The patient groups receiving the probiotic differed between the two studies – the first consisting of antibiotic treated patients with various infectious diseases, the second of patients with non-typhoid salmonellosis. Thus, it is not surprising that the effect of the probiotic differed between the studies.

The dose of the probiotic strain was lower in the study of patients on antibiotics, 10^{10} CFU/day compared to the study of patients with salmonellosis (5×10^{10} CFU/day) and also compared to other studies using *L. plantarum* 299v, where $2-5 \times 10^{10}$ CFU/day has often been used [280, 286]. It could be that a higher dose would have contributed to a reduction not only of loose stools, but also of diarrhoea in the antibiotics-treated patients. However, it is also possible that the increased risk of loose stools observed with intake of this probiotic strain by patients with salmonellosis was a side effect of too high a dose in the *Salmonella* study.

The time when administration of the probiotic is started could be very important. In the study on antibiotic-treated patients, when *L. plantarum* 299v had some positive effect against loose stool and nausea, the probiotic was given prophylactically to patients who had not yet developed any gastrointestinal symptoms, whereas the patients with salmonellosis had already had gastroenteritis for more than a week in most cases.

L. plantarum was administered differently in the two studies, in a fruit drink in the study of antibiotic-treated patients and as a freeze dried powder in the study of patients with salmonellosis. The viability of bacteria was checked for both preparations. The powder contained skim milk, but patients with known hypersensitivity to milk products were not included in the study. However, both blueberries and oats, which were parts of the fruit drink, are known to have an effect on the gut. Oat is rich in starch and β -glycan and may exert effects by itself in a way similar to so called prebiotics. A prebiotic is defined as "a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, and thus improves health" [381]. An animal study found an increase in short chain fatty acids and bifidobacteria, and a decrease in faecal pH and coliforms when comparing an oat-based with an oat-free diet [382]. An *in vitro* study of oat bran fermentation by a mixed bacterial culture found an increase of bifidobacteria and lactobacilli, and a decrease in clostridia [383]. Studies on the effect of blueberries are scarce, but they have been shown *in vitro* to have an effect against the pathogen *Giardia duodenalis* [384]. Blueberries have also been found to decrease counts of caecal *Enterobacteriaceae*, and also to decrease disease activity, bacterial translocation and inflammation in a rat colitis model [385]. Thus, it is possible that oat and blueberries consumed together with *L. plantarum* 299v by patients on antibiotics, modified the mechanisms whereby *L. plantarum* 299v contributed to an increased risk of loose stool after clearance of *Salmonella* in patients with salmonellosis.

The prevalence of nausea was lower in the *L. plantarum* group in the study of patients on antibiotics, but tended to be higher with *L. plantarum* treatment in the *Salmonella* study. This difference could relate to the factors discussed above, e.g. differences between populations studied, different doses of the probiotic and different formulas.

Gender-related findings in the studies of treatment with L. plantarum 299v

The influence of gender on the outcomes measured was studied in both treatment studies and several gender-related differences were found, especially in the study of patients with salmonellosis, where the influence of gender was also more thoroughly investigated. In the study involving antibiotic-treated patients, women

had significantly more nausea and abdominal pain than men during treatment with antibiotics. The same symptoms were more pronounced in women as compared to men at study start in the study of patients with salmonellosis. The differences in the latter study remained when excluding women reporting menstruation at that time, and there were no significant differences between women and men before the onset of *Salmonella* which could explain the findings. Thus, it seems that women are more prone to suffer from abdominal pain and nausea during gastrointestinal infection and antibiotic treatment. Gastric emptying is slower in women, which may be related to differences in autonomic tone [386]. This could possibly partly explain the increased symptoms in women.

Women tended to clear *Salmonella* more rapidly than men, but had a longer diarrhoeal phase. There are few studies of gender differences in salmonellosis, but a recent study describes increased *Salmonella*-related morbidity in females as compared to males in their mid-adult years [387]. A possible explanation could be that women exert a stronger inflammatory response than men, which clears the bacteria but increases symptoms. However, in contrast to a previous study [388], we found a positive correlation between fever and time to *Salmonella* clearance, and prolonged carriage was also associated with longer duration of gastrointestinal symptoms. Furthermore, asymptomatic carriers of *Salmonella* mostly clear the bacteria within a short period of time [89]. Thus, a more symptomatic response is not related to a shorter period of *Salmonella* carriage.

Female mice clear *S. Enteritidis* significantly faster than male mice. The gene *Slc11a1* has been found to be of importance in the early defence in salmonellosis, but also enhances persistence of these bacteria [389]. However, the gender related differences in time to clearance in mice were not dependent on the presence of this gene [390].

The IL12/IFN- γ pathway is believed to be very important for the control of *Salmonella* [391] and deficiencies in this pathway are associated with severe *Salmonella* infections. Testosterone has been found to increase IL-10 production in mice [392] and antigen presenting cells from female mice secrete IL-12, but not IL-10, during activation, whereas the opposite is true for male mice [393]. Other studies, however, found no differences [394]. During the first four weeks after *Salmonella* clearance in our study, when the study preparations were still administered, women had a higher risk of loose stools, nausea and flatulence than men. Women also had more abdominal pain than men, but this was mainly due to a higher risk of abdominal pain in women in the *L. plantarum* group.

We also analysed the effect of *L. plantarum* in women and men separately. Women taking *L. plantarum* had more fever and abdominal pain than women on placebo. The increased risk of fever that was noted in women in the *L. plantarum* group may be related to the fact that women in this group significantly more often had fever at

study start. We cannot explain the increased risk of abdominal pain among women receiving *L. plantarum*. This strain has previously been shown to reduce abdominal pain in patients with irritable bowel syndrome [286].

Men in the *L. plantarum* group had more diarrhoea and tended to have more loose stools than men in the placebo group, but the most evident effect was a decreased risk of hard stools in men receiving *L. plantarum*. There were no significant differences between men in the *L. plantarum* and placebo groups regarding these symptoms at inclusion. Possible reasons for why *L. plantarum* could be effective in the treatment of constipation were discussed above. However, it is unclear why these effects of *L. plantarum* were observed in men only.

Persistence of gastrointestinal symptoms after salmonellosis may in some patients represent early stages of post-infectious irritable bowel syndrome (IBS) [86]. IBS is more common in women than in men [86] and may be associated with low grade inflammation in the gut [395]. Reduced IL-10 production has been found in irritable bowel syndrome [396, 397], and gender related differences in the balance between IL-10 and IL-12 could play a role in the more prolonged gastrointestinal symptoms observed in women.

In conclusion, lactobacilli are part of the normal oral and faecal microbiota of the majority of adults, but most infants are not stably colonised in the gut by these bacteria. Lactobacilli are believed to be beneficial for health and are commonly used as probiotics. However, in our studies on the probiotic effects of *L. plantarum* 299_v, this strain was not found to be of major benefit in the prevention of antibiotic associated diarrhoea or in the treatment of non-typhoid salmonellosis. Given the different properties of different lactobacilli down to the strain level, it is very possible that other probiotic bacteria, or combination thereof, would prove to be more useful. The most interesting findings in the treatment studies, especially in the study of patients with *Salmonella* gastroenteritis, were the gender related differences in symptoms, some of which were related to intake of *L. plantarum* 299_v and some which were not.

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