Lactobacillus characterization and effects on oral biofilm composition

Nelly Romani Vestman
To my family

“Life isn’t about waiting for the storm to pass...
It’s about learning to dance in the rain”

Vivian Green
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## ACKNOWLEDGMENTS

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Abstract

The human body is home for millions of bacteria. The largest microbial community is located in the gastro-intestinal (GI) tract, including the oral cavity with >700 identified taxa. Lactobacillus, which is normal inhabitant of the GI tract, contributes to health by possible biofilm and immune modulation. Breast milk is a claimed source for transmittance of Lactobacillus to infants’ GI tract, but there is limited information if breastfeeding affects lactobacilli in the oral cavity. The objectives of Papers I and II of this dissertation were to compare infant oral microbiota by feeding mode, and to characterize oral lactobacilli including potential probiotic properties of the dominant Lactobacillus species.

Two cohorts with a total of 340 healthy 3- to 4-month-old infants were investigated. Saliva and oral mucosal swab samples were collected. Bacteria were characterized by culture-dependent and -independent methods, including 16S rRNA genes sequencing, quantitative PCR, and the Human Microbe Identification Microarray (HOMIM). Inhibition of growth and adhesion were also tested.

Multivariate modeling of HOMIM-detected oral bacteria clustered breastfed infants separately from formula-fed infants, and linked breastfed infants to a more health-associated flora. Lactobacilli were essentially detected in breastfed infants only. Lactobacillus gasseri was most prevalent out of six identified Lactobacillus species. Infant isolates of L. gasseri bound to saliva gp340 and MUC7 and adhered to gingival epithelial cells. Infant isolates also inhibited adhesion of Streptococcus mutans to saliva-coated hydroxyapatite, and inhibited growth of S. mutans, Streptococcus sobrinus, Actinomyces naeslundii, Actinomyces oris, Candida albicans and Fusobacterium nucleatum in a concentration-dependent fashion.

Papers III and IV aimed to assess persistence of probiotic Lactobacillus reuteri, if persistence is necessary for a regrowth of mutans streptococci (MS), and if L. reuteri intake affects oral microbiota composition.

Two well-documented L. reuteri strains (DSM 17938 and PTA 5289) were used in two double-blind, randomized controlled trials. In the first, 62 subjects (test=32, placebo=30) with high counts of MS were exposed to L. reuteri for 6 weeks. Exposure followed full-mouth disinfection with chlorhexidine. In the second study, 44 healthy subjects (test=22, placebo=22) consumed the L. reuteri for 12 weeks. Saliva and biofilm samples were collected before, during and up to 6 months after exposure. Analyses included culture, strain-specific PCR and 454-pyrosequencing targeting the hypervariable region V3-V4 of the 16S rRNA gene.
*L. reuteri* test strains were detected in the mouth of approximately two thirds of test participants during intake. However, their presence decreased gradually when consumption stopped. Subjects with detectable *L. reuteri* had slower regrowth of MS compared to non-carriers.

Pyrosequencing yielded a total of 812,547 high-quality sequencing reads. Firmicutes, Proteobacteria, Bacteroidetes, Fusobacteria and Actinobacteria were the major bacterial phyla recovered. Exposure to *L. reuteri* strains did not affect overall phylotype abundance, but multivariate modeling clustered 12-week-treated test subjects separately from those who received placebo. Exposure to the test strains was strongly associated with presence and increased levels of *F. nucleatum* and *Streptococcus* spp.

In conclusion, the oral microbiota differed by feeding mode in infants. One third of breastfed infants had lactobacilli in the mouth, while only single formula-fed infant had it. *L. gasseri*, predominant in infants, displayed probiotic characteristics *in vitro*. Retention of probiotic *L. reuteri* was a prerequisite for delay of MS regrowth after disinfection. However, probiotic bacteria may not be beneficial for all, since *L. reuteri* DSM 17938 and PTA 5289 were retained in only 2 of 3 consumers. Finally, the altered microbiota after 12 weeks consumption of *L. reuteri* indicates that intake of probiotic bacteria, or at least *L reuteri*, has an impact on oral ecology. However, this finding needs further investigation.
Sammanfattning på svenska


Laktobaciller förekommer i många hälsoprodukter med påstådd probiotisk effekt. Andra delen av denna avhandling syftade till att bedöma om intag av tabletter med den probiotiska arten Lactobacillus reuteri påverkar ekologin i mikrofloran i munnen, om arten etablerar sig hos alla vid exponering, och om etablering är nödvändig för probiotisk effekt (mätt som hämmad återväxt av kariesassocierade mutansstreptokocker efter antimikrobiell behandling). Två stammar L. reuteri (DSM 17938 and PTA 5289) användes i två dubbelblinda, randomiserade studier. I båda studierna intog deltagarna i testgruppen tabletter med L. reuteri-stammarna och de i kontrollgruppen identiska tabletter utan bakterier. I den första studien deltog 62 deltagare (32 test, 30 kontroll) i 6 veckor och i den andra 44 personer (22 test, 22 placebo) under 12 veckor. Saliv och biofilmsprover samlades in vid

Sammanfattningsvis visar dessa studier att amning är associerad med att ha probiotiska laktobaciller i munnen men bara vissa etablerar arten i munnen. Hos vuxna försenade *L. reuteri* återkolonisation av mutansstreptokocker efter antibakteriell behandling, och påverkade ekologin i bakteriefilmerna i munnen. Även hos vuxna ledde exponering till etablering bara hos vissa individer.
List of Publications

This dissertation is based on the following papers, which are referred in the text by their roman numbers (I-IV):


IV. **Nelly Romani Vestman**, Tsute Chen, Pernilla Lif Holgerson, Carina Öhman, Ingegerd Johansson. 454 Pyrosequencing characterization of the oral microbiota after 12-week supplementation with *Lactobacillus reuteri* DSM 17938 and PTA 5289. Manuscript.

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### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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</thead>
<tbody>
<tr>
<td>CFU</td>
<td>Colony forming units</td>
</tr>
<tr>
<td>CHX</td>
<td>Chlorhexidine</td>
</tr>
<tr>
<td>C-section</td>
<td>Caesarean section</td>
</tr>
<tr>
<td>ECC</td>
<td>Early childhood caries</td>
</tr>
<tr>
<td>FITC</td>
<td>Fluorescein isothiocyanate</td>
</tr>
<tr>
<td>FMD</td>
<td>Full-mouth disinfection</td>
</tr>
<tr>
<td>GI</td>
<td>Gastro-intestinal</td>
</tr>
<tr>
<td>HA</td>
<td>Hydroxyapatite</td>
</tr>
<tr>
<td>HOMD</td>
<td>Human oral microbiome database</td>
</tr>
<tr>
<td>HOMIM</td>
<td>Human microbe identification microarray</td>
</tr>
<tr>
<td>H$_2$O$_2$</td>
<td>Hydrogen peroxide</td>
</tr>
<tr>
<td>LAB</td>
<td>Lactic acid bacteria</td>
</tr>
<tr>
<td>MFGM</td>
<td>Milk fat globule membrane</td>
</tr>
<tr>
<td>MRS</td>
<td>The Man, Rogosa and Sharpe's agar</td>
</tr>
<tr>
<td>MS</td>
<td>Mutans streptococci</td>
</tr>
<tr>
<td>OTU</td>
<td>Operational taxonomic unit</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative PCR</td>
</tr>
<tr>
<td>RCT</td>
<td>Randomized control trial</td>
</tr>
<tr>
<td>RFLP</td>
<td>Restriction fragment length polymorphism</td>
</tr>
<tr>
<td>S-layer</td>
<td>Surface layer</td>
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</table>
Introduction

The human gastro-intestinal (GI) microbiota

The human body is home to microbial ecosystems (microbiota) whose genomic content is estimated to be 100-fold greater than the human genome (Wilson, 2009). Indeed, only 10% of the cells in our body are of human origin. The largest microbial community is located in the gastro-intestinal (GI) tract, including the oral cavity, esophagus, stomach and the intestines (Tancrede, 1992). The human microbiota play a crucial role for health with influences on physiology, immune system, and metabolism (Aziz et al., 2013; Cho & Blaser, 2012).

The current opinion is that approximately 10 phyla are found in the GI tract, with dominance of Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria and Fusobacteria, and genera Bacteroides, Eubacterium, Clostridium, Ruminococcus, Bifidobacterium, Lactobacillus and Streptococcus (Eckburg et al., 2005; Tap et al., 2009). However, a core microbiome with species in the genera Bacteroides, Prevotella and Ruminococcus are found in most subjects (Arumugam et al., 2011), and then “personal” patterns with anatomical site and time variations are found (Costello et al., 2009).

The oral microbiota consist of protozoa, yeast, mycoplasma, archea and bacteria, and the bacteria are most abundant. Among the 600-700 bacterial taxa found in the oral cavity, the phyla Firmicutes, Bacteroidetes, Proteobacteria, Actinomyces and Fusobacteria comprise >95% of detected species (Dewhirst et al., 2010). Some species are described as “true” commensal bacteria, such as Streptococcus sanguinis, Streptococcus mitis, Streptococcus oralis, Actinomyces naeslundii, Fusobacterium nucleatum, Haemophilus parainfluenzae, Eikenella corrodens and some species of Prevotella (Aas et al., 2005; Kilian et al., 2006), whereas others may be opportunistic, such as the mutans streptococci (MS) (Loesche, 1986). According to Aas et al. (2005), most sites in the oral cavity harbor 20-30 species, and the number of species per subject ranges from 34 to 72. However, the reported number of species per subject may increase with use of newer techniques (Zaura et al., 2009).

In a similar fashion to the GI tract, the oral cavity offers distinct niches due to varying environmental conditions (Figure 1). Segata et al. (2012) compared biofilm composition at seven oral sites, and found three distinct types: (i) at the buccal mucosa, gingiva and hard palate, (ii) in saliva and at the tongue, and (iii) supra- and subgingival dental biofilms. In general, the oral microbiota exist in a harmonious relationship with the host, but this can be distorted and disease can occur, such as dental caries or periodontitis.
Development of the GI microbiota

It is commonly accepted that the GI tract of a fetus is essentially sterile and that bacteria appear within hours after birth (Cho & Blaser, 2012). The infant GI microbiota approach that of adults after about 1 year of age (Palmer et al., 2007). Culture studies indicate that in the first week of life the GI tract of most infants is colonized by facultative anaerobes, such as enterobacteria (Eschericia coli), enterococci (Enterococcus faecalis and Enterococcus faecium), alpha hemolytic streptococci and coagulase-negative staphylococci (Adlerberth & Wold, 2009; Rotimi & Duerden, 1981). As the facultative anaerobic bacteria multiply and oxygen is consumed an anaerobic environment develops and strict anaerobic bacteria, such as Bifidobacterium, Clostridium and Bacteroides, are facilitated (Rotimi & Duerden, 1981). DNA-based evaluations have confirmed the picture, but have also added information about non-cultivable anaerobes, such as ruminococci (Del Chierico et al., 2012; Palmer et al., 2007).

In the oral cavity, mucosal surfaces, such as the tongue and the buccal and palatal epithelium, offer colonization sites in early infancy. In addition,
saliva provides binding receptors, nutrients, enzymes, antibodies and innate immunity components. These factors form a basis for bacteria growth and adhesion (Scannapieco, 1994).

Development of the indigenous oral microflora begins with colonization by facultative anaerobic genera, such as viridans streptococci (S. mitis and Streptococcus salivarius) (Carlsson et al., 1970a; Kononen, 2000; Smith et al., 1993) and Actinomyces (Sarkonen et al., 2000). These initial colonizers open up for subsequent colonizers by environmental modifications and species-species interactions (Kolenbrander & Andersen, 1986). Hence, anaerobic Veillonella spp. and the Prevotella melaninogenica group are commonly detected slightly later (between 2 and 6 months of age), as is F. nucleatum (key bacteria in coaggregation) (Kolenbrander & Andersen, 1986; Kolenbrander, 2000), Phorphyromonas catoniae, non-pigmented Prevotella spp. and Leptotrichia spp. (Kononen, 1999; Kononen, 2000) (Figure 2).

At tooth eruption, saliva-coated hard tissues offer new epitopes for bacteria attachment. This novel situation leads to colonization of health-associated S. sanguinis around the age of 9 months (Carlsson et al., 1970b; Caufield et al., 2000). S. sanguinis are associated with delayed colonization of Streptococcus mutans (Caufield et al., 2000), and is more prevalent in situations with healthy teeth (Becker et al., 2002). When initial acquisition of S. mutans occurs is debated. Caufield et al. (1993) suggest a window of infectivity for S. mutans at a median age of 26 months (range 9-44 months), whereas others report that S. mutans may be acquired at any time from around 6 months to 3 years of age (Tanner et al., 2002; Wan et al., 2001;
Wan et al., 2003). Tanner et al. (2002) reported that S. mutans was present in 55% of tooth biofilm samples and 70% of tongue scrapings from 6 to 18-month-old children. Timing of S. mutans colonization is clinically relevant because early colonization correlates with more caries later (Kohler et al., 1988).

**Factors influencing infant colonization**

Establishment of the GI microflora in early infancy is influenced by: (i) maternal factors, such as transmission during delivery, feeding, gestational age and maternal illness; (ii) environmental factors, such as antibiotic, prebiotic and probiotic consumption, and (iii) host factors, including geno- and phenotypical traits (Figure 3) (Adlerberth & Wold, 2009; Cho & Blaser, 2012; Marques et al., 2010). Maternal factors are probably most influential due to contacts during birth, feeding and nursing. Interestingly, infants whose mothers clean the pacifier by licking, develop an oral microflora that is associated with less allergy (Hesselmar et al., 2013), and children, whose mothers have high levels of cariogenic MS acquire those bacteria at younger age and in higher numbers than when mothers have low levels (Kohler et al., 2003).

![Figure 3. Schematic drawing of factors that may influence transmission and GI colonization.](image)
**Mode of delivery**

Vaginally delivered infants have been reported to have more diverse GI microbiota while infants born by caesarean section (C-section) have higher numbers of *Clostridium difficile* and delayed acquisition of *Bacteroides, Bifidobacterium* and *E. coli* in the GI tract (Adlerberth & Wold, 2009; Penders et al., 2006). Dominguez-Bello et al. (2010) showed that children born vaginally acquired GI bacteria mimicking their mother’s vaginal microbiota with dominance of *Lactobacillus, Prevotella* or *Sneathia* spp. In contrast, infants delivered by C-section harbored bacteria similar to those found on skin, with dominance of *Staphylococcus, Corynebacterium*, and *Propionibacterium* spp. It has been reported that infants born by C-section have increased risk of asthma, obesity and type 1 diabetes (Neu & Rushing, 2011).

In the **oral cavity**, mode of delivery has been more sparsely studied. Li et al. (2005) concluded that infants delivered by C-section acquired MS 11.7 months earlier than vaginally delivery infants. Nelun Barfod et al. (2011) reported that the prevalence of health-related streptococci and lactobacilli were significantly higher in vaginally-delivered infants than in C-section delivered infants. In line with this, Lif Holgerson et al. (2011) found that the microarray detected microbiota differed distinctly between vaginally-delivered and C-section-born infants at 3-months of age. In contrast to these results, Pattanaporn et al. (2013) found that after adjustment for mother’s gestational age, MS score, feeding practice, child’s age and tooth brushing habits, both levels of MS and early childhood caries (ECC) were higher in vaginally-born than C-section-delivered children. Thus, more studies are needed to elucidate the association between type of delivery on the oral microbiota composition and disease outcome.

**Mode of feeding**

Breastfed infants are reported to harbor significantly higher counts in the GI tract of *Bifidobacterium* and *Lactobacillus* and lower counts of *Bacteroides, Clostridium coccoides* group, *Staphylococcus*, and Enterobacteriaceae compared with formula-fed infants (Bezirtzoglou et al., 2011; Fallani et al., 2010; Harmsen et al., 2000). Diet was also strongly influential on microbiota variation when development of the intestinal microbiota was followed longitudinally from birth to 2.5 years of age (Koenig et al., 2011). During the latest decade, it has been claimed that breast milk harbors a natural bacterial inoculum, including staphylococci, streptococci, micrococi, lactobacilli, enterococci, lactococci and bifidobacteria (Cabrera-Rubio et al., 2012; Martin et al., 2007; Martin et al., 2009; Ward et al., 2013). Further, it has been shown that lactobacilli from milk of healthy mothers, *i.e. Lactobacillus*
gasseri and Lactobacillus fermentum have in vitro characteristics in line with defined probiotic strains (Martin et al., 2005).

Little is known about the effect of type of infant feeding on colonization of the oral cavity. Studies on the relation between type, pattern and frequency of feeding, and S. mutans acquisition and development of ECC are partly inconclusive. On the one hand, human milk contains fermentable lactose. On the other hand, it contains innate immunity peptides/proteins, which affect bacterial metabolism. Thus, milk inhibits adhesion of S. mutans (Wernersson et al., 2006) and promotes adhesion of commensal Actinomyces oris (own unpublished data) to saliva-coated hydroxyapatite (HA) in vitro. This suggests protection against establishment of cariogenic species. Thus, to date there is no consensus on the association between breastfeeding and caries risk (Ribeiro & Ribeiro, 2004; White, 2008). The present dissertation contributes knowledge on the effect on the oral microbiota by mode of infant feeding.

The genus Lactobacillus

Lactobacilli are Gram-positive, non-spore-forming, catalase negative, aerotolerant or anaerobic, acid tolerant organisms with a low DNA G+C content (Lebeer et al., 2008). The genus Lactobacillus belongs to the phylum Firmicutes, class Bacilli, order Lactobacillales, and family Lactobacillaceae (http://www.ncbi.nlm.nih.gov/taxonomy). Lactobacilli are members of the lactic acid bacteria (LAB) since their main end product from carbohydrate metabolism is lactic acid. They are the largest genus in the LAB group with over 100 species reported (Canchaya et al., 2006; Claesson et al., 2007). However, the number of species continuously changes due to reclassifications and identification of new species. At the time of writing (August 2013) the Lactobacillus genus contained 172 species (NCBI taxonomy database).

The natural habitats of lactobacilli span from plants, dairy and meat products, sourdough breads, and fermented foods to various niches in animals and humans. Lactobacilli are part of human’s normal microflora in the oral cavity (Ahrne et al., 2005; Kõll-Klais et al., 2005), vagina (Antonio et al., 1999), stomach (Ryan et al., 2008), small intestine (Molin et al., 1993), large intestine (Ahrne et al., 1998; Molin et al., 1993; Reuter, 2001), and milk (Martin et al., 2003). The interest in lactobacilli is basically due to (i) their suggested health-promoting properties, (ii) that inclusion in foods improves nutritional and other quality aspects, and (iii) their pronounced lactic acid formation.
In the oral cavity, lactobacilli are traditionally associated with caries development and progression (Byun et al., 2004; van Houte, 1980). This is partly based on their ability to generate low pH from carbohydrate fermentation and to tolerate low pH, and partly that they are found in elevated numbers in caries-diseased subjects. Nevertheless, lactobacilli have lately been recognized for potential beneficial traits in a general and oral perspective (Goh & Klaenhammer, 2009). Lactobacilli as probiotics will be reviewed later in this chapter.

Establishment and colonization

Information on lactobacilli establishment in the GI tract of neonates is contradictory. Some report low GI colonization rates in infancy (Balmer & Wharton, 1989; Ellis-Pegler et al., 1975; Matsumiya et al., 2002), while others find lactobacilli in considerably higher quantities (10⁷–⁹ CFU/g) in infant feces (Ahrné et al., 2005; Hall et al., 1990; Kleessen et al., 1995). Chen et al. (2007) detected lactobacilli in all fecal samples of Chinese neonates using qPCR. Ahrné et al. (2005) found that lactobacilli could be cultured from 21% of stool samples from 1-week-old Swedish infants. The frequency increased to 45% by 6 months of age, dropped to 17% by 12 months and increased again by 18 months of age. In the first 2 months Lactobacillus rhamnosus and L. gasseri were most common. At 6 months of age L. rhamnosus was most abundant followed by Lactobacillus paracasei, Lactobacillus plantarum, L. fermentum and L. gasseri (Ahrné et al., 2005).

In the oral cavity lactobacilli have been detected by culture in early infancy (Carlsson & Gotheors, 1975). Thus, at the ages 34 days and 7 months, 24 and 47% of the infants, respectively, harbored lactobacilli (Plonka et al., 2012). Thereafter the numbers decreased. Some authors find that lactobacilli do not become regularly present in the mouth until the age of 2 to 3 years, and then only in approximately half of the children and at rates varying from 2x10³ to 4x10⁴ CFU/mL of saliva (Carlsson et al., 1975; Köhler et al., 1984). Most studies find an association between total number of lactobacilli and caries status. Interestingly, Kanasi et al. (2010) reported that in pre-school children L. gasseri, L. fermentum, and Lactobacillus vaginalis were associated with childhood caries, whereas Lactobacillus acidophilus, L. rhamnosus and Lactobacillus reuteri were inversely associated with caries.

In adults, lactobacilli form a minor portion of both the intestinal (Dal Bello et al., 2003; Kimura et al., 1997; Lebeer et al., 2008) and oral (Marsh & Martin, 1999) microbiota. Thus, in adults Lactobacillus constitutes around 0.01-0.6%, and 1% of the total bacterial counts in feces and the oral cavity,
respectively. Common *Lactobacillus* species identified from healthy human are described in Table 1.

**Table 1.** *Lactobacillus* species found at different human sample sites.

<table>
<thead>
<tr>
<th>Saliva</th>
<th>Feces</th>
<th>Milk</th>
<th>Vagina</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L. acidophilus</em></td>
<td><em>L. acidophilus</em></td>
<td><em>L. oris</em></td>
<td><em>L. crispatus</em></td>
</tr>
<tr>
<td><em>L. casei</em></td>
<td><em>L. casei</em></td>
<td><em>L. casei</em></td>
<td><em>L. gasseri</em></td>
</tr>
<tr>
<td><em>L. crispatus</em></td>
<td><em>L. crispatus</em></td>
<td><em>L. crispatus</em></td>
<td><em>L. iners</em></td>
</tr>
<tr>
<td><em>L. fermentum</em></td>
<td><em>L. curvatus</em></td>
<td><em>L. fermentum</em></td>
<td><em>L. jensenii</em></td>
</tr>
<tr>
<td><em>L. gasseri</em></td>
<td><em>L. delbrueckii</em></td>
<td><em>L. gasseri</em></td>
<td></td>
</tr>
<tr>
<td><em>L. oris</em></td>
<td><em>L. gasseri</em></td>
<td><em>L. gastricus</em></td>
<td></td>
</tr>
<tr>
<td><em>L. paracasei</em></td>
<td><em>L. paracasei</em></td>
<td><em>L. plantarum</em></td>
<td></td>
</tr>
<tr>
<td><em>L. plantarum</em></td>
<td><em>L. plantarum</em></td>
<td><em>L. reuteri</em></td>
<td></td>
</tr>
<tr>
<td><em>L. rhamnosus</em></td>
<td><em>L. reuteri</em></td>
<td><em>L. rhamnosus</em></td>
<td></td>
</tr>
<tr>
<td><em>L. salivarius</em></td>
<td><em>L. ruminis</em></td>
<td><em>L. salivarius</em></td>
<td></td>
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<tr>
<td></td>
<td><em>L. sakei</em></td>
<td><em>L. vaginalis</em></td>
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</tbody>
</table>

1 Data from Teanpaisan and Dahlén (2006) and Kõll-Klais et al. (2005).
2 Data from Heilig et al. (2002) and Walter et al. (2001).
3 Data from Jeurink et al. (2013).
4 Data from Vásquez et al. (2002).

**Lactobacillus taxonomy**

Originally, lactobacilli were organized into three groups based on phenotypic characteristics: (i) homofermentative species with exclusive lactic acid production via the glycolytic Embden-Meyerhof pathway, *e.g.* *L. acidophilus* and *L. gasseri*; (ii) heterofermentative species which produce lactic acid, acetic acid, formic acid, carbon dioxide and ethanol via the phosphoketolase pathway, *e.g.* *L. plantarum* and *L. casei*; (iii) facultative heterofermentative species, which use both pathways, *e.g.* *L. fermentum* and *L. reuteri* (Kandler, 1983). This classification has expanded by molecular assessments (Collins et al., 1991; Singh et al., 2009). To the best of my knowledge, the phylogenetic structure published by Salvetti et al. (2012) is the latest. They recognized 15 lactobacilli groups based on 16S rRNA gene sequence alignment (Table 2). Note that species belonging to the same cluster do not always share phenotypic characteristics. Although the 16S rRNA gene is considered as the gold standard for molecular taxonomy and widely used to characterize a bacterial community, it should be emphasized that 16S rRNA genes identification may be insufficient if closely related species are evaluated. Thus, in some cases, 16S rRNA genes sequencing analysis is restricted to group classification.
Table 2. Phylogenetic groups of lactobacilli according to Salvetti et al. (2012), with examples of Lactobacillus species in each group.

<table>
<thead>
<tr>
<th>Phylogenetic group</th>
<th>Lactobacillus species</th>
</tr>
</thead>
<tbody>
<tr>
<td>L. delbrueckii</td>
<td>L. delbrueckii, L. acidophilus, L. gasseri, L. iners, L. johnsonii</td>
</tr>
<tr>
<td>L. salivarius</td>
<td>L. salivarius, L. agilis, L. animalis</td>
</tr>
<tr>
<td>L. reuteri</td>
<td>L. reuteri, L. fermentum, L. oris, L. vaginalis</td>
</tr>
<tr>
<td>L. buchneri</td>
<td>L. buchneri, L. otakiensis, L. parabuchneri</td>
</tr>
<tr>
<td>L. alimentarius</td>
<td>L. alimentarius, L. crustorum, L. farcininis</td>
</tr>
<tr>
<td>L. brevis</td>
<td>L. brevis, L. hampmesii, L. koreensis</td>
</tr>
<tr>
<td>L. collinoides</td>
<td>L. collinoides, L. kimchicus, L. odoratitofui</td>
</tr>
<tr>
<td>L. fructivorans</td>
<td>L. fructivorans, L. florum, L. homohichioi</td>
</tr>
<tr>
<td>L. plantarum</td>
<td>L. plantarum, L. paraplantarum, L. pentosus</td>
</tr>
<tr>
<td>L. sakei</td>
<td>L. sakei, L. curvatus, L. fuchuensis, L. graminis</td>
</tr>
<tr>
<td>L. casei</td>
<td>L. casei, L. paracasei, L. rhamnosus</td>
</tr>
<tr>
<td>L. coryniformis</td>
<td>L. coryniformis, L. bifermantans, L. renini</td>
</tr>
<tr>
<td>L. manihotivorans</td>
<td>L. manihotivorans, L. nasuensis, L. porcinia</td>
</tr>
<tr>
<td>L. perolens</td>
<td>L. perolens, L. harbinensis, L. shenzhenensis</td>
</tr>
<tr>
<td>L. vaccinostercus</td>
<td>L. vaccinostercus, L. oligofermentans</td>
</tr>
</tbody>
</table>

Lactobacillus identification techniques

A variety of biochemical and molecular identification tools have been proposed for lactobacilli identification. The classic approach using carbohydrate fermentation patterns such as API 50 CH strips (BioMérieux Inc, Marcy l’Etoile, France) is today suggested as a complement to molecular genetic techniques with superior discriminatory power (Boyd et al., 2005). Table 3 lists methods commonly used for human lactobacilli identification, including (i) denaturing gradient gel electrophoresis (DGGE), (ii) sequence analysis of 16S rRNA or other genes, (iii) restriction fragment length polymorphism (RFLP), (iv) multiple locus sequencing typing (MLST), (v) pulse-field gel electrophoresis (PFGE), (vi) randomly PCR amplified fragment pattern (RAPD), and (vii) quantitative polymerase chain reaction (qPCR). No method satisfies all situations, and method(s) selection should be adjusted to purpose.
Table 3. Approaches for identification of *Lactobacillus*.
Abbreviations are explained in the text.

<table>
<thead>
<tr>
<th>Approach</th>
<th>Principle</th>
<th>Comments</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>API 50</td>
<td>Identify species based on carbohydrate fermentation profile</td>
<td>May discriminate to species level. Risk for misidentification/uninterpretable data</td>
<td>(Boyd et al., 2005)</td>
</tr>
<tr>
<td>DGGE</td>
<td>PCR technique with electrophoresis DNA separation in gel with denaturing agent</td>
<td>Discriminates to species level. Suitable for mixed populations</td>
<td>(Temmerman et al., 2004)</td>
</tr>
<tr>
<td>Sequences</td>
<td>DNA fragments of 16S rRNA or other genes are amplified, sequenced and compared with known sequences</td>
<td>Discriminates to species level</td>
<td>(Drake et al., 1996)</td>
</tr>
<tr>
<td>RFLP</td>
<td>Total DNA or amplifies sections are digested by restriction enzymes, and separated by electrophoresis</td>
<td>Discriminates to species level. Species unique-bandng pattern</td>
<td>(Yavuz et al., 2004)</td>
</tr>
<tr>
<td>MLST</td>
<td>Automated DNA sequencing to map alleles present in various loci in house-keeping genes</td>
<td>Discriminates to species and strain level. Used to differentiate isolates with highly related genotypes</td>
<td>(De las Rivas et al., 2006)</td>
</tr>
<tr>
<td>PFGE</td>
<td>Periodically switching voltage in electrophoresis to separate large DNA fragments from restriction digests</td>
<td>Discriminates to strain level</td>
<td>(McCartney, 2002)</td>
</tr>
<tr>
<td>RAPD</td>
<td>PCR amplification of random DNA segments with arbitrary primers</td>
<td>Discriminates to species and strain level</td>
<td>(Kwon, 200c)</td>
</tr>
<tr>
<td>qPCR</td>
<td>DNA amplification in real time</td>
<td>Less false positives, quantitative data</td>
<td>(Kao et al., 2007)</td>
</tr>
</tbody>
</table>

**Lactobacillus as a probiotic**

According to the World Health organization and Food and Agriculture Organization of the United Nations (WHO/FAO) (WHO/FAO, 2007), live microorganism that confer a health benefit to the host when administrated in adequate amounts are referred to as probiotics. This definition is debated since heat-killed strains exert similar beneficial effects as live bacteria (Tanzer et al., 2010). Many bacterial strains with health beneficial properties belong to *Lactobacillus* or *Bifidobacterium* (Collado et al., 2009).

To be considered a probiotic bacterium the following criteria ought to be fulfilled: (i) it should be isolated from the same species as it is intended for, (ii) it should survive through the GI tract, (iii) it should have the ability to adhere to the host tissues, (iv) it should be non-pathogenic, non-toxic, and free of other significant adverse effects, (v) it should have a substantiated beneficial effect on the host, and (vi) it should be stable during storage and
compatible with the product it is intended for. For a specific oral application these criteria may need modifications to suit local prerequisites.

**Probiotics: Possible mechanisms of actions**

Mechanisms underlying probiotic effects are not completely understood, and the mechanisms cannot be generalized as different strains may differ. Most research in the area has focused on the GI tract. The claimed mechanisms underlying GI beneficial effects include: (i) secretion of antimicrobial substance, (ii) competitive adherence to mucosa and epithelium (iii) strengthening of the GI-epithelium barrier, and (iv) modulation of the immune system (Bermudez-Brito *et al*., 2012; Collado *et al*., 2009; Reid *et al*., 2010b). Since the mouth represents the first part of the GI tract, it has been claimed that mechanisms of action in the oral cavity may be similar to those described for the intestine (Haukioja, 2010; Meurman, 2005; Meurman & Stamatova, 2007). Thus, probiotic bacteria are suggested to interact directly in oral biofilms (microbe-microbe interactions), or have indirect effects (microbe-host interactions) (Figure 4). Studies related to microbe-microbe interactions are generally focused on the inhibition of pathogenic bacteria and may involve competition for specific adhesion sites (competitive exclusion), coaggregation, competition for nutrients, and production of antimicrobial compounds, such as bacteriocins, hydrogen peroxide (H$_2$O$_2$) and acids. Aspects related to microbe-host interactions, which are beyond the scope of this dissertation, address diverse mechanisms, including immune-stimulation and reduction of inflammation.

![Figure 4. Proposed mechanisms for direct and indirect probiotic effects, i.e. microbe-microbe interactions, and microbe-host interactions.](image-url)
Competition for adhesion sites between probiotic and oral bacteria has been studied using host ligand-coated HA as a tooth model (Haukioja et al., 2008; Jalasvuori et al., 2012; Marttinen et al., 2013). In this model, probiotic bacteria reduce adhesion of \textit{S. mutans} and adhere to salivary agglutinin gp340, the main saliva receptor for \textit{S. mutans} (Carlen & Olsson, 1995). Probiotic strains also inhibit growth of putative pathogens, such as \textit{S. mutans}, \textit{Streptococcus sobrinus}, and periodontal pathogens \textit{in vitro} (Hasslof et al., 2010; Koll et al., 2008; Soderling et al., 2011; Teanpaisan et al., 2011) and \textit{in vivo} (Caglar et al., 2007; Nikawa et al., 2004; Yli-Knuuttila et al., 2006). The inhibitory effect has been attributed to production of bacteriocins, H$_2$O$_2$ and organic acids, and possibly coaggregation (Keller et al., 2011; Lang et al., 2010; Twetman et al., 2009a).

\textit{Adhesion and colonization of probiotics}

Adhesion is the initial step for probiotics to play their biological role, but exact mechanisms for this have not been elucidated. It likely involves non-specific (hydrophobicity) and specific host ligand-bacterial adhesin interactions. S-(surface) layer proteins are suggested to act as adhesins for host attachment of lactobacilli (Åvall-Jääskeläinen & Palva, 2005). Removal of the S-layer protein decreases lactobacilli adhesion to various host targets (Zhang et al., 2013; Åvall-Jääskeläinen & Palva, 2005), but beyond involvement of lectin binding, the molecular mechanisms are not understood. All lactobacilli do not express the S-layer protein, which suggests additional binding mechanisms (Åvall-Jääskeläinen & Palva, 2005). A permanent alteration of the GI and oral microbiota composition by probiotics is considered unlikely (Fooks & Gibson, 2002; Kim et al., 2013) and hence permanent colonization of the host is also considered unlikely. Persistence and recovery of probiotic lactobacilli in the oral cavity are evaluated in the present dissertation.

\textit{Probiotics and health implications}

Randomized controlled trials (RCT) have provided evidence for treatment and prevention of acute diarrhea and antibiotic-induced diarrhea, as well as prevention of cow milk allergy in infants and young children (Goldin & Gorbach, 2008). Other suggested applications include gastroenteritis, inflammatory bowel syndrome, various cancers, immunosuppressed states, urogenital and respiratory tract infections, liver diseases, \textit{Helicobacter pylori} infection, and prevention or alleviation of allergies or atopic diseases in infants (WHO/FAO, 2007). However, to date no product or bacterial strain has been approved for an official health claim by the European Food and Safety Authority (EFSA).
In the recent decades, probiotics have been suggested for oral health purposes too. Cagetti et al. (2013), in a systematic review, reported that around two thirds of studies published on probiotics and caries risk factors showed that probiotics may reduce salivary MS. *L. reuteri* DSM 17938 and PTA 5289, *L. rhamnosus* GG and LB21, *L. acidophilus*, *L. paracasei*, and strains of *Bifidobacterium* have been used. Few studies have evaluated long-term effects of probiotics on caries development. One report, including 594 one-to-six-year-old children, showed that milk containing *L. rhamnosus* GG reduced caries development (Nase et al., 2001). Likewise, Stecksn-Blicks et al. (2009) found similar effects from *L. rhamnosus* LB21 containing milk when given to 248 one to four-year-old children, although a confounding effect from fluoride, which was in the test product also, cannot be excluded. Further, an RCT in elderly showed protective effects on root caries development using *L. rhamnosus* LB21 (Petersson et al., 2011). In contrast, Taipale et al. (2013), did not find any effect on caries development when giving 126 four-year-old children *Bifidobacterium animalis* subsp. *lactis* for 22 months, and supplementation with *L. paracasei* F19 in early childhood did not affect caries development in later childhood (Hasslof et al., 2013).

Taken together, clinical recommendation for use of probiotic bacteria for caries prevention is still not justified (Cagetti et al., 2013; Haukioja, 2010; Meurman, 2005; Meurman & Stamatova, 2007; Twetman & Keller, 2012). Similarly, there is not yet sufficient evidence to substantiate prevention of gingivitis or periodontal disease by probiotics (Yanine et al., 2013). However, some benefits on amounts of dental plaque and gingival inflammation (Krasse et al., 2005; Twetman et al., 2009b; Vivekananda et al., 2010) and from adjunct treatment in chronic periodontitis (Teughels et al., 2013) have been reported. Single studies have suggested positive effect of probiotics on oral candida infection (Hatakka et al., 2007) and halitosis (Iwamoto et al., 2010).

**Saliva and biofilm formation**

Each day about 0.5 to 1.5 liters of saliva is secreted into the mouth from the major salivary glands (parotid, submandibular and sublingual) and numerous minor salivary glands. Saliva is mainly water, but contains approximately 0.5% proteins, enzymes, hormones, electrolytes and other compounds. Whole saliva also contains crevicular fluid, leukocytes, epithelial cells, bacteria, viruses, food debris, and various blood components (Helmerhorst & Oppenheim, 2007). Salivary constituents have a multitude of functions in relation to health of the host. Of special importance is the multifunctionality of most salivary proteins. For bacteria salivary proteins/peptides, nutrients and electrolytes are important.
This dissertation studied lactobacilli adhesion and adhesion inhibition of potential oral pathogens using two model ligands in saliva: gp340 and the mucins MUC7. Gp340 (also called agglutinin) (Ericson & Rundegren, 1983) is a large, highly glycosylated protein that interacts with bacteria through carbohydrate and peptide receptors. It is the major host ligand for *S. mutans*, but it also interacts with several commensal streptococci (Loimaranta et al., 2005) and lactobacilli (Haukioja et al., 2008), and pathogens, e.g. *H. pylori*. In addition, human saliva contains two mucin-type glycoproteins: the high molecular weight, gel-forming mucin MUC5B, and the low molecular weight, soluble mucin MUC7 (Rayment et al., 2000). Saliva mucins bind a variety of bacterial species, including *S. mutans* (Liu et al., 2000) and *Lactobacillus* (result from present dissertation).

In contrast to gp340 and MUC7, some host ligands do not bind bacteria in the fluid phase, but only when hidden receptors are exposed upon surface attachment, e.g. statherin and acidic proline-rich proteins (Gibbons & Hay, 1988; Niemi & Johansson, 2004).

**Biofilm formation**

Oral biofilms are structurally and functionally organized microbial communities embedded in a matrix of polymers of bacterial and salivary origin (Marsh, 2004). Oral biofilms are found on tooth and mucosal surfaces. The stages in biofilm formation are: (1) formation of an acellular, 0.1 to 1 micrometer thick film (the acquired pellicle) with a host and tissue-specific composition, (2) reversible attachment of pioneer species through weak physicochemical interactions between charged molecules of the bacterial cell and the host, (3) irreversible anchoring of bacteria to host receptors in the acquired pellicle or epithelial cell membranes, (4) co-adhesion to attached bacteria by second layer colonizer, (5) multiplication of attached bacteria and synthesis of exopolymers forming the biofilm matrix, and (6) de-attachment to a planktonic stage with possibility to colonize new niches before space and nutrients become limited (Kolenbrander, 2000; Marsh, 2004; Marsh et al., 2011).

**Human milk and milk fat globule membrane**

Breast milk has a fundamental role in nutrition, metabolism and immunological events related to infant’s health. It also contains peptides, proteins, glycoproteins, glycolipids, oligosaccharides and a population of bacteria (including lactobacilli) that affect bacterial colonization and metabolism (Lonnerdal, 2010). Human milk as a source of bacteria was reported during the latest decade (Martin et al., 2003; Martin et al., 2009;
Martin *et al.*, 2012), and recent metagenomic analyses confirmed this (Cabrera-Rubio *et al.*, 2012; Hunt *et al.*, 2011; Ward *et al.*, 2013).

The dominant constituents in milk are lipids, lactose, oligosaccharides and proteins. Description of milk constituents is beyond the area of the dissertation, but one bioactive component named Milk Fat Globule Membrane (MFGM), studied in Paper II, deserves attention. In milk, fat is secreted from the glands in small spherical globules that are surrounded by a membrane, known as MFGM (Liao *et al.*, 2011). MFGM is rich in phospholipids, gangliosides, cholesterol and many other biologically active proteins (Liao *et al.*, 2011).

MFGM has shown exceptional nutritional and defense functions in the newborn (Cavaletto *et al.*, 2008; Charlwood *et al.*, 2002). Thus, it has been suggested that hydrolytic products from the triglyceride core of the globule can lyse enveloped bacteria, protozoa, and viruses in the stomach of neonates (Hamosh *et al.*, 1999). The glycoproteins in MFGM are also assumed to act as viral and bacterial ligands and prevent their attachment to the intestinal mucosa of the infant (Hamosh *et al.*, 1999). MFGM proteins comprise 1-4% of the total milk protein (Cavaletto *et al.*, 2008), and includes alpha-lactalbumin, lysozyme precursor, beta-casein, clusterin, lactotransferrin, polymeric immunoglobulin receptor precursor, and human milk fat globule EGF-factor 8 protein (Charlwood *et al.*, 2002; Lonnerdal, 2013). Many of these proteins are glycosylated and/or have innate immunity functions (Charlwood *et al.*, 2002; Lonnerdal, 2013). It has been reported that MFGM adheres to *L. reuteri* (Bisson *et al.*, 2010), but not *L. acidophilus* or *L. gasseri* (Clare *et al.*, 2008).
Aims

The overall aim of the present dissertation was to characterize naturally occurring *Lactobacillus* from infants and determine the effects of probiotic *L. reuteri* DSM 17938 and PTA 5289 on the oral biofilm composition.

The specific aims were the following:

- To compare the oral microbiota in 3-month-old breastfed and formula-fed infants.
- To identify the most prevalent *Lactobacillus* species in infants by feeding regimen and to evaluate probiotic traits of the most prevalent *Lactobacillus* species.
- To determine the persistence of *L. reuteri* DSM 17938 and PTA 5289 in saliva during and after a 6- and 12-week supplementation.
- To assess whether the persistence of *L. reuteri* DSM 17938 and PTA 5289 in saliva is important for delay of regrowth of MS after a full-mouth disinfection (FMD) with chlorhexidine (CHX).
- To characterize the complex microbiota in the oral cavity after 12-week supplementation with *L. reuteri* (DSM 17938, and PTA 5289) using the 454-pyrosequencing technique.

It was hypothesized that oral lactobacilli have possible oral health benefits (probiotic) characteristics and that probiotic *L. reuteri* DSM 17938 and PTA 5289 induce shifts in the oral microbiota composition.
Materials and methods

All studies followed the guidelines of the Declaration of Helsinki. The regional ethics committee of Umea University (Papers I, II, III and IV) and the University of Copenhagen (Paper III) approved the studies. Before entering the studies, subjects signed an informed consent form (in case of children, their parents) and confirmed that they understood they could withdraw from the project at any time with no consequences.

This section gives an overview of materials and major methods used in the studies. For more detailed information, see individual papers.

Study populations and study designs

The general characteristics of the participants included in each paper are listed in Table 4.

**Paper I**: comprised a cohort of 207 3-month-old infants recruited from a longitudinal study on early colonization and determinants in young children.

**Paper II**: included a cohort of 133 4-month-infants recruited from an ongoing double-blind RCT (NCT00624689, total 240) evaluating a new infant formula. Details from the parent study will be reported separately.

**Paper III**: is a double-blind RCT performed in young adults recruited among dental and nursing students at the University of Umeå and Copenhagen. Sixty-two participants were randomly allocated to a test (n=32) or a control (n=30) group. Before onset of the intervention, subjects received a FMD including a two-day treatment with professional cleaning, flossing, and application of CHX varnish. Between the two days participants rinsed with a CHX solution. Intervention lasted for six weeks, and the participants took two lozenges per day. Saliva samples were taken at different occasions as indicated in Figure 5.

**Paper IV**: is a double-blind RCT including 44 adults recruited among students and staff at Umeå University. Volunteers were randomized to a test (n=22) and a control (n=22) group. Treatment lasted for 12 weeks, participants took two lozenges per day, and saliva and tooth biofilm samples were collected at six occasions as showed in Figure 5. Compliance was monitored by counting lozenges left in returned containers. Compliance was acceptable if ≤15% of lozenges were left.
Table 4. Participant characteristics and inclusion and exclusion criteria. Age presented as mean values. Abbreviations are in list of abbreviations.

<table>
<thead>
<tr>
<th>Paper</th>
<th>Subjects</th>
<th>Age</th>
<th>Inclusion Criteria</th>
<th>Exclusion criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>207 infants</td>
<td>3.7 mo</td>
<td>Healthy 3-months infants born at Lycksele and Umeå University Hospital, Sweden.</td>
<td>Chronic illness.</td>
</tr>
<tr>
<td>II</td>
<td>133 infants</td>
<td>4 mo</td>
<td>Birth weight: 2,500-4,500 g, full term and exclusively breast-or formula-fed at recruitment.</td>
<td>Chronic illness.</td>
</tr>
<tr>
<td>III</td>
<td>62 adults</td>
<td>23 yr</td>
<td>Self reported healthy status, no active caries or periodontitis, salivary MS counts ≥10⁵ CFU/mL saliva.</td>
<td>Antibiotic, smoking or probiotic intake 2 mo prior to the study.</td>
</tr>
<tr>
<td>IV</td>
<td>44 adults</td>
<td>28.6 yr</td>
<td>Self reported healthy status.</td>
<td>Antibiotic, smoking or probiotic intake 3 mo prior to the study.</td>
</tr>
</tbody>
</table>

Figure 5. Study designs and sampling in papers III and IV.
Bacterial and fungal strains

Bacterial and fungal strains (clinical isolates and type strains) that were used are described in the respective paper.

Saliva, mucosa and dental biofilm sampling

In infants - Whole saliva was collected using a suction set (Unomedical AB, Malmö, Sweden) modified to fit our purpose. Saliva was immediately placed in ice-chilled containers and cultivated within hours. Oral biofilm samples were obtained by careful swabbing of the mucosa of the cheeks, tongue, and alveolar ridges using sterile cotton swabs. Samples were kept at -80ºC.

In adults – Chewing-stimulated whole saliva was collected into ice-chilled test tubes. In Paper III, saliva was stored at -80ºC for 3-9 months until analyses. In Paper IV, saliva was spread on Petri plates immediately, and the remaining saliva was centrifuged and the pellets stored at -80ºC. Supragingival biofilm (Paper IV) was collected with sterile toothpicks, and stored in Tris-EDTA (TE) buffer at -80ºC. Parotid and submandibular saliva, and milk were collected from healthy donors for *in vitro* analyses in Paper II.

Epithelial cells

Human primary gingival epithelial cells (HGEPp.05) were purchased from CELLnTEC (CELLnTEC Advanced Cell Systems AG, Bern, Switzerland). Cells were cultured in CnT-24 cell culture medium (CELLnTEC) at 37°C in a 5% CO2 incubator.

Test products

In Paper II, formula-fed infants received either a standard infant formula or a formula with addition of MFGM fraction. Both formulas were provided by Semper AB, Sundbyberg, Sweden. Purified MFGM fraction (LACPRODAN® MFGM-10, Arla Foods Ingredients, Viby, Denmark) was diluted in buffer (1 mg/mL) and used in the *in vitro* experiments.

In Papers III and IV, test lozenges contained *L. reuteri* (DSM 17938 and PTA 5289; 1 x 10⁸ CFU of each strain, BioGaia AB, Stockholm, Sweden), isomalt, hydrogenated palm oil, peppermint and menthol flavor, peppermint oil, and sucralose (http://www.biogaia.com). Except for the lactobacilli, placebo and test lozenges were identical in composition, appearance and taste.

The major methods and investigated parameters in this dissertation are summarized in Table 5.
Table 5. Major methods and investigated parameters used. Lbc=lactobacilli, other abbreviations are in list of abbreviations.

<table>
<thead>
<tr>
<th>Paper</th>
<th>Samples</th>
<th>Methods</th>
<th>Investigated parameters</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Saliva</td>
<td>Selective culture</td>
<td>Tentative Lbc identification and quantification</td>
</tr>
<tr>
<td></td>
<td></td>
<td>RFLP</td>
<td>Lbc isolate identification</td>
</tr>
<tr>
<td></td>
<td></td>
<td>16S rRNA sequence</td>
<td>Lbc isolate identification</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Agar overlay test</td>
<td>Bacteria growth inhibition</td>
</tr>
<tr>
<td></td>
<td>Oral swabs</td>
<td>qPCR</td>
<td>Detection and quantification of (L. ) gasseri</td>
</tr>
<tr>
<td>II</td>
<td>Saliva</td>
<td>Selective culture</td>
<td>Tentative Lbc identification and quantification</td>
</tr>
<tr>
<td></td>
<td></td>
<td>16S rRNA sequence</td>
<td>Lbc isolate identification</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Agar overlay test</td>
<td>Bacteria growth inhibition</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Adhesion HA</td>
<td>Adhesion to host ligands coated HA</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Aggregation</td>
<td>Aggregation with host ligands</td>
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<tr>
<td></td>
<td></td>
<td>Cell adhesion</td>
<td>Adhesion to human epithelial cells</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Western blot</td>
<td>Identification of gp340 and MUC7</td>
</tr>
<tr>
<td></td>
<td>Oral swabs</td>
<td>qPCR</td>
<td>Detection and quantification of (L. ) gasseri</td>
</tr>
<tr>
<td>III</td>
<td>Saliva</td>
<td>Selective culture</td>
<td>Tentative Lbc identification and quantification of (L. ) reuteri</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Strain-specific PCR</td>
<td>(L. ) reuteri 17938 and 5289 identification</td>
</tr>
<tr>
<td>IV</td>
<td>Saliva</td>
<td>Selective culture</td>
<td>Tentative Lbc identification and quantification of (L. ) reuteri</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Strain-specific PCR</td>
<td>(L. ) reuteri 17938 and 5289 identification</td>
</tr>
<tr>
<td>VI</td>
<td>Tooth biofilm</td>
<td>454-pyrosequencing</td>
<td>Oral microbiota diversity</td>
</tr>
</tbody>
</table>

Lactobacilli identification

Culture-dependent methods

Rogosa agar

Isolates that grew anaerobically on Rogosa agar at 37°C after 48-72h were tentatively considered lactobacilli (Figure 6). Up to 30 isolates from each plate were typed using molecular methods (Figure 7).

Identification of \(L. \) reuteri DSM 17938 and PTA 5289

Two agar media were optimized by adjusting growth temperature, pH value and bile concentration, and tested for selective growth using 12 \textit{Lactobacillus} strains commonly found in the oral cavity. Rogosa agar containing vancomycin (50 mg/L) and ampicillin (2 mg/L) at pH 5.5 with cultivation for
72 h at 37°C under anaerobic conditions was chosen for the isolation and preliminary identification of *L. reuteri* DSM 17938 and De Man Rogosa agar (MRS) containing vancomycin (50 mg/L) at pH 6.3 and enriched with 0.5% porcine bile with cultivation for 72 h at 37°C (Paper III) or 40°C (Paper IV) under anaerobic conditions was selected for tentative identification of *L. reuteri* PTA 5289. Saliva was cultivated on both selective media and isolate identifications were confirmed by strain-specific PCR.

**Culture-independent methods**

*Restriction fragment length polymorphism (RFLP)*

After bacteria DNA purification, a 1420-bp fragment of the 16S rRNA gene was amplified using the primers 16S-LF and 16S-R. Aliquots of the 16S PCR product were digested for 1.5 hours at 37°C using the enzymes *Hinfl* and *MsiI* and the digests were separated by gel electrophoresis and visualized using ultraviolet light.

*16S rRNA genes sequencing*

The 16S rRNA PCR amplification product was sequenced and identified by comparison to the Human Oral Microbe Database (HOMD) and the National Center for Biotechnology Information (NCBI) database (http://www.homd.org; http://blast.ncbi.nlm.nih.gov/Blast.cgi). Unique sequences with ≥98.5% similarity were used for identification to the species level (Figure 7).

![Figure 7. Lactobacilli identification in infants before testing probiotic traits.](image)

*qPCR for identification and quantification of *L. gasseri***

Bacterial DNA was extracted using the Gen Elute Bacterial Genomic DNA kit (Sigma Aldrich, St. Louis, MO) according to the instructions from the
manufacturer. Extracted DNA was dissolved in TE buffer, and DNA concentration was measured using a NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, DE). The qPCR standard curve reaction mixture consisted of *L. gasseri* ATCC 29601 genomic DNA ranging from 2.5 ng/μL to 2.5 fg/μL, Roche SYBR Green master mix 2X, primers LactoF and LgassR and PCR grade water. Amplification and detection of DNA were performed using a Roche Lightcycler 480.

**Strain specific PCR for identification of *L. reuteri* DSM 19738 and PTA 5289**

DNA extracted from saliva samples was added to a PCR reaction employing PureTaq Ready-To-Go PCR Beads. *L. reuteri* DSM 17938 and PTA 5289 detection was performed with strain-specific primers targeting a gene encoding a cell surface protein (GenBank DQ074924), and a gene encoding an unknown protein (GenBank NZ_ACLB01000038), respectively. Presence of PCR products was verified by electrophoresis.

**Evaluation of probiotic traits**

Five *L. gasseri* isolates (B1, B16, L10, A241, A274) and the *L. gasseri* type strain CCUG 31451 were analyzed for probiotic traits by different *in vitro* test as described below.

**Growth Inhibition**

Serial dilutions of broth cultures of lactobacilli ranging from $10^3$ to $10^9$ CFU/mL were prepared and cast with MRS agar in Petri dishes (MRS-lactobacilli agar). A layer of M17 agar was cast over the MRS-lactobacilli agar. Aliquots of *S. mutans*, *S. sobrinus*, *A. naeslundii* and *A. oris* were stamped into the top layer with a Steers replicator. For *F. nucleatum* and *Candida albicans*, the top layers were Fastidious Anaerobe agar (FAA) and Difco™ Sabouraud Maltose agar, respectively. Agar plates without lactobacilli were used as negative control. Growth was scored: 0 = no growth (complete inhibition); score 1 = moderate growth (slight inhibition); and score 2 = same or more growth as the control (no inhibition).

**Adhesion**

*Adhesion to saliva and milk-coated HA*

Adhesion of bacteria to host ligands was examined using an HA assay (Figure 8). HA beads, a model for tooth enamel, were coated with host ligands (e.g. milk, parotid- or submandibular/sublingual saliva or MFGM). Coated beads were washed and blocked with bovine serum albumin (BSA), and then incubated with radiolabelled *Lactobacillus* or *S. mutans*. 22
Thereafter unbound cells were washed away and the amount of bound microorganisms was determined by scintillation counting.

**Figure 8. Principle of HA-assay.** Radiolabelled bacteria were bound to test ligand coated HA-beads, and enumerated by scintillation counting.

**Binding of planktonic bacteria (aggregation assay)**

Equal amounts of *Lactobacillus* and test substance (saliva, milk, MFGM fraction) were incubated at 37°C on a glass slide. The size of visible aggregates was rated on a scale from 0 to 4 under microscopic inspection.

**Western blot for identification of saliva ligands for L. gasseri**

*Lactobacillus* was suspended in potassium chloride (KCl) buffer and incubated under slow rotation with parotid or submandibular/sublingual saliva. Bacteria were separated from unbound salivary components by centrifugation. To release the bound proteins, the bacterial pellets were boiled with sodium dodecyl sulfate (SDS) and separated on a Tris-HCl gel. Proteins were transferred to a polyvinylidene difluoride (PVDF) membrane, followed by incubation with monoclonal antibodies against gp340 (mAb143) or MUC7 (LUM7-2). Membranes were washed and incubated with HRP-conjugated anti-mouse for gp340 or HRP-conjugated anti-rabbit for MUC7 and presence of proteins was detected using Super Signal West Dura Extended Duration Substrate.

**Adhesion to human epithelial cells**

Human primary gingival epithelial cells (HGEPp.05) were cultured on glass slides at different concentrations at 37°C in a 5%-CO2 incubator. Cells were then fixed and the slides were blocked in bovine serum albumin. Fluorescein isothiocyanate (FITC)-labeled *L. gasseri* concentration was adjusted to OD600 = 0.2 and incubated with the epithelial cells for 2 h. After incubation, the slides were washed 300 times in buffer changed every 100 dips and mounted for microscopic evaluation. All images were evaluated using a Zeiss imager Z1 upright microscope and software Zen 2011 with 400x optical magnification.
Mutans streptococci levels in saliva

The salivary levels of MS were estimated using Dentocult SM Strip mutans (Orion Diagnostica, Espoo, Finland) according to the manufacturer’s instructions. The strips were rotated on the back of the tongue and cultivated for 48h at 37°C under semi-anaerobic conditions. The levels of bacteria were estimated by quantification of CFU/cm² using a specific device described by Twetman and Frostner (1991).

Oral microbiota diversity

Two techniques were used in this dissertation to analyze the overall microbiota in oral cavity: the Human Microbe Identification Microarray (HOMIM) and 454-pyrosequencing. Both target the 16S rRNA gene.

Human microbe identification microarray

Microarray identification of infant oral microbiota was performed at the HOMIM facility at The Forsyth Institute, Cambridge, MA. Purified DNA from 52 randomly selected breastfed infants (out of 184) and 21 (all available) formula-fed infants was assayed using 422 oligonucleotide probes targeting >300 bacterial species (http://mim.forsyth.org/homim.html). HOMIM procedures, including amplification of 16S rRNA genes, labeling of PCR products, hybridization and signal detection, were as described (Colombo et al., 2009). The lower detection limit was approximately $10^4$ bacterial cells. Hybridization signals were either used as a dichotomous variable, i.e. detected (scores $\geq 1$) versus not detected (score 0), or as a continuous variable using the 6-level scale. A schematic illustration of HOMIM is given in Figure 9.
Figure 9. Overview of steps performed in the microarray analysis (HOMIM).
**454-Pyrosequencing**

For Paper IV, amplicon processing and 454-pyrosequencing was conducted at Lund University Sequencing Facility (Faculty of Science, Lund, Sweden). After DNA extraction from each biofilm sample, the V3-V4 hypervariable regions of the 16S rRNA genes were PCR amplified using the forward primer 347F and reverse primer 803R (Nossa et al., 2010). Pyrosequencing was performed on a 454 Life Sciences Genome Sequencer FLX+ machine. Unique barcode sequences were included for sample identification. Briefly, amplicon fragments are bound to beads (one fragment/bead), PCR amplified (emPCR), and placed in Picotitre plate wells with DNA polymerase and chemicals for light signaling upon hybridization. The sequencing reaction is based on flows of nucleotides, which, when complementary to the single stranded DNA on the bead, add to the sequence while light is generated. By evaluating the reads in the flow diagram (intensity of light signals), sequences of the template can be determined (Ronaghi et al., 1998). A schematic drawing of the 454 process is shown in Figure 10.

**Pyrosequencing data analysis**

Only reads with >300 nucleotides after primer sequences removal and filtering and removal of chimeras were kept for further analyses. Cleaned reads were processed using QIIME (Quantitative Insights Into Microbial Ecology) software and clustered into operational taxonomic units (OTUs) at a sequence similarity of 97% against HOMD 16S rRNA genes (http://www.homd.org). Rarefaction curves were calculated to compare microbial richness. For phylogenetic comparisons between bacterial communities (β-diversity), principal coordinate analysis (PCoA) of the unweighted and weighted normalized UniFrac were calculated after exclusion of samples with <10,000 reads.
Figure 10. Overview of the pyrosequencing technique.
**Statistical analyses**

Descriptive statistics were calculated and differences between groups tested with parametric or non-parametric tests depending on data characteristics and distribution.

In Papers I, II and IV, group differences in per cent boys, vaginal delivery, positive for lactobacilli were tested with a Chi² test. Differences in group means for continuous data, such as total bacterial counts (log10), anthropometric measures and taxa abundance, were tested using analysis of variance (ANOVA) followed by a Bonferroni corrected post hoc test or student t-test. A *p*-value <0.05 was considered statistically significant.

In Papers III, IV the non-parametric Mann-Whitney U-test was used for comparisons of lactobacilli and MS counts.

Generalized linear modeling (Paper II) was used to adjust means for potential confounders before testing differences between group means of lactobacilli counts (log10) in saliva and swabs. *L. gasseri* detected in swabs was additionally adjusted for amount of DNA.

Data from the microarray (Paper I) were analyzed as detected (signal levels >1) versus not detected (score 0). Odds ratios to have a species/cluster were estimated by logistic regression, including the potential confounder mode-of-delivery. The False Discovery Rate was used to account for multiple testing.

PASW Statistics, (IBM Corporation Route 100, Somers, New York, USA) was used for these analyses.

**Partial least square analysis (Paper I, II, IV)**

Multivariate partial least square modeling (PCA, PLS) was performed using SIMCA P+, version 12.0, Umetrics AB, Umeå, Sweden. PCA/PLS modeling defines the maximum separation between class members. It is suitable for data where the number of observations is small, and where the x variables covary. $R^2$- and $Q^2$-values give the explanatory ($R^2$) and predictive ($Q^2$) power of the model tested. Clustering of subjects is displayed in a score loading plot and the importance of each X-variable by correlation coefficients in a column loading plot or a value for the variable importance in projection (VIP).
Results

A summary of the results from Papers I-IV is presented here. More detailed information is given in the individual papers.

Studies on the oral microbiota in infants (I, II)

Cohort characteristics

Paper I: Among the 207 three-month-old infants, 146 (70.5%) were exclusively breastfed, 38 (18.4%) partially breastfed and 23 (11.1%) exclusively formula-fed. Two infants received antibiotics after birth and approximately half of the mothers who delivered by caesarean section received intravenous antibiotics. No other medication or probiotic products were given to these infants.

Paper II: Among the 133 four-month-old infants 43 (32.3%) were breastfed and 90 (67.7%) were exclusively formula-fed with either a standard formula (34 out of 90) or a formula supplemented with MFGM (47 out of 90). These proportions were in line with the plan to recruit twice as many formula as breastfed infants. Two infants received antibiotics (one at birth and one at 3 months of age), and 25 infants were given probiotic drops.

In both studies, mode of delivery, probiotic and antibiotic consumption were handled as possible confounders. In neither study, proportion boys/girls, body weight and length at birth or screening differed by feeding mode.

Lactobacilli in the oral cavity by feeding mode

Lactobacilli colonized the oral cavity of breastfed infants significantly more frequent than formula-fed infants (Figure 11). Thus, in Paper I, viable lactobacilli were cultivated in saliva from 32.4% and 27.0% of fully and partially breastfed infants, respectively, but in none of the formula-fed infants. Counts of lactobacilli (CFU/mL saliva) were significantly higher in exclusively breastfed than in partially breastfed infants (p=0.005).

Similarly, in Paper II, lactobacilli were cultured in saliva of 34.1% of the breastfed infants compared with 4.7% and 9.3% of the standard and MFGM-enriched formula-fed infants, respectively. CFU lactobacilli/mL saliva differed significantly between breastfed, standard- and MFGM-formula-fed infants (p<0.001). qPCR analyses of oral swabs showed that breastfed infants had significantly higher levels of \textit{L. gasseri} than infants fed a standard formula (p=0.004).
**Lactobacillus species in breastfed infants**

*Lactobacillus* isolates from infant saliva were identified from 16S rRNA gene sequences as: *L. gasseri*, *L. fermentum*, *L. reuteri*, *L. casei/rhamnosus*, *L. paracasei*, and *L. plantarum*. *L. gasseri* was the most prevalent species (Figure 12). It was detected in 78.8% of all infants harboring lactobacilli. *L. gasseri* was also detected in oral swabs (29.8% of all infants) by qPCR. Most infants (85%) harbored only one *Lactobacillus* species.

**Potential probiotic properties of *L. gasseri***

Five *L. gasseri* isolates and the *L. gasseri* type strain were found to have the following potential probiotic traits:

- **Growth inhibition of oral bacteria** - *L. gasseri* inhibited growth of *F. nucleatum* strains ATCC 25586 and UJA11-a, *A. naeslundii* genospecies 1 strains ATCC 35334 and ATCC 29952, *A. oris* strains T14V and M4366, *S. mutans* strains Ingbritt, NG8, LT11 and JBP,
S. sobrinus strains OMZ176 and 6715, and C. albicans strains ATCC 10231, ATCC 28366, GDH3339, GDH18 and CA1957, in a concentration-dependent fashion.

- Adhesion to and aggregation by saliva and milk - L. gasseri adhered to submandibular/sublingual (27.3% cells bound) and parotid (20.2% cells bound) saliva-coated HA. There was less binding to purified bovine MFGM fraction (13% cells bound), and no binding to human milk. L. gasseri bound avidly to purified gp340 and it depleted saliva of gp340. A similar pattern was observed for the host ligand MUC7. Aggregation of L. gasseri cells by saliva or milk followed a similar pattern as adhesion to saliva-coated HA. Thus, highest aggregation was observed for submandibular/sublingual saliva, followed by parotid saliva, MFGM and human milk.

- Adhesion inhibition of S. mutans - Adhesion of S. mutans strain Ingbritt to parotid and submandibular/sublingual saliva-coated HA decreased significantly after pre-incubation of saliva with L. gasseri. This was a consequence of reduced gp340 in both saliva types and MUC7 in submandibular/sublingual saliva after L. gasseri incubation.

- Adhesion to human epithelial cells - FITC-labeled L. gasseri strains adhered to gingival epithelial cells. Bacteria were only observed adjacent to epithelial cells (Figure 13).

**Figure 13.** Adhesion of L. gasseri to human epithelial cells. The field of view contains human gingival epithelial cells and fluorescently stained L. gasseri strains A274 (upper picture) and B1 (lower picture). Optical magnification was 400x (upper picture) and 200x (lower picture).
Clustering of infants by feeding mode-associated microbiota

Overall, 81 species and 12 bacterial clusters (aggregate of ≥3 species) in 7 phyla were detected using the HOMIM. On average each infant had 22 detected species/cluster. *Streptococcus* cluster III, *Streptococcus anginosus/intermedius*, *S. oralis/Streptococcus* spp. HOT 064, *Streptococcus parasanguinis* I and II, *Gemella haemolysans*, *Veillonella atypica/parvula* were detected in >90% of the infants.

Multivariate modeling of the HOMIM detected phylotypes clustered breastfed infants separately from formula-fed infants whereas partially breastfed infants were scattered among both groups (Figure 14). Breastfed infants still clustered apart from formula-fed infants when modeling was restricted to vaginally delivered infants that were exclusively breast or formula-fed.

![Figure 14](image.png)

**Figure 14.** Partial least square discriminant analysis clustering of infants by feeding mode using all HOMIM microarray signals, saliva cultivated bacteria and individual characteristics as the X- block and mode of feeding as Y.

Microbiota in breastfed versus formula-fed infants

Of the 93 species/clusters detected, 25 were identified only in oral samples of exclusively breastfed compared with 14 species/clusters detected only in formula-fed infants. However, mean numbers of species detected were higher in formula-fed than in breastfed infants (p<0.005). *Actinomyces gerencseriae* and *Streptococcus australis* were detected more frequently in breastfed compared to formula-fed infants. When including only exclusively breastfed and exclusively formula-fed infants, breastfeeding was associated with having *Streptococcus* Cluster II and III, *Actinomyces* cluster I, *Lactobacillus* cluster I, while being formula-fed was associated with having *Prevotella* spp. HOT 308, and *Aggregatibacter actinomycetemcomitans*. 
Studies on *L. reuteri* effects in adults (III, IV)

*L. reuteri* treatment and viable lactobacilli

The proportions of cultivable lactobacilli (CFU/mL saliva) in test participants increased and differed significantly from control participants at most occasions during exposure in both study III and IV. However, at the follow-up samplings, no difference was seen between test and control groups (Figure 15).

![Figure 15. Viable lactobacilli in saliva in the test (*L. reuteri* exposed) and control group. * = p<0.05 (Paper IV).](image)

Persistence of *L. reuteri* DSM 17938 and PTA 5289

*L. reuteri* DSM 17938 was identified by strain specific PCR in 38.7-46.6% (Paper III) and 61.9-71.4% (Paper IV) of the participants during exposure. Presence of the *L. reuteri* strains decreased gradually when consumption stopped. Thus, at 6-months follow-up, the test strain could not be recovered (Figure 16).

![Figure 16. *L. reuteri* detection before, during and after exposure (Paper IV).](image)

Delayed regrowth of MS when *L. reuteri* persists

After FMD with CHX the levels of MS (CFU/cm²) increased significantly in subjects lacking detectable *L. reuteri* DSM 17938 (red circles in Figure 17), whereas a less pronounced, non-significant increase occurred in subjects harboring the strain DSM 17938 (blue triangles in Figure 17).
454-Pyrosequencing determined microbiota

After sequence cleaning and removal of single reads, 752,175 sequences were clustered into 4,925 OTUs at 97% taxonomic identity. OTU taxonomic assignment against HOMIM database resulted in 9 phyla, 17 classes or 107 clusters (genus or higher level of identification). For identification to the species level, 20.1% of sequences (i.e. 448 named species) were assigned at 98.5% taxonomic identity. 1.6% of the sequences were undefined.

Firmicutes was the most prevalent phylum, followed by Proteobacteria, Bacteroidetes, Fusobacteria and Actinobacteria. These five phyla corresponded to >90% of all sequences, and they were found in all samples (Figure 18).

At the genus level Streptococcus were the most common (32.1% abundance), followed by Veillonella and Fusobacterium (8.8% and 5.5% abundance, respectively). Further, 15 more genera were present with >1%, whereas the remaining genera reached <1% abundance. Among the identified species, Abiotrophia defectiva was most abundant (mean 3.04 %) followed by
Granulicatella adiacens and adiacens paraadiacens, Capnocytophaga leadbetteri and Capnocytophaga sputigena (mean 1.4%). The remaining species were present in <1%.

**Individuality in oral microbiota composition**

The individual variation in OTUs was reflected in individually differing proportions of phyla, classes and genera. For example, *Streptococcus* abundance ranged from 18 to 55%, *Veillonella* from 2 to 16% and *Fusobacteria* from 6 to 13%. OTUs richness showed by rarefaction curves in control subjects did not differ between sampling occasions indicating stability of their oral microbiota.

**Effects of L. reuteri exposure on oral microbiota composition**

PCoA of weighted UniFrac (Figure 19) and PLS-DA employing bacterial taxa clustered 12-week-treated test samples together (except for one subject) whereas other samples were scattered. Interestingly, the non-clustering subject had not carried the *L. reuteri* strains during the entire study period. Species strongly associated with being in the test and the control group are presented in Figure 20.

**Figure 19.** Three-dimensional weighted normalized UniFrac PCoA clustering of participants in Paper IV. Red dots denote subjects with 12-week exposure to *L. reuteri*.

**Figure 20.** PLS column plot illustrating species associated with the *L. reuteri*-treated (to the right) and control (to the left) groups.
Discussion

Effects of infant feeding on oral microbiota

The initial microbial colonization of the GI tract and oral cavity of the neonate is considered to play an important role in health, but it can also predispose for disease later in life (Sjogren et al., 2009; Turnbaugh et al., 2009; West et al., 2009). Breastfeeding has been reported to have an impact on the composition of the initial GI microbiota. The two first papers in this dissertation show that type of feeding in infancy is important for the oral microbiota as well. The main findings were that the oral microbiota discriminated infants according to their type of feeding, and that breastfeeding was associated with a more health-associated oral microflora. Studies on the oral microbiota composition by type of feeding are scarce. However, reports on intestinal microbiota have associated a predominance of beneficial species, i.e. Bifidobacterium, with breast-feeding (Bezirtzoglou et al., 2011; Fallani et al., 2010). Recently, Azad et al. (2013), used pyrosequencing to study fecal samples from 4-month-old infants. They found high variability of microbial profiles in all infants, and a spectra dominated by Actinobacteria and Firmicutes. However, in the formula-fed infants there was often an overrepresentation of the pathogen C. difficile.

A novel finding was that at age 3-4 months, lactobacilli were frequently found in saliva from breastfed infants but rarely in formula-fed infants. The effect of diet on the presence of lactobacilli in the GI tract is controversial. In line with our results, some investigators have shown that breastfed infants harbor more Lactobacillus in the GI tract than formula-fed infants (Balmer & Wharton, 1989; Lönnerdal, 1999). On the contrary, Adlerberth and Wold (2009), reported that Lactobacillus did not characterize breastfed infants, albeit it may favor particular species, such as L. rhamnosus. Since the association between breastfeeding and the presence of oral lactobacilli was confirmed in two independent study groups (Papers I, II), this finding is considered valid.

Human breast milk has, through its content of microorganisms including lactobacilli and streptococci, been proposed to provide a continuous inoculum of bacteria to the oral cavity and other parts of the GI tract (Martin et al., 2003). Thus, the finding that lactobacilli, but also Streptococcus species included in the HOMIM cluster I, were more common in breastfed infants may reflect such a continuous inoculation. Further, milk-provided nutrients (Kunz et al., 2000) and binding epitopes for protein-protein or lectin interactions (Wernersson et al., 2006) may support the establishment of transmitted bacteria. The concerted action of these different mechanisms
probably determines the role milk plays in acquisition of oral bacteria in early infancy, including *Lactobacillus*, *Bifidobacterium* and *Streptococcus*, (Cabrera-Rubio *et al.*, 2012; Hunt *et al.*, 2011; Ward *et al.*, 2013).

The present studies cannot substantiate that milk is a major source for lactobacilli acquisition in the mouth of breastfed infants. However, the hypothesis is supported by the facts that several studies have demonstrated transmission of bacteria from mother to infant through breastfeeding (Albesharat *et al.*, 2011; Martin *et al.*, 2003; Martin *et al.*, 2012), and the fact that dominance of *L. gasseri* in the infants’ mouth is in line with recovery of this species from milk and intestinal samples from breastfed infants (Ferreira *et al.*, 2011; Kirtzalidou *et al.*, 2011; Rodrigues da Cunha *et al.*, 2012). Still, it should be considered that *L. gasseri* is common in the vaginal microflora of fertile women, and that vaginally delivered infants acquire bacteria from their mother’s vaginal microbiota during birth (Dominguez-Bello *et al.*, 2010). In this perspective, it should be noted that the findings reported in Papers I and II were consistent when analyses were stratified or adjusted for mode of delivery. In addition, *L. gasseri* is a common colonizer of the mouth and other parts of the GI tract of adults, and bacteria may be transferred from the parents through near contact, such as licking on the pacifier. Thus, to find out if milk is a major source for transmission of lactobacilli in early infancy, a follow-up study where lactobacilli in milk and from the infant’s mouth are matched at the clonal level is needed.

Six different lactobacilli species were identified in the mouth of the examined breastfed infants, with an outstanding dominance of *L. gasseri*. This is well in line with previous studies from infant intestines (Ahrne *et al.*, 2005; Solis *et al.*, 2010) and healthy oral cavity in adults (Köll-Klais *et al.*, 2005). This finding raises the thought that *L. gasseri* may have probiotic functions in the mouth/GI tract of infants. Probiotic traits for *L. gasseri* have been shown *in vitro*. Thus, production of several bacteriocins, with the most well-characterized gassericin A from *L. gasseri* LA39 (Kawai *et al.*, 1998), but also gassericin T and KT7, have been identified. All gassericins have been demonstrated to antagonize pathogens, such as species of *Clostridium* (Arakawa *et al.*, 2009). *L. gasseri* also produces organic acids, which inhibit non-specific pathogens through intracellular acidification, and H2O2 (Selle & Klaenhammer, 2013). Further, *L. gasseri* have been shown to reduce mutagenic enzymes in feces (Pedrosa *et al.*, 1995), stimulate macrophages and lymphocytes, and modulate the immune system through the toll receptors (Gomez-Llorente *et al.*, 2010). In addition to these traits, *L. gasseri* was found (Papers I, II) to inhibit growth of several opportunistic oral bacterial species and counteract attachment of *S. mutans* in a tooth-mimicking model. Together, these traits may be beneficial for oral and
general health by modulating oral/GI biofilms or programming the immune system to immunotolerance of the commensal microflora.

**Lactobacilli acquisition and persistence in the oral cavity varies among individuals**

Both previous studies, and the present studies, support the hypotheses that exposure to probiotic lactobacilli does not lead to permanent colonization of the host, and that some subjects do not retain them at all. Thus, only one third of the breastfed infants in the studies in Papers I and II harbored the genus and two thirds of adults in Papers III and IV retained ingested *L. reuteri*. This is in accordance with Dommels et al. (2009), who studied survival of *L. reuteri* 17938 in feces, and found detectable levels of *L. reuteri* in 11 out of 13 volunteers after a 3-week intervention period. Similarly, Caglar et al. (2009) and Iniesta et al. (2012) found that approximately half of the participants retained ingested *L. reuteri* in the oral cavity after a short intervention period, and Yli-Knuuttila et al. (2006) found that *L. rhamnosus* LGG was present in 66% of the participants after a two-week treatment. After termination of exposure most published studies report that the test strains are lost rapidly. The present studies identified *L. reuteri* PTA 17938 in approximately 20-30% of the participants one month after termination of exposure, but after 6 months all had lost the strain. The difference between the present results and earlier studies may relate to a longer exposure period (up to 12 weeks) and the new molecular identification method used here.

Ability to adhere to host tissues is considered important for lactobacilli to exert their function (WHO/FAO, 2007). *L. gasseri* isolates from feces and human milk have been shown to adhere to intestinal epithelial cells and intestinal mucus (mainly MUC2) (Ferreira et al., 2011; Rodrigues da Cunha et al., 2012). Similarly, *L. gasseri* isolates from infant saliva were here shown to attach to oral epithelial cells, glandular saliva and purified salivary glycoproteins, and this may contribute to persistence in the mouth upon transmission. Epithelial cells coated with salivary components are the only surfaces in the mouth that offer adhesion sites for bacteria in early infancy.

The presently found individuality in acquisition of lactobacilli in breastfed infants and adults with daily consumption emphasizes the importance of host factors for bacterial retention and hence biological effects (Marsh & Devine, 2011). Host factors that may contribute to the variability of *Lactobacillus* retention may include binding to or aggregation by salivary agglutinins or other bacteria in the fluid phase and subsequent clearance (Loimaranta et al., 2005). Phenotypic variation in the host receptors may also add to variations in the retention of lactobacilli. Orally isolated lactobacilli adhere to saliva gp340 (Haukioja et al., 2008) and mucins
Polymorphism in gp340 has been shown to correlate with adhesion capacity of \textit{S. mutans} (Jonasson \textit{et al.}, 2007), but if these polymorphisms are related to individuality in lactobacilli retention remains to be explored. Phenotypical variation could also relate to the ABO blood group antigen system. Several salivary glycoproteins and mucosal linings carry ABO blood group antigens (Makivuokko \textit{et al.}, 2012). ABO phenotypical variation has been linked to the human intestinal microbiota composition (Makivuokko \textit{et al.}, 2012), and strain specific ABO antigen binding of \textit{Lactobacillus} spp. reported (Uchida \textit{et al.}, 2006; Watanabe \textit{et al.}, 2012). Thus, Lam 29 from \textit{Lactobacillus mucosae} ME-340 binds to histone H3 and blood group antigens A and B (Watanabe \textit{et al.}, 2012) and \textit{L. plantarum} LA 318 recognizes human A and B blood group antigens (Kinoshita \textit{et al.}, 2008).

**Persistence of the probiotic \textit{L. reuteri} could be reflected in its effect on the oral cavity**

The present and previous results suggest that retention of the selected probiotic species at the site of action should be taken into account when effects of probiotics are to be evaluated. In fact, this may help in understanding why some studies show effects on MS (Caglar \textit{et al.}, 2006; Caglar \textit{et al.}, 2007; Caglar \textit{et al.}, 2009; Nikawa \textit{et al.}, 2004) and others do not (Cildir \textit{et al.}, 2012; Lexner \textit{et al.}, 2010), and why some patients benefit from probiotic treatment and others do not. Data in Paper III illustrate that analysis of retention of the specific probiotic strain(s) at the site of action (here the oral cavity) may reveal results that otherwise might remain hidden. Thus, regrowth of MS was assessed in two publications that analyzed results from the same trial, \textit{i.e.} Keller \textit{et al.} (2012) and Paper III. Subjects included in the study had high levels of MS and received a FMD with CHX before \textit{L. reuteri} exposure. CHX treatment before probiotic consumption has been suggested to induce a more stable colonization of the probiotic than by the test bacteria alone (Aminabadi \textit{et al.}, 2011). In the first publication, Keller \textit{et al.} (2012) did not find any significant differences in MS regrowth between a test and a control group using a chair side kit for MS detection and a DNA-DNA checkerboard technique as an estimate of 19 bacterial strains associated with oral health and disease. It was speculated that the inclusion criteria (subjects with high levels of MS) could explain the outcome (Hasslöf, 2013). In Paper III, the presence of the actual test strains (\textit{L. reuteri} strains 17938 and 5289) was traced by selective culture media and strain-specific PCR. This allowed stratification of subjects by the presence of \textit{L. reuteri} DSM 17938 at different time points. Now, it was revealed that re-establishment of MS was related to the presence of \textit{L. reuteri} DSM 17938. Further, among the seven subjects whose dental biofilms were characterized by pyrosequencing (Paper IV), the effects on microbiota pattern deviated in
one subject after 12 weeks treatment. This subject was the only one among
the seven who never had detectable *L. reuteri* test strains in the mouth. In
general, studies have not considered the presence of the used probiotic
strains when interpreting data. Based on the present results this should be
urged.

**L. reuteri effects on oral biofilm composition**

Oral biofilms are structured microbial communities on hard and soft
surfaces in the body. *In vitro*, probiotic lactobacilli, including *L. reuteri*, have
the ability to be part of oral biofilms, but also to affect attachment of other
bacteria to biofilms (Jalasvuori et al., 2012; Soderling et al., 2011). Most
studies that relate probiotic intake to biofilm composition have targeted
single species, often *S. mutans* as a risk marker for dental caries, but both
newer data (Aas et al., 2008; Tanner et al., 2011) and the ecological plaque
hypothesis (Marsh, 2006), suggest that, at least for dental caries, a more
complex approach should be taken. One such approach would be to evaluate
the entire microbiota simultaneously using 454-pyrosequencing. To the best
of my knowledge, the role of probiotic bacteria on the multi-species dental
biofilm using the 454-pyrosequencing metagenome sequencing approach
has not been evaluated previously.

The main finding was that subjects in the test and the control group were
clustered distinctly after 12-week treatment by PLS-DA, and that the PCoA
Unifrac phylogenetic-based matrix plot aggregated 6 out of 7 treated subjects
compared with control subjects and baseline samples that were scattered. A
small Unifrac distance implies that the composition of the bacteria
communities was similar, which was the case for 6 out of 7 subjects in the
test group (Paper IV). The interpretation of this result is that treatment leads
to a more homogenous microbiota and that 12-week exposure to *L. reuteri*
strains DSM 17938 and PTA 5289 affects the overall oral microbiota
composition.

Somewhat unexpectedly, no difference was observed between the control
and *L. reuteri*-exposed group for the number of phylotypes (richness) or the
total number of sequences at different taxonomical levels. However, the
findings are in line with studies reporting a lack of changes in GI microbiota
in healthy subjects with or without probiotic administration (Kim et al.,
2013), and that feces phylotype composition assigned to the species level did
not differ between female monozygotic twin pairs consuming fermented milk
containing a consortium of probiotic bacteria (McNulty et al., 2011).

The number of OTUs that could be defined to the species level based on 454
sequences were quite limited due to read-length restrictions. Still, a cross
sectional comparison between the test and control group after a 12-week
exposure, and a longitudinal difference between baseline and 12-week measures, confirmed that being in the test group was strongly associated with increased levels of *F. nucleatum* and *Streptococcus* spp. Increased abundance of *F. nucleatum* has previously been associated with consumption of *L. reuteri* (Keller et al., 2012) using a DNA-DNA hybridization technique. *F. nucleatum* is suggested to act as a bridge-organism that facilitates colonization of other bacteria by coaggregation (Kolenbrander, 2000). Coaggregation between *F. nucleatum* and *Streptococcus* spp. and various other oral bacteria has been demonstrated *in vitro* (Kang et al., 2005; Keller et al., 2011).

Single studies have applied pyrosequencing on the microbiota in other parts of the GI tract than the mouth. One such study, which used the same *Lactobacillus* strain as in Papers III and IV, in colicky infants observed an increase of Bacteroidetes in infants with decreased colicky symptoms, but no effect on the global composition (Roos et al., 2013).

The present findings are interesting and certainly noteworthy, but further knowledge is needed to understand the biological relevance of this finding.

**Material and methodological considerations**

Different microbiological methods were chosen to examine various aspects of the oral lactobacilli. Culture of saliva with a *Lactobacillus*-selective medium was used to isolate strains for subsequent species identification by 16S rRNA gene amplification and sequence analysis. The aim was to evaluate possible probiotic characteristics of the isolates. Having found that *L. gasseri* was the dominant species, a targeted quantitative assay was used for that species in the mucosal swab samples to focus on detection of that species alone. Thus, the different techniques were used sequentially to address different questions. qPCR had the advantage that samples could be taken and stored frozen before analysis.

The 16S rRNA gene was targeted to identify *Lactobacillus* species from culture isolates. This gene has been recognized as the gold standard for molecular taxonomy, and it is widely used to characterize bacterial community composition in various ecological niches including the human oral microbiome (Dewhirst et al., 2010). 16S rRNA genes have well-conserved regions, but the variations in the variable regions are not sufficient to discriminate very closely related species, such as some lactobacilli species. Therefore, the usefulness of 16S rRNA gene sequence similarity in distinguishing *L. gasseri* from *L. acidophilus* and *Lactobacillus johnsonii* is limited (Dahllöf et al., 2000). Based on this limitation, we additionally targeted two other genes: *rpoB* (which encodes an RNA
polymerase beta subunit) (Dahllöf et al., 2000) and hsp60 (which encodes a 60-kDa heat shock protein) (Blaiotta et al., 2008) to confirm L. gasseri identification (data not published).

In Paper I, lactobacilli in saliva were detected exclusively in breastfed infants, but HOMIM-positive signals for Lactobacillus Cluster I including L. casei, L. paracasei and L. rhamnosus were positive for both feeding groups. One possible reason could be the differences in sample origin since saliva was used for culture, whereas swab samples that included bacteria from the dorsum of the tongue were used for the microarray assay. Even though we have not compared these two sites with the same microbiological assay, a number of studies have described site-based differences in the oral microbiota (Segata et al., 2012; Tanner et al., 2006). L. gasseri was not detected in the microarray analysis of a mucosal swab, despite the fact that a valid probe was included. However, L. gasseri was detected by qPCR. Accordingly, the microarray has a detection threshold of about $10^4$ cells, and it can be less sensitive than qPCR and selective culture. Since most infants had $<10^4$ CFU/mL saliva of lactobacilli, qPCR was performed to evaluate whether the difference between saliva and microarray detection was due to method sensitivity or sample origin. It was confirmed that L. gasseri was present in oral swabs and that infants with salivary lactobacilli were five times more likely to have L. gasseri detected in the mucosal swabs.

The selection of probiotic bacteria with the highest chance of response in the host is challenging. According to Reid et al. (2010a), one philosophy for selection of certain strains is whether they are naturally occurring in target sites or if they have a set of characteristics suited for application at a target site. Therefore, two well-documented L. reuteri strains isolated from the oral cavity (PTA5289) and human milk (DSM 17938), which are considered safe and well tolerated, and with an “identical” and available placebo, were selected. In general, L. reuteri is a normal habitant of humans (Reuter, 2001). Interestingly, the whole genome of L. reuteri 17938 has been characterized as part of the Human microbiome project, and candidate genes have supported probiotic features involved in survivance and persistence of the strain in the GI tract (Saulnier et al., 2011). In clinical and in in vitro studies, both tested strains have demonstrated positive effects in GI tract and oral health (Haukioja, 2010; Indrio et al., 2008; Jones & Versalovic, 2009).

A strength of the present dissertation is the use of the fairly new next-generation pyrosequencing technique. This technique is increasingly applied in microbial diversity studies by targeting the 16S rRNA gene or other selected genes. Most studies do taxonomic determination to the genus level, but if appropriate genes are selected the method can even be used for large-
scale clonal diversity distinction. The advantage of the technique lies in the ability to multiplex a large number of samples in the same run. Thus, thousands of sequences can be obtained simultaneously from a single sample (Keijser et al., 2008). This provides the power to comprehensively study oral bacterial community composition (Zaura, 2012). The major advantage over traditional Sanger sequencing using cloning makes pyrosequencing less labor intense.

The benefits, however, are accompanied by some disadvantages. First, the current technologies cannot sequence the entire length of the 16S rRNA gene, which results in limited taxonomic resolution (Pinto & Raskin, 2012). In Paper IV, we sequenced the hypervariable regions V3-V4 of the 16S rRNA gene from which substantial taxonomic information could be obtained (Kim et al., 2011). In silico evaluations have predicted that the V3–V4 and V4–V5 regions would provide the highest classification accuracy for the pyrosequencing technology as well as the lowest base error rate in 454-pyrosequencing (Claesson et al., 2010). In pyrosequencing, data interpretation may be disturbed at different steps, e.g. PCR or sequencing errors or chimeras, which can lead to overestimation of sample richness. Further, taxa could be missed due to the high detection limit (10⁶ CFU/mL) (Lagier et al., 2012). Meanwhile, the present limitation in read lengths (here 450 base pairs), has been improved to around 700 bp, and it will probably increase soon to 1200 bp. The latter would allow alignments of virtually the entire 16S rRNA gene, which will greatly improve taxonomic resolution to the species level.
Conclusions and future perspectives

This doctoral dissertation will help to expand our knowledge in understanding the dynamics of early colonization in infants’ oral cavity and in evaluating the influence of probiotic bacteria on biofilm ecology. The main conclusions are the following:

- The infant oral microbiota differs between breastfed and formula-fed infants. Interestingly, breastfed infants showed a more health-associated oral flora compared to the formula-fed infants. Lactobacilli were cultured significantly more frequently in breastfed infants. Further, *L. gasseri* was the most predominant species of six *Lactobacillus* species identified, and it displayed probiotic characteristics *in vitro* with possible oral health benefits. A future aim of interest would be to study the presence of lactobacilli in the oral cavity in a longitudinal study, recruiting infants immediately after delivery. It would also be of interest to study the lactobacilli origin by analyzing the mother’s milk and vagina samples. Further, it would be of interest to explore the role of lactobacilli, especially *L. gasseri*, in a complex biofilm model.

- Consumption of *L. reuteri* DSM 17938 and PTA 5289 may not necessarily result in retention of the strains in the oral cavity. Further, effects of probiotics may be restricted to subjects carrying the probiotic strains. Since adhesion of lactobacilli to oral tissues is important for the exertion of probiotic functions, this would be a fascinating topic for further studies. Indeed, there is an individuality in bacterial adhesion that could be influenced by phenotypic host receptors, *e.g.* gp340 variation. It could be of interest to study these size variants of gp340 in relation to *Lactobacillus* adhesion in the oral cavity. ABO blood group antigens have been shown to provide a host-specific binding site for bacteria in the GI tract. It would also be of interest to test if lactobacilli retention could be related to the ABO blood group antigens.

- *L. reuteri* DSM 17938 and PTA 5289 do not affect phylotype abundance and richness, respectively, but they do shift the oral microbiota composition. The biological relevance of species associated with *L. reuteri* exposure is a very important issue that remains to be elucidated. Thus, there is still need of new studies, combining improved multiplex sequencing and highly sensitive molecular techniques, to target specific species, to confirm and expand the present results.
We still know very little about how best to introduce a specific strain to a
given microbial community. An alternative option that recently gained
renewed interest is microbiota transplantation. Indeed, it has been showed
that exposure of the infant to parent saliva may reduce the risk for allergic
development (Hesselmar et al., 2013). Further, fecal microbial
transplantation has been proposed as a treatment for recurrent *C. difficile*
infection with highly successful outcome (van Nood et al., 2013). In order to
search for more natural approaches, and since the benefits of probiotics may
be limited to some individuals, this new strategy should be of interest. Thus,
a complete microbiota transplantation of a set of well-established organisms
from one person to another could be beneficial to the recipient. This would
be a highly interesting topic for further studies with focus on the oral cavity.

In addition, dental biofilms consist not only of ecological communities of
many bacterial species, but also bacteria-derived components and external
components. It would be of interest to study the dynamics in the biofilm
upon exposure to probiotic bacteria using metabolomics technique.

In summary, lactobacilli may play an important role during the initial oral
bacterial colonization. Breast milk may be a source of *L. gasseri* which may
play a beneficial role on infants’ oral health. Probiotic *L. reuteri* DSM 17938
and PTA 5289 shift the oral biofilm composition, and their persistence in the
oral cavity is possible, but only in individual cases.
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