

Probiotic Lactobacilli in the context of dental caries as a biofilm-mediated disease

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To my family

‘As is a tale, so is life: not how long it is, but how good it is, is what matters’

Seneca

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Abstract

Background: The World Health Organization defines probiotics as 'live microorganisms which, when administered in adequate amounts, confer a health benefit to the host'.

Traditionally, probiotic microorganisms have been used to prevent or treat gastrointestinal tract diseases. In the last 15 years, there has been increasing interest of a possible probiotic impact on the oral microbiota and dental caries. Dental caries is a multifactorial disease, and the causative factor in the oral microbiota includes a shift from a balanced microflora to a microflora that includes more aciduric species such as mutans streptococci (MS), non-mutans streptococci, and *Actinomyces*. MS is considered an opportunistic pathogen although several other bacteria also contribute to the disease. Early acquisition of MS is associated with early development of caries; therefore a desirable complement to other prophylactic measures would be a MS colonization inhibitor.

Objective: To better understand how selected strains of probiotic lactobacilli interact with MS *in vitro* and *in vivo* and to study the impact of probiotic lactobacilli on caries development during childhood.

Material and methods: The *in vitro* properties of probiotic lactobacilli were studied with regard to (i) acid production from sugars and sugar alcohols, (ii) growth inhibition capacity on clinical isolates and reference strains of MS as well as *Candida albicans* and (iii) the capacity to co-aggregate with MS. A randomized controlled trial (RCT) tested the short-term effect of intervention with two *Lactobacillus reuteri* strains on MS, which was evaluated after treatment with chlorhexidine. The re-growth patterns of MS and 19 other selected strains were also evaluated. In the second clinical study we investigated the long-term effect on MS prevalence and dental caries after an intervention with *Lactobacillus paracasei* ssp. *paracasei* F19 (LF19) between 4 and 13 months of age.

Results: The results from the *in vitro* testing showed that strains of probiotic lactobacilli differed in their fermentation patterns, inhibition capacity and their capacity to co-aggregate, which should be kept in mind in the translation to clinical research. The clinical study on short-term effects of two *L. reuteri* strains on MS and other oral strains showed no effect on re-growth patterns after intervention. The clinical study on long-term effects of LF19 showed no effect on the prevalence of MS. Furthermore, the clinical follow-up at 9 years of age showed no differences in either decayed, missing, and filled surface (dmfs) or DMFS between the probiotic and placebo groups. Evaluation of saliva samples showed no signs of oral colonization with LF19 in the study group.

Conclusion: The *in vitro* testing showed potentials of the selected probiotic *Lactobacillus* strains for interference with MS and *C. albicans*. The results from the clinical studies showed no such effect on MS or dental caries. Evidence regarding the effectiveness of specific probiotic applications in the prevention of dental caries is limited and does not allow for conclusions concerning the use of probiotic bacteria as a preventive measure.

List of publications

This thesis is based on the following publications, which will be referred to by the corresponding Roman numerals:

- I.** Hedberg M, **Hasslöf P**, Sjöström I, Twetman S, Stecksén-Blicks C. Sugar fermentation in probiotic bacteria – an *in vitro* study. *Oral Microbiol Immunol* 2008; 23:482-485.
- II.** **Hasslöf P**, Hedberg M, Twetman S, Stecksén-Blicks C. Growth inhibition of oral mutans streptococci and candida by commercial probiotic lactobacilli – an *in vitro* study. *BMC Oral Health* 2010; 2:10-18.
- III.** Keller MK, **Hasslöf P**, Stecksén-Blicks C, Twetman S. Co-aggregation and growth inhibition of probiotic lactobacilli and clinical isolates of mutans streptococci: an *in vitro* study. *Acta Odontol Scand* 2011; 69:263-268.
- IV.** Keller MK*, **Hasslöf P***, Dahlén G, Stecksén-Blicks C, Twetman S. Probiotic supplements (*Lactobacillus reuteri* DSM 17938 and ATCC PTA 5289) do not affect regrowth of mutans streptococci after full mouth disinfection with chlorhexidine: a randomized controlled multicenter trial. *Caries Res* 2012; 46:140-146.
*Authors contributed equally to the paper.
- V.** **Hasslöf P**, West CE, Karlsson Videhult F, Brandelius C, Stecksén-Blicks C. Early intervention with probiotic *Lactobacillus paracasei* F19 has no long-term effect on dental caries. *Accepted* DOI: 10.1159/000350524.

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Abbreviations

ATCC: American Type Culture Collection

C-section: Caesarean section

CHX: Chlorhexidine

CFU: Colony forming units

DMFS: Decayed, missing, filled surface

DNA: Deoxyribonucleic acid

GIT: Gastrointestinal tract

LF19: *Lactobacillus paracasei* ssp. *paracasei* strain F19

MRS: de Man, Rogosa, and Sharpe (medium)

MS: Mutans streptococci

RCT: Randomized controlled trial

Svensk sammanfattning

Probiotika definieras som 'levande mikroorganismer som när administrerade i tillräcklig mängd ger en hälsovinst för värden'. Forskning har visat att vissa tillstånd och sjukdomar kan förebyggas med probiotiska bakterier, t.ex. antibiotika associerad diarré, virusorsakad diarré hos barn och allergiska sjukdomar. Under de senaste 15 åren har intresset vuxit för att studera om probiotiska bakterier även kan användas för att behandla och förebygga sjukdomar i munhålan. Karies är en multifaktoriell sjukdom som uppstår när syror producerade av bakterier på tandytan löser ut tandens mineraler. Framför allt mutans streptokocker (MS) är viktiga men även flera andra bakterier har en del i utvecklingen av kariessjukdomen. Höga nivåer av MS i den orala floran har visat sig vara en god prediktor för karies hos barn. Även om karies minskat kraftigt sedan 1970-talet har ungefär 20% av 6-åringarna i Sverige lagat en eller flera tänder. Traditionella metoder för att förebygga karies är tandborstning med fluortandkräm och kostrådgivning. Eftersom vissa mikroorganismer är en del i kariessjukdomen har kontroll på dessa en central roll i förebyggandet av sjukdomen. I avhandlingen ingår fem delarbeten varav tre är utförda i laboratoriemiljö och två är utförda som kliniska studier. Laboratoriearbetena undersöker specifika egenskaper hos utvalda probiotiska laktobaciller såsom förmågan att fermentera olika sockerarter och sockeralkoholer, växthämning av MS och förmågan att co-aggregera med MS. De undersökta laktobacillerna fermenterade sockerarterna olika effektivt med varierande pH-sänkning som följd. Alla probiotiska laktobaciller hade förmåga att hämma växt av MS i laboratoriemiljö. *Candida albicans* hämmades också men inte lika uttalat. Förmågan att co-aggregera med MS visade små skillnader mellan de olika probiotiska laktobacillerna som undersöktes. I en klinisk studie undersöktes om två utvalda stammar av probiotiska laktobaciller; *L. reuteri* PTA 5289 och *L. reuteri* DSM 17938 hade förmåga att påverka återväxten av MS efter behandling med klorhexidin. Försöket visade att de två laktobacillerna inte påverkade återväxten av MS. I den andra kliniska studien undersöktes en grupp 9-åriga barn som fått gröt med den probiotiska *L. paracasei* F19 (LF19) under avvänjningen (4–13 månaders ålder). Studien inkluderade analys av saliven och dessa barn hade samma mängd MS som en obehandlad kontrollgrupp, dvs. interventionen hade inte hämrat kolonisation av MS. LF19 hade inte heller installerats i munfloran hos dessa barn. Kariesförekomsten hos barnen som fått gröt med probiotiska LF19 skiljde sig inte åt jämfört med kontrollgruppen. Sammanfattningsvis visade studierna att i laboratoriemiljö kan olika typer av probiotiska laktobaciller hämma växt av MS och *C. albicans* och co-aggregera med MS. De kliniska studierna visade att de stammar som undersökts inte hade någon effekt på vare sig förekomst av MS eller på kariesutveckling. Det finns inte vetenskapligt stöd för att rekommendera användning av probiotiska laktobaciller i kariesförebyggande syfte.

Introduction

Bacteria colonize epithelial surfaces in certain parts of the body and there is a constantly on-going competition for space and resources between bacterial species. An adult carries 10 times as many microbial as mammalian cells and the total weight of bacteria colonizing the human body is approximately 1.25 kg, including 1000 g in the gastrointestinal tract (GIT) and 20 g in the mouth. The ability of microorganisms to selectively colonize a niche reflects their evolutionary adaptation (Reid et al., 2011). The properties of different habitats are selective and, therefore, microflora of the skin, mouth, and digestive tract differ (Marsh et al., 2011). This difference (tropism) depends on the fact that the demand for physiological properties such as pH, redox potential, oxygen, and nutrition requirements vary between species. Compared to other parts of the body, the oral cavity, the colon, and the vagina are more densely colonized with microorganisms. Bacteria are ubiquitous and the vast majority is harmless or beneficial to the host. Our understanding with regard to the effect of microbes on human health has gradually developed from pathogens inducing infections to a mutually beneficial interaction with indigenous microorganisms that contribute to normal human physiology and immune homeostasis (Rautava et al., 2012). Commensal microorganisms have key roles in our physiology, including immune-responses and metabolism, as well as in disease (Blaser et al., 2013). Some diseases are associated with alterations in the microbiota, including obesity, malnutrition, and a variety of inflammatory diseases of the skin, mouth, and intestinal tract (Costello et al., 2012). 'The human body can be viewed as an ecosystem and human health can be construed as a product of ecosystem services delivered in part by the microbiota' (Costello et al., 2012). The human microbiome is much more complex than first realised. It is thought that only about 40–50% of the bacteria in the oral cavity have been discovered using culturing techniques (Kroes et al., 1999, Paster et al., 2001). Molecular techniques have broadened our knowledge, and today more than 700 species have been isolated from the oral cavity (Aas et al., 2005). Probiotics are defined as 'live microorganisms which when administered in adequate amounts confer a health benefit to the host' (FAO/WHO). Traditionally, probiotic microorganisms (mainly *Lactobacillus* and *Bifidobacterium* strains) have been used to prevent or treat diseases in the GIT. In the past 15 years, there has been increased interest in possible probiotic effects in the oral cavity. The studies included in this thesis investigated the possible use of already commercially used probiotic bacteria in the beginning of the GIT, i.e. the oral cavity.

Background

Resident microflora in the GIT

The GIT and accessory digestive organs (teeth, tongue, salivary glands, liver, gall bladder, and pancreas) constitute the digestive system. Bacteria are divided into mutualistic (benefiting themselves and the host), commensal (benefiting themselves but not the host), pathogenic (benefiting themselves by harming the host), and opportunistic bacteria (Marsh et al., 2009, Reid et al., 2011). The composition of the normal microbiota in the GIT plays an important role in the health of the host because it is involved in nutrition, pathogenesis, and development of the immune system (Bezirtzoglou et al., 2011). Another important trait of the normal microflora is that it protects the body from invasion by pathogens, so called colonization resistance. The oral cavity containing various microenvironments (cheeks, palate, tongue, tooth surfaces, gingival areas, and saliva), each with its own microbiota (Aas et al., 2005), offers good conditions for many microorganisms. Under normal conditions, saliva contains about 10^6 – 10^9 colony forming units (CFU)/mL saliva. The bacterial counts are low in the upper GIT, i.e. gastric aspirates contain 0– 10^3 CFU/mL, but increase in the proximal small intestine (10^3 – 10^5 CFU/mL) and in the colon (10^{11} to 10^{12} CFU/mL). So far, 700 species have been isolated from the oral cavity (Aas et al., 2005) with about 100–200 species being present in one individual (Paster et al., 2006).

Colonization of the GIT

The colonization of the human gut and mouth starts at birth and continues throughout life with distinct, age-specific changes (Mitsuoka, 1992). Important factors that decide which bacteria can colonize are genetic factors and the order in which the bacteria are ingested, and environmental factors such as general hygiene. The newborn child is colonized with bacteria from the mother's skin, oral cavity, and gut, by so-called vertical transmission. The first bacteria to colonize the gut include species such as *Escherichia coli*, *Clostridium* spp., *Streptococcus* spp., *Lactobacillus* spp., *Bacteroides* spp., and *Bifidobacterium* spp. (Servin, 2004). One important determinant for the colonization pattern is the mode of delivery. Children born vaginally are exposed to bacteria in the birth canal, whereas children born by caesarean section (C-section) are exposed to bacteria from people present at the delivery as well as organisms from instruments and equipment used during the delivery. Studies have demonstrated that vaginally born children have a more diverse gut microbiota (Biasucci et al., 2008) as well as a more diverse oral microflora early in life compared to children born with C-section (Li et al., 2005, Lif Holgerson et al., 2011, Nelun Barfod et al., 2011).

Among the environmental factors that influence the intestinal microbiota of infants, the type of feeding is considered the most important one (Mackie et al., 1999). It has been demonstrated that the oral microbiota of breastfed 3-month-old babies differed from formula-fed babies of the same age (Lif Holgerson et al., 2011). Breast-fed babies were colonized with more *Lactobacillus* species in contrast to formula-fed babies. The differences in the colonization patterns between vaginally born, children born with C-section, and whether they were breast-fed or formula-fed seem to disappear after weaning (Magne et al., 2006). However, the long-term consequences of the differences in the early oral microbiota remain to be evaluated.

The development of the microbiota in the oral cavity

The oral cavity is the beginning of the GIT and provides different and varying habitats for bacteria as it consists of both soft shedding tissues of the mucosa (mucosa, tongue, cheek, palate) and hard non-shedding surfaces (teeth) tissues, which are all embedded in saliva. The human mouth contains a great microbial diversity and harbours both viruses, fungi, protozoa, archaea, and bacteria (Wade, 2013). The epithelial surfaces in the oral cavity in pre-dentate infants are colonized by various bacterial species; predominant species include *S. oralis*, *S. mitis*, and *S. salivarius*. During the first months of life, anaerobic bacteria such as *Veillonella* and *Prevotella* spp. are added (Smith et al., 1993, Pearce et al., 1995, Fejerskov and Kidd, 2008). Eruption of the first primary teeth initiates a considerable change in the composition of the oral flora where species, including *S. mutans*, *S. sanguinis*, and *Actinomyces* ssp., become established. The flora becomes more complex during childhood and reaches stability in teenagers. Results from different studies on which bacteria exist are difficult to compare because of differences in the age of the study participants, diet, sampling techniques and locations, as well as differences in the microbial evaluation methodology (McFarland, 2000). However, it has been suggested that the ‘true’ commensals of the oral cavity include *S. mitis*, *S. oralis*, *Actinomyces naeslundii*, *Fusobacterium nucleatum*, *Haemophilus parainfluenzae*, *Eikenella corrodens*, and some species of *Prevotella* (Kilian et al., 2006).

The major mechanism by which children acquire mutans streptococci (MS) is through vertical transmission by saliva from their mothers or primary care takers (Kohler et al., 2003). Transmission increases with high maternal salivary levels of MS (Caufield et al., 1993, Plonka et al., 2012). A discrete ‘window of infectivity’ has been suggested to exist during the age of 2 years, where the child is most vulnerable for colonization with MS (Caufield et al., 1993). However, acquisition of MS is possible before and after this period (Straetemans et al., 1998, Law and Seow, 2006, Okada et al., 2010, Hansson et al., 2012). It has been demonstrated in a longitudinal study of children and

their parents that early-acquired strains persist into young adulthood (Kohler et al., 2003). Risk factors observed for early colonization of *S. mutans* include high maternal *S. mutans* levels, low infant birth weight, early tooth emergence, low salivary IgA antibody levels, preterm birth, and delivery by C-section (Li et al., 2005).

The normal microflora in the oral cavity of an adult contains a wide variety of microbes, which are mainly anaerobic bacteria (McFarland, 2000). The microflora in adults maintains stability over time (microbial homeostasis) (Marsh et al., 2009). Dietary components, in particular sugars, increase bacterial growth (Kilian et al., 2006, Reid et al., 2011). The microflora is site-specific with different inhabitants on the tongue, buccal surfaces, and teeth, but a few bacteria such as certain *Streptococcus* spp. and *Veillonella* spp. are present in most individuals and colonize most sites (Paster et al., 2006). The microbiota is affected by oral hygiene and interaction with host tissues and secretions (Kolenbrander et al., 2010). As in the intestine, the resident oral microflora is important for excluding pathogens that try to colonize, termed 'colonization resistance' (McFarland, 2000). Important mechanisms in colonization resistance are occupation of attachment sites, production of inhibitory compounds, and development of environments that are not conducive to the establishment of invading organisms (Marsh et al., 2011).

The microflora in the oral cavity does not itself cause diseases as long as it exists in equilibrium and in balance with the host (Marsh et al., 2011). However, it is also the normal microbiota that is responsible for the two most common diseases in the oral cavity, i.e. dental caries and periodontal diseases (Wade, 2013). Bacteria cause these diseases, but they are not infectious diseases in a classical way. Rather, they are the result of complex interactions between commensal microbiota, host susceptibility, and environmental factors such as diet and smoking (Wade, 2013).

The oral microbiota has also been shown to contribute to or be associated with systemic diseases. Bacteria from the oral cavity can spread directly or hematogenously with a subsequent up-regulation of cytokines and inflammatory mediators (Soder et al., 2012). An association (but not a causal relationship) between poor oral hygiene and increased cancer mortality has been shown (Soder et al., 2012). Further, periodontal diseases have been shown to increase the risk of low birth weight (Boggess et al., 2006) and preterm delivery (Offenbacher et al., 2006).

Saliva

Saliva is constantly secreted from salivary glands, about 0.5-1 L per day. Important functions of the saliva include lubrication of the oral tissues, assistance with swallowing, speech, and taste, but it also buffers acids, which hinders changes in the pH. The saliva is also important for protection against microbes. Without saliva, or with decreased saliva secretion, the oral mucosa becomes vulnerable to bacterial, viral, and fungal infections. The parotid, submandibular, and sublingual glands produce 90% of the saliva, while the buccal glands produce 10%. Saliva consists of water, immunoglobulin, proteins, and glycoproteins. (Wong, 2008). Saliva is the main nutritional source for bacteria in the oral cavity, but it is also the most important defence mechanism against microbes (Marsh et al., 2009). Lactoperoxidase, lysozyme, agglutinin and lactoferrin are examples of antibacterial proteins in saliva (Wong, 2008). Further, saliva contains calcium and phosphate ions that are crucial for the re-mineralization phase after an acid challenge on dental hard tissues (Selwitz et al., 2007).

Pellicle formation, adhesion, and colonization

The pellicle is a 1- to 10- μm -thick layer of absorbed salivary proteins and other macromolecules on the enamel surface. The pellicle structure is important for microbial colonization on teeth. Further, the pellicle protects the enamel from direct access of acids to the dental surface. Adhesion is a key factor in colonization, illustrated schematically in Figure 1. Adherence of bacteria includes both specific and non-specific mechanisms. Initially, weak electrostatic attractive forces mediate attraction, but there are also specific molecular interactions between bacterial adhesins and host receptors from the saliva (Marsh et al., 2009). Colonization is a complex process as it involves interaction between bacteria and their environments as well as bacterial interactions. Primary colonizers have receptors for new binding opportunities with secondary colonizers in the oral biofilm (Nobbs et al., 2011). In the case of specific adherence, the presence of a receptor on the host cell or another microbe and a matching adhesion molecule on the bacterium is the key requirement in the first attachment.

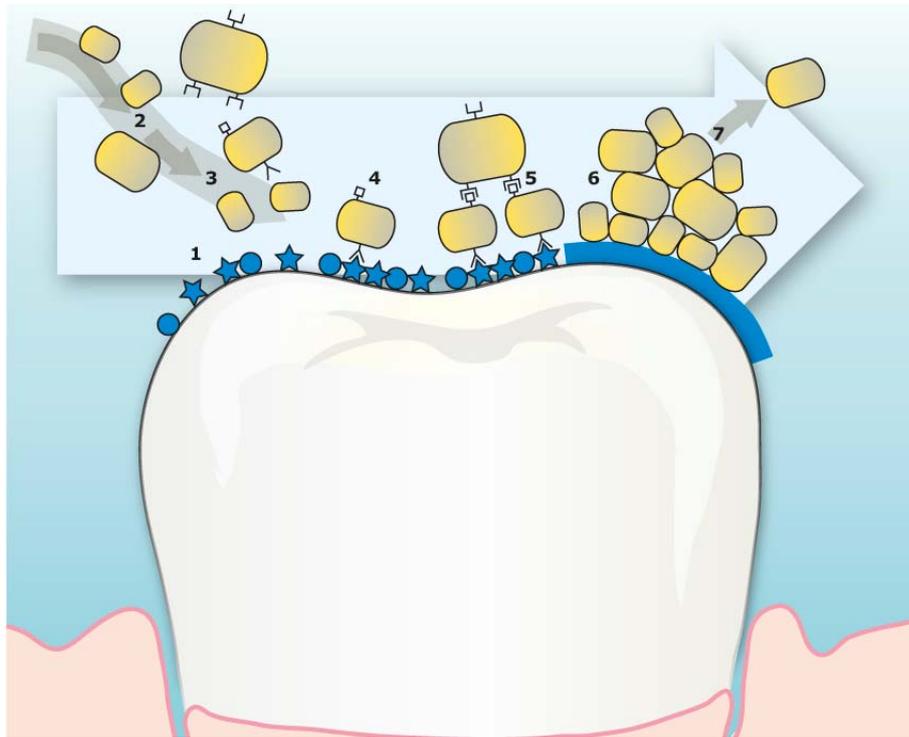


Figure 1. Schematic overview of pellicle formation, adhesion and colonization, adapted after Marsh et al (Marsh et al., 2009). **1.** Pellicle formation. **2.** Bacteria are transported passively to the tooth surface. **3.** Bacteria may be held reversibly by weak electrostatic forces of attraction. **4.** Attachment becomes irreversible mediated by adhesins on the bacteria and receptors in the pellicle. **5.** Secondary colonizers attach to primary colonizers (co-adhesion). **6.** Growth of bacteria results in biofilm maturation **7.** De-attachment may occur.

The interaction between bacteria of genetically distinct cell types in suspensions is termed co-aggregation (Kolenbrander et al., 2006). When it occurs in already attached cells in a biofilm, it is termed co-adhesion. The principal mechanisms of co-aggregation and co-adhesion are the same (Kolenbrander, 2000). Many authors have demonstrated that patterns of interaction between different strains of oral bacteria and co-aggregation involves the recognition of carbohydrate structures on one organism by lectin-like protein adhesins on the compatible partner (Nobbs et al., 2011). One special bacterium in this phenomenon is *F. nucleatum* that has been demonstrated to co-aggregate with all species of bacteria examined so far; it has been hypothesized to act as a bridge between anaerobes and aerobes (Kolenbrander, 2000).

Biofilms

A biofilm is defined as a matrix-enclosed microbial community attached to a surface (Costerton et al., 1978). The principal pieces of the biofilm are micro-colonies and the exopolysaccharide layer. The matrix usually consists of products produced by microbes, but it also contains molecules from the host. Depending on which bacterial species involved, the exopolysaccharide layer comprises 75–90% of the biofilm and the micro-colonies accounts for 10–25% (Costerton et al., 1999). Bacterial cells that settle and produce a biofilm adopt a different phenotype that differs from their planktonic counterparts (Costerton et al., 1999). Bacteria living in biofilms are usually remarkably less sensitive to antimicrobial agents and host defence mechanisms; communities of interacting species can be more pathogenic than pure cultures (Marsh et al., 2009). Biofilms are found in different parts of the body, e.g. on the surface of teeth, crypts of the tongue, in the vagina, or on prosthesis (artificial joints or heart valves). Examples of biofilm-mediated diseases are osteomyelitis, endocarditis, pneumonia, otitis media, periodontitis, and dental caries.

Dental plaque

A dental plaque is a biofilm on teeth and has been defined as the microbial community that develops on the tooth surface, embedded in a matrix of polymers of bacterial and salivary origin (Marsh et al., 2009). A large number of bacteria are involved and there is fierce competition for nutrients and attachment sites. Plaque formation is a highly dynamic process and attachment, growth, removal, and reattachment of bacteria may occur at the same time (Marsh and Bradshaw, 1995).

Bacterial interaction

Bacteria in a habitat interact in a synergistic and/or antagonistic manner. Antagonism includes the production of antimicrobial substances and the competition for space and resources. Nutritional resources are a focal point in microbial competition. However, bacteria can also interact and a food chain can be formed. An example of that is that *Veillonella* ssp. can reduce the cariogenic potential of other plaque bacteria because they use lactate and convert it to weaker acids (Marsh et al., 2009). In an environment such as the oral cavity that provides multiple ecological niches, competition can lead to a selection for variants that are better suited to colonize these niches (Hibbing et al., 2010). In densely colonized nutrient rich environments as the oral cavity, it has been proposed that antimicrobial production might be of greater importance for bacterial growth and survival than in habitats with lower counts of microorganisms (Hibbing et al., 2010).

Pathogenesis of dental caries

Dental caries forms through complex interactions between acid-producing bacteria, fermentable carbohydrates, and many host factors, including teeth and saliva (Selwitz et al., 2007). Over time, it leads to the loss of tooth structure, may lead to infection in the surrounding tissues, and eventually results in the loss of the whole tooth. Figure 2 shows a historical overview of different plaque hypotheses.

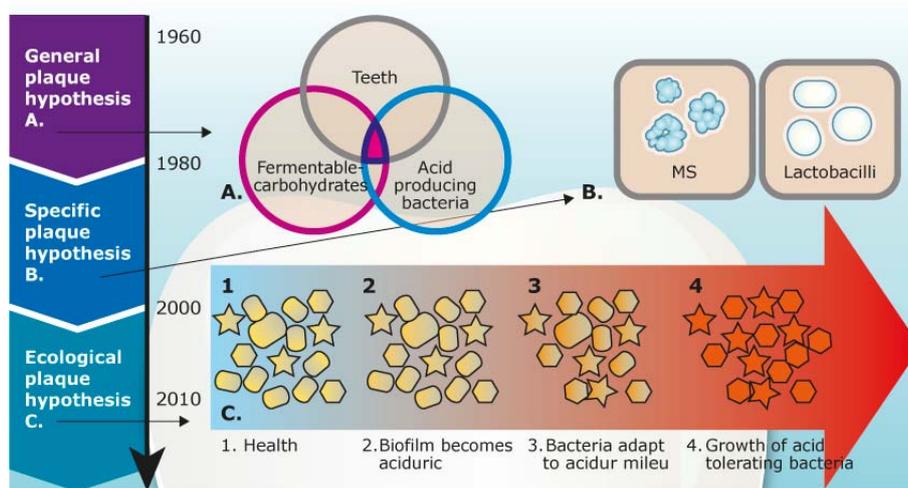


Figure 2. Historical overview of the different plaque hypotheses. Adapted from Svensäter et al., (Svensäter, 2008).

It has been known since the late 1800s that microorganisms and fermentable carbohydrates are required for the development of dental caries. Miller proposed in 1881 ‘the chemo-parasitic theory’ (Miller, 1890). It was hypothesized that microorganisms (parasites) in the oral cavity metabolize dietary starch and produce organic acids that dissolve tooth minerals. This could be addressed as the ‘non-specific or general plaque hypotheses’ where all microorganisms in the plaque were collectively considered as being pathogenic in the caries process. Fitzgerald and Keyes (Fitzgerald and Keyes, 1960) proved the strong causal relationship of certain specific microorganisms such as streptococci and lactobacilli for the development of caries in hamsters. Later, in 1976, Loesche described ‘the specific plaque hypotheses’ (Loesche, 1976). In this view, it was important to eliminate specific, but not all, pathogens with antimicrobial treatment, for example chlorhexidine (CHX).

The non-specific and specific plaque hypotheses have now been convicted with 'the ecological plaque hypothesis' (Marsh, 1994, Marsh, 2003). It was proposed that the switch from homeostasis to disease in the oral cavity is driven by a change in the environmental conditions. The change could be started with an increased intake of fermentable carbohydrates, change in oral hygiene habits, change in fluoride exposure, or reduced salivary flow. The latter could prolong periods with low pH conditions, thereby favouring an overgrowth of acid-tolerant bacterial species such as MS (Marsh, 2003). The microflora on the tooth surfaces changes with carious lesion development from dominance of non-mutans streptococci and *Actinomyces* to a dominance of MS and other non-mutans bacteria, including lactobacilli and bifidobacteria (Takahashi and Nyvad, 2011). In this view, MS is associated with dental decay but is not necessarily the causal agent (Taipale et al., 2012). According to the ecological plaque hypothesis, treatment should attempt to control rather than eliminate the plaque flora (Marsh, 2004).

Microorganisms use sucrose, glucose, fructose, or other mono- and disaccharides to produce lactic, acetic, formic, and propionic acid. According to the 'ecological plaque hypothesis', acid production is an environmental determinant of the oral microflora through acid-induced adaptation and selection (Takahashi and Nyvad, 2011). In addition, acid production is the direct causative factor of the demineralization of the tooth surface and a plaque pH below 5.5 is critical for enamel demineralization. The acids produced by the microorganisms result in a local pH drop and subsequent release of calcium, phosphate, and carbonate from the tooth (demineralization). The demineralization can be followed by the uptake of calcium, phosphate, and fluoride (remineralization) when the pH is restored by saliva.

The importance of sugar consumption for the development of dental caries is high and epidemiological studies have shown a positive correlation between the availability of sugars in various countries and caries prevalence (Sreebny, 1982) as well as between the access to fermentable sugars and caries (Sheiham, 1984, Moynihan and Petersen, 2004). Although all fermentable carbohydrates cause acid production in cariogenic bacteria, sucrose is the carbohydrate most related to caries (Kidd, 2005).

Microorganisms associated with dental caries

There is a consensus that the acids from microbial fermentation of diet carbohydrates result in an imbalance that may lead to changes in the microbial composition of the oral biofilm with an overgrowth of acid-tolerating species. Nyvad et al. concluded 'Many oral bacteria cannot be cultivated and, therefore,

conclusions are drawn on an incomplete picture' (Nyvad et al., 2013). Future analysis with molecular techniques will add more knowledge regarding these questions. Some examples of microorganisms that have been associated with dental caries are described below.

Mutans streptococci

MS comprise mainly of the species *S. mutans* and *S. sobrinus*. On the basis of an extensive research, these species have been considered as the major pathogens of dental caries (van Houte, 1994). MS can induce caries formation in animals fed a sucrose-rich diet and MS is found to be frequently isolated from carious lesions (Hamada and Slade, 1980, Loesche, 1986). The association between presence of MS in plaque or saliva in preschool children and dental caries have been systematically reviewed and it was concluded that MS is associated with an increased risk for dental caries (Thenisch et al., 2006). According to evaluations with cultivation techniques, MS constitute about 30% of the total cultivable flora in cavitated lesions in dentin (Loesche et al., 1984, Boue et al., 1987), but MS comprise only 2% or less of the total streptococci population (Nyvad and Kilian, 1990). MS have several virulence factors that include important traits for their ability to colonize tooth surfaces. MS can adhere either to salivary agglutinin or to other bacteria, the extracellular matrix, and epithelial cell surface receptors using ionic and lectin-like interactions (Mitchell, 2003). *S. mutans* express several adhesins, including antigen I/II, and cell wall-associated glycosyltransferases. The main virulence factor is the production of acids. MS can scavenge dietary sugars very effectively and rapidly convert them to acid by using fermentation products (mainly lactate). *S. mutans* can metabolize a wider variety of carbohydrates than any other gram-positive microorganism investigated so far (Mitchell, 2003).

Lactobacilli

Lactobacilli are a part of the normal microbiota in the oral cavity, GIT, and vagina and the same species can be found at all sites (i.e. there are no specific strains that only colonize the oral cavity) (Maukonen et al., 2008). In the oral cavity, lactobacilli comprise about 1% of the cultivable microbiota. Commonly isolated strains include *L. casei*, *L. paracasei*, *L. plantarum*, *L. rhamnosus*, *L. fermentum*, *L. acidophilus*, and *L. salivarius* (Ahrne et al., 1998, Marsh et al., 2009). In contrast to MS, lactobacilli have poor adherence properties and have traditionally been considered as having an opportunistic role in the caries disease, i.e. they are isolated from deep carious lesions, but are not responsible for initiating the carious lesion (Becker et al., 2002). High levels of lactobacilli have been associated with high caries activity and high sugar consumption (Larmas, 1992). Recent studies have complicated the role of lactobacilli in the oral cavity. In an analysis of the microbiota from children

with and without early childhood caries, it was shown by using DNA probes and polymerase chain reaction analysis that *L. gasseri*, *L. fermentum*, and *L. vaginalis* were associated with caries, while *L. acidophilus* was negatively associated with caries (Kanasi et al., 2010). Simark-Mattsson and colleagues isolated lactobacilli from subjects with different levels of dental caries (Simark-Mattsson et al., 2007). They showed that naturally occurring lactobacilli inhibited the growth of MS; the effect of lactobacilli was most marked in subjects without dental caries, who were lacking *S. mutans*.

Bifidobacteria (*Bifidobacterium dentium* and *B. longum*) have also been isolated from carious lesions together with MS and lactobacilli, suggesting that these bacteria are associated with caries pathogenesis (Nakajo et al., 2010, Beighton et al., 2010).

C. albicans is both a commensal and an opportunistic microorganism, a persistent member of the oral microbiota in children with caries (de Carvalho et al., 2006), and considered to have a significant contribution to caries pathogenesis (Marchant et al., 2001, Klinke et al., 2009). *C. albicans* has a strong predictive value for caries in children (Ollila and Larmas, 2008) and has a substantial growth response to sucrose exposure (Sissons et al., 2007).

Dental caries is a public health concern

Oral health is an essential component of general health that affects the ability to eat and speak and the self-esteem (Drum et al., 1998). It is problematic with comparisons of epidemiological data, as varying criteria may have been used for caries registration and whether x-rays are included in the examination or not. However, worldwide, dental caries affects about 60–90% of school-aged children and the majority of adults (Petersen et al., 2005). Dental caries remains the single most common chronic childhood disease despite the decline in its prevalence (Petersen, 2009). In Sweden, the DMFT score for 12-year-olds is 1, compared to an average score of 2.6 in the European region, of 3.0 in America, and of 1.7 in Africa (Petersen et al., 2005). Between the ages of 3 and 6 years, approximately 20% of the children develop dentin caries (Socialstyrelsen, 2005), and when the child reaches adulthood, about 40% experience dentin caries (Socialstyrelsen, 2005). The distribution is skewed, i.e. a small group of children has a high number of affected teeth (Bankel et al., 2006, Stecksén-Blicks et al., 2008). There is a clear correlation between caries early in life and future caries development (Isaksson et al., 2013). These facts motivate a focus on the prevention of dental caries among preschool children. Caries is most frequently discovered on predilection sites such as fissures, proximal surfaces, and the gingival parts of free smooth surfaces. Approximal initial carious lesions among teenagers constitute 80–90% of the total number of carious lesions (Skold et al., 1995, Forsling et al., 1999).

Prevention of dental caries

Oral hygiene together with use of fluorides and restriction of the frequency of sugar intake constitute the basic elements for prevention of dental caries. CHX-treatment has been used for biofilm control and has been proven to effectively reduce the number of MS. However, this reduction is often only temporary as re-colonization usually occurs (Twetman, 2004).

Probiotic bacteria

The development of different antibiotics has been regarded as very important for the decline in human mortality and morbidity in infectious diseases (Cotter et al., 2013). Because of an increasing resistance to antibiotics, however, research on complementary ways of manipulating the microflora has emerged. Proposed alternatives to antibiotic treatment include the use of plant-derived compounds, antimicrobial peptides from various sources, and probiotics (Cotter et al., 2013). The knowledge about the importance of the composition of the intestinal microbiota for development of the immune system and the links between intestinal microbiota and diseases such as allergies, atopic eczema, and obesity has increased (Holt et al., 1997, Ley et al., 2006). In addition, the understanding of the importance of the resident microflora in protecting the body from pathogens led to increased interest in probiotic therapy. The term 'probiotic' was introduced by Lilli and Stillwell in 1965 as opposed to antibiotic (Lilly and Stillwell, 1965). Probiotics is the appellation on live microorganisms that have a positive impact on health, which, through different means, compete with pathogenic bacteria in different parts of the body. Because most of the probiotic products are consumed orally, it is feasible that the consumed probiotic bacteria also attach to oral surfaces (Haukioja et al., 2006). During the past 15 years, interest has increased on a possible probiotic impact on the oral microbiota and the biofilm-mediated disease dental caries (Meurman, 2005), as well as periodontal diseases and halitosis (Gupta, 2011, Keller et al., 2012).

The Nobel Prize laureate Élie Metchnikoff (1845–1916) introduced the concept of probiotics into the scientific community and ascribed the beneficial effects of fermented dairy products to changes in the microbial balance in the gut. Most microorganisms used as probiotics are bacterial species, but also some fungal species have been used. *Lactobacillus* spp. and *Bifidobacterium* spp. and the fungal *Saccharomyces* spp. are among the most used. Requirements on microorganisms intended for probiotic use include that they should be of human origin and have shown to be non-pathogenic, non-toxic, and easy to culture. When screening probiotic strains for their application to improve gut health, desirable properties include acid resistance, adherence to host epithelial cells, and *in vitro* antagonism of pathogenic microorganisms

(Dunne et al., 2001). The biological activities of different probiotic bacteria differ even in strains within the same species (Haukioja et al., 2006).

Many beneficial effects on health have been attributed to probiotics. Traditionally, probiotic microorganisms have been used for the prevention and treatment of gastrointestinal infections or diseases (Guandalini, 2011, Whelan and Quigley, 2013). The strongest evidence available on the beneficial effects of probiotic lactobacilli and bifidobacteria is related to the prevention or treatment of infections in the lower part of the GIT (Sullivan and Nord, 2005, Doron and Gorbach, 2006). As an example, the use of probiotic bacteria in the prevention of infectious diarrhoea in children and adults and antibiotic-associated diarrhoea in children has been evaluated in reviews, which concluded that specific strains can reduce the number of days with diarrhoea (Allen et al., 2003, Johnston et al., 2007). Furthermore, probiotics have been used to treat or prevent urogenital and respiratory tract infections and to prevent allergies and atopic diseases in infants (Saxelin et al., 2005, Minocha, 2009, Reid, 2012, Williams and Tang, 2012).

Safety of probiotics

Regular consumption of probiotic bacteria is considered as 'generally regarded as safe' (GRAS) by health authorities around the world (Salminen et al., 1998).

Prebiotics

The definition for prebiotics is: 'A non-digestible food ingredient that benefits the host by selectively stimulating the favourable growth and/or activity of one or more indigenous probiotic bacteria' (Thomas et al., 2010). Human milk contains oligosaccharides and is an example of a natural prebiotic.

Use of probiotics in the oral cavity

In the last 15 years, several *in vitro* and *in vivo* studies have been conducted on possible effects of probiotic bacteria in the oral cavity. When used as an oral probiotic, it is desirable that the bacteria have the ability to survive in saliva and studies by Haukioja et al. have shown that different strains of probiotic bacteria have this ability (Haukioja et al., 2006).

This research on the oral clinical implications has mostly used probiotic lactobacilli that are known to enhance gut microbiota and the immunological response from the immunological parts of the intestine. However, the desired properties on probiotic bacteria that are aimed for use in the oral cavity may be different from those for their use in the lower parts of the GIT. It has been shown that good adhesion to intestinal mucus does not correlate with good adherence to oral surfaces (Haukioja et al., 2006). *In vitro* studies have shown

that some commonly used probiotic lactobacilli can interfere with *S. mutans* biofilm formation (Soderling et al., 2011) and probiotic lactobacilli can inhibit the adherence of *S. mutans* to hydroxyapatite (Haukioja et al., 2008a).

The oral health measures targeted in RCT studies included occurrence of MS, *C. albicans*, dental caries, gingivitis, and halitosis have been reviewed (Meurman, 2005, Twetman and Steckslen-Blicks, 2008, Keller et al., 2012). Recently, Hallstrom et al., showed that daily intake of *L. reuteri* ATCC 55730 and ATCC PTA 5289 not affected the plaque accumulation, inflammatory reaction or composition of the biofilm during experimental gingivitis (Hallstrom et al., 2013). This result was in contrast to other clinical studies on possible effects of *L. reuteri* on established inflammation (Krasse et al., 2006, Twetman et al., 2009). Most clinical studies on probiotics with oral health-related variables as outcome have been of short-term and measured changes in MS counts. Their results are summarized in Table 1. The effect on *C. albicans* was studied in a Finnish study and it was demonstrated that elderly people who consumed *L. rhamnosus* GG had lower levels of *C. albicans* in saliva compared to an untreated control group (Hatakka et al., 2007).

Only few studies have been conducted with dental caries as an outcome; however, all of them propose a beneficial effect on caries development. One report suggested that *L. rhamnosus* GG could reduce the incidence of caries in young children (Nase et al., 2001). A study on *L. rhamnosus* LB21 suggested similar effects, although a confounding effect from the fluoride, which had been added to the test product, could not be excluded (Steckslen-Blicks et al., 2009). In addition, one RCT in elderly showed beneficial effects on root caries development with *L. rhamnosus* LB21 (Petersson et al., 2011).

Table 1. Clinical studies with MS or dental caries as outcome.

Author, year	Strain	Design, duration	Age (yrs), N	MS	Caries
(Nase et al., 2001)	<i>L. rhamnosus</i> GG	RCT, 7 m	1-6, 594	↓	↓
(Ahola et al., 2002)	Lactobacilli mix	RCT, 3 w	18-35, 74	↓	-
(Nikawa et al., 2004)	<i>L. reuteri</i> ATCC 55730	Crossover, 2 w	20, 40	↓	-
(Montalto et al., 2004)	Lactobacilli mix	RCT, 45 d	23-37, 35	↔	-
(Caglar et al., 2005)	<i>Bifidobacterium animalis</i> ssp. lactis DN-173010	Crossover, 2 w	21-24, 21	↓	-
(Caglar et al., 2006)	<i>L. reuteri</i> ATCC 55730	RCT, 2 w	21-25, 120	↓	-
(Caglar et al., 2007)	<i>L. reuteri</i> ATCC 55730	RCT, 3 w	21-24, 80	↓	-
(Caglar et al., 2008)	<i>Bifidobacterium lactis</i> BB-12	Crossover, 10 d	20-24, 40	↓	-
(Caglar et al., 2009)	<i>L. reuteri</i> ATCC 55730 <i>L. reuteri</i> ATCC PTA 5289	RCT, 10 d	20, 20	↓	-
(Cildir et al., 2009)	<i>Bifidobacterium animalis</i> ssp. lactis DN-173010	Crossover, 2 w	12-16, 24	↓	-
(Stecksen-Blicks et al., 2009)	<i>L. rhamnosus</i> LB21	C RCT, 21 m	1-5, 174	↔	↓
(Lexner et al., 2010)	<i>L. rhamnosus</i> LB21	RCT, 2 w	12-15, 20	↔	-
(Singh et al., 2011)	<i>L. acidophilus</i> La5 <i>Bifidobacterium lactis</i> Bb-12	Crossover, 10 d	12-14, 40	↓	-
(Jindal et al., 2011)	* Probiotic mixture	RCT, 14 d	7-14, 150	↓	-
(Marttinen et al., 2012)	** Probiotic mixture	Crossover, 2 w	20-30, 13	↔	-
(Chuang et al., 2011)	<i>L. paracasei</i> GMNL-33	RCT, 2 w	20-26, 70	↓	-
(Cildir et al., 2012)	<i>L. reuteri</i> ATCC PTA 5289 <i>L. reuteri</i> DSM 17938	RCT, 25 d	4-12, 19	↔	-
(Pettersson et al., 2011)	<i>L. rhamnosus</i> LB21	RCT, 15 m	58-84, 160	↔	↓
(Taipale et al., 2012)	<i>Bifidobacterium animalis</i> BB-12	RCT, 15 m	2 m to 2 yrs, 106	↔	↔
(Juneja and Kakade, 2012)	<i>L. rhamnosus hct 70</i>	RCT, 9 w	12-15, 40	↓	-
(Sudhir et al., 2012)	<i>L. acidophilus</i>	RCT 3w	10-12, 40	↓	-

**L. rhamnosus*, *Bifidobacterium longum*, *Saccharomyces cerevisiae*

***L. rhamnosus* GG, *L. reuteri* SD2112, *L. reuteri* PTA 5289

RCT= randomized controlled trial; C RCT = cluster randomized controlled trial; Crossover = randomized crossover trial; d= days, w= weeks, m= months

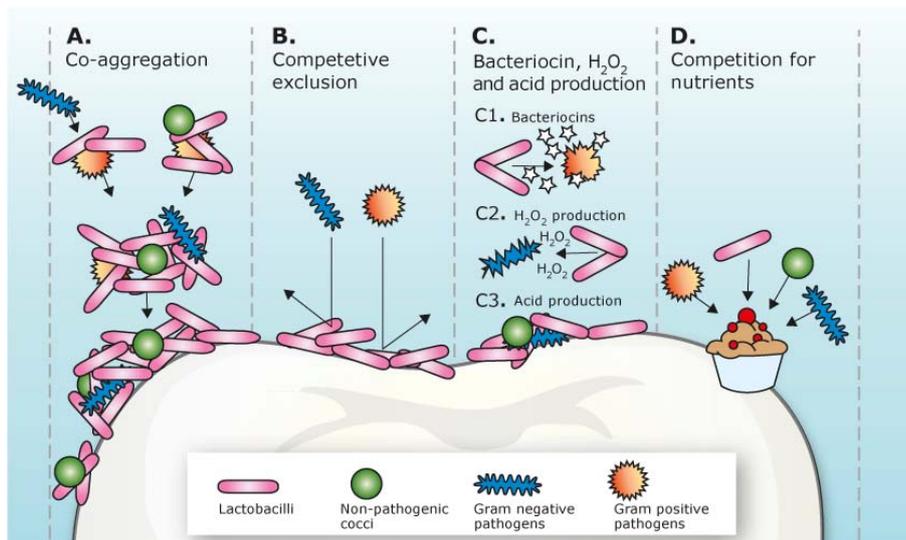


Figure 3. Proposed mechanisms of local effects on microorganisms by probiotic lactobacilli. Adapted from Reid et al., (Reid et al., 2011).

Proposed mechanisms of action for probiotic bacteria

Probiotic bacteria are thought to mediate positive effects through both systemic and local mechanisms. These mechanisms could be divided into 1) interference with other bacteria; the ability to exclude or inhibit pathogens, 2) modulate host immune responses resulting in both local and systemic effects, and 3) influence/enhance the function of the intestinal epithelial barrier (Servin, 2004). The local effect is the effect that probiotic lactobacilli have when they interact with other bacteria in the biofilm and hamper growth by pathogens through production of hydrogen peroxide, bacteriocins, and organic acids. While organic acids lower the pH, which promotes the growth of acid-tolerating bacteria, the production of bacteriocins may inhibit the growth of other pathogenic species. Proposed mechanisms of local effects on microorganisms by probiotic lactobacilli are illustrated in Figure 3. Systematic effects are thought to be mediated through immunological pathways. Each probiotic strain is associated with a unique profile of cytokines secreted by lymphocytes, enterocytes, or dendritic cells interacting with the particular bacterium (Minocha, 2009). Immunological effects are seen in the mucosa, i.e. increased IgA production, stimulated macrophage activity, and increased phagocytosis. This results in a better resistance in the mucosa, which may counteract bacterial translocation. It has been concluded that there is more evidence of the proposed mechanisms of action from *in vitro* and animal studies but less from clinical studies (Goldin and Gorbach, 2008). It is important to notice that the effects of one probiotic bacterium should not be

generalized because different strains exert different effects (Ezendam and van Loveren, 2006, Minocha, 2009).

Colonization with probiotic lactobacilli

Colonization and survival of probiotic bacteria in the target biofilm is a desirable property. The first contact probiotics that have been consumed in food have with the human mucosa takes place in the oral cavity. It has been shown that *L. rhamnosus* GG is regularly recovered from saliva samples during interventions with this bacterial strain (Saxelin et al., 2010). It is difficult to alter the composition of the adult oral microbiota (Kilian et al., 2006) and so far, it seems unlikely that probiotic candidates are able to permanently colonize the host (Yli-Knuuttila et al., 2006, Caglar et al., 2009, Saxelin et al., 2010). It has been suggested, however, that exposure early in life may facilitate a permanent incorporation into the oral microbiota (Meurman, 2005). Furthermore, it has been hypothesized that it could be easier to alter caries-associated microbiota at the time of colonization compared to later in life when the microbiota has been firmly established (Devine and Marsh, 2009).

Aims

The general aim of this thesis was to explore some *in vitro* properties of selected strains of probiotic lactobacilli that are considered as important for interference with MS and dental caries. Furthermore, to investigate both the short-term and long-term clinical effects of three strains of probiotic lactobacilli. The long-term goal is to find new ways of preventing dental caries.

The more specific aims were as follows:

Study I: To assess the acid production from various sugars and sugar alcohols by six probiotic *Lactobacillus* strains.

Study II: To investigate the ability of eight probiotic *Lactobacillus* strains to inhibit growth of MS and *C. albicans in vitro*.

Study III: To investigate the *in vitro* abilities of eight probiotic *Lactobacillus* strains to co-aggregate and inhibit growth of oral MS strains isolated from adults with contrasting levels of caries.

Study IV: To investigate the effectiveness of two probiotic strains (*L. reuteri* DSM 17938 and *L. reuteri* ATCC PTA 5289) in inhibiting the re-colonization of MS after a full mouth disinfection with CHX. Further, to analyse changes of other oral species associated with oral health and disease.

Study V: To study if an intervention with probiotic *Lactobacillus paracasei* ssp *paracasei* strain F19 (LF19) during weaning had an effect on occurrence of MS, lactobacilli, and dental caries at 9 years of age. Further, to investigate if LF19 was incorporated into the oral microbiota.

Null hypotheses

The null hypothesis for **Study I, II, and III** was that there were no differences between the tested strains. For **Study IV**, the null hypothesis was that the levels of MS would not differ in comparison with a placebo treated control group at baseline or at designated follow-ups. The null hypothesis for **Study V** was that there were no differences between the probiotic and placebo groups in the frequency of caries or colonization by LF19, MS, and/or lactobacilli.

Materials and methods

The methods are described in the respective materials and methods section of each paper. A brief summary is presented below. Studies **I**, **II**, and **III** were *in vitro* studies. **Study IV** was a two centre double-blinded RCT with two parallel arms. **Study V** was a follow-up on a double-blinded RCT with two parallel arms eight years after the intervention.

Table 2. Overview of study populations in **Studies IV** and **V**.

Study	Invited	Consented	Male/female	Mean age, range (yrs)
Study IV	69	62	11/51	23 (19–35)
Study V	171	118	52/66	9

Lactobacillus strains

An overview of the probiotic strains is given in Table 3. They were provided by the companies, except for *L. acidophilus* La5, which was isolated from A-fil®, Arla Sweden.

Table 3. Overview of examined probiotic *Lactobacillus* strains.

Strain	Origin	Company	Product	Target
<i>L. plantarum</i> 299v	Isolate from human colon	Probi AB, Sweden	Fruit drink	GIT
<i>L. plantarum</i> 931	Isolate from healthy woman from northern Sweden	Essum, Sweden	Fruit drink	GIT
<i>L. rhamnosus</i> GG	Isolate from human intestinal tract	Valio Ltd, Finland	Yogurt Fermented milk	GIT
<i>L. rhamnosus</i> LB21	Isolate from human intestine, 5-day-old infant	Essum, Sweden	Yogurt	GIT
<i>L. paracasei</i> F19	Isolate from human colon	Arla Ltd, Sweden	Yogurt Porridge	GIT
<i>L. reuteri</i> PTA 5289	Oral isolate from Japanese woman	BioGaia, Sweden	Chewing gum, lozenges	GIT Oral
<i>L. reuteri</i> ATCC 55730*	Isolate from Peruvian mother's milk	BioGaia, Sweden	Chewing gum, Gruel, lozenges, drops	GIT Oral
<i>L. acidophilus</i> La5	Diary cultures	Arla Ltd, Sweden	Fermented milk	GIT

*now named *L. reuteri* DSM 17938

Test products

In **Study IV**, lozenges containing *L. reuteri* DSM 17938 and ATCC PTA 5289 (10^8 CFU of each strain) were used. Identical lozenges without bacteria were used as placebo. The lozenges were provided by BioGaia AB, Lund, Sweden.

In **Study V**, porridge supplemented with *Lactobacillus paracasei* ssp *paracasei* strain F19 (LF19) was used. The daily dose was 10^8 CFU. The porridge was provided by Semper AB, Stockholm Sweden, and the same porridge but without bacteria was used as placebo.

Sugar fermentation experiment (Study I)

Six of the probiotic strains were evaluated in a fermentation assay. After cultivation on de Man, Rogosa, Sharpe (MRS) agar (Oxid, Hampshire, England) at 37°C in anaerobic atmosphere for 48 hours, a distinct colony was transferred to a modified MRS broth without addition of carbohydrates. After preparation, the optical density of each bacterial suspension was adjusted to 1.0 (650 nm). Nine dietary sugars and three sugar alcohols were prepared in 2% aqueous solutions and sterile filtered. A solution of bromocresol purple was used as indicator for pH changes. The fermentation assay was performed in microtitre plates, where the solution of different carbohydrates, bacterial solutions, and pH indicator were mixed together and incubated at 37°C in anaerobic condition or in aerobic conditions. Changes in the pH were recorded after 24, 48, and 72 hours.

Growth inhibition experiments (Studies II and III)

Eight probiotic *Lactobacillus* strains were used in an agar-overlay growth inhibition experiment testing the possible growth inhibition of five MS strains and five *C. albicans* strains. After initial cultivation of the lactobacilli, a distinct colony was transferred to 4.5 mL MRS broth and further incubated overnight. The broth cultures of lactobacilli were then serially diluted in 10-fold steps. Undiluted suspensions and cell suspensions corresponding to approximately 10^9 , 10^7 , 10^5 , and 10^3 CFU/mL were used in the experiment. Briefly, 1 mL of the suspensions was added to molten MRS agar. The plates were incubated overnight. On the following day, a second layer of either M17 agar or Sabouraud dextrose agar was casted on top of the MRS agar. Suspensions of MS (grown in Todd-Hewitt broth) and *C. albicans* (grown in Sabouraud Maltose broth) were prepared and the optical density was measured at 500 nm and adjusted to 0.2. The suspensions of MS and *C. albicans* were stamped on the plates using a Steers replicator. The plates were then incubated overnight. The result of the assay was scored according to Simark-Mattson (Simark-Mattsson et al., 2007), i.e. score 0 = complete inhibition (no visible colonies), score 1 = slight inhibition (at least 1 visible colony, but definitely smaller amounts than in the control plate), and score 2

= no inhibition (similar growth as on the control plate). For the estimation of acid production by lactobacilli, the surface pH of the plates was measured before and after the final incubation of each *Lactobacillus* strain. The same method for evaluation of growth inhibition was used in **Study III**. In the experiment, MS strains were isolated from patients who were caries-free (n = 3) or caries-active (n = 5).

Co-aggregation experiment (Study III)

Lactobacilli and MS strains were cultivated in MRS or Brain Heart Infusion - broth in anaerobic incubator at 37° for 24 hours. Thereafter, the bacteria were aerobically harvested by centrifugation, washed twice in phosphate buffered saline and suspended. After this preparation, co-aggregation was investigated spectrophotometrically (Genesis 10 uv; Thermo Scientific, Madison, Wisconsin, USA) according to Collado et al. (Collado et al., 2007a). The optical density was adjusted to 0.5 at 600 nm (approximately 10⁸ cells/mL). Equal volumes of lactobacilli and MS strains were mixed and incubated at 37°C. The suspensions were measured after 1, 2, and 4 hours of incubation.

Re-colonization (Study IV)

Students from the medical faculties at Umeå University and University of Copenhagen were invited to participate. Inclusion criteria were uncompromised general health (including being a non-smoker), no active oral disease or need for treatment concerning caries or periodontitis, salivary MS counts ≥ 10⁵ CFU/mL, no regular medication (except for contraceptives). Of the 168 students that were screened, 69 met the inclusion criteria. Seven of those did not give consent to participate; thus, 62 participants were allocated to a test group and a control group. After inclusion, full mouth disinfection with CHX was performed according to Eberhard et al. (Eberhard et al., 2008). The intervention lasted for 6 weeks and the test product was lozenges containing 1 × 10⁸ CFU of each strain of *L. reuteri* (DSM 17938 and ATCC PTA 5289). Placebo lozenges were identical to the test lozenges, except that they did not contain bacteria. During the intervention, participants consumed two tablets per day. Compliance with the protocol was assessed during the intervention in interviews. Stimulated whole saliva samples were collected at five occasions; at baseline immediately after full mouth disinfection, and after 1, 6, and 12 weeks. Dentocult® SM Strip mutans (Orion Diagnostica, Helsinki, Finland) were used to evaluate the levels of salivary MS. The checkerboard DNA-DNA hybridization method was used to determine the presence and levels of 19 oral species (Wall-Manning et al., 2002, Lexner et al., 2010).

Follow-up study (Study V)

Study V was a follow-up study of a previous double-blinded RCT. The aim of the original study (West et al., 2008) was to explore if an intervention with LF19 to healthy term infants of 4–13 months of age could have an effect on gut microbial composition, T cell function, Th1/Th2 immune balance, and eczema incidence. A total of 179 participants were randomized to consume cereals with (n = 89) or without (n = 90) LF19. The probiotic intervention consisted of at least one serving of cereals containing 10^8 CFU per day. Compliance in the original study was excellent, as evaluated by presence of LF19 in stool samples (evaluated by randomly amplified polymerase chain reaction). The intervention resulted in a reduced risk of eczema of 50% in the probiotic group (West et al., 2009). At 9 years of age, the study population was re-examined with regard to the prevalence of both eczema and dental caries. Saliva was further analysed for the amount of MS, lactobacilli, and total microbial count (total viable count) with conventional techniques, while the presence of LF19 was analysed by using randomly amplified polymerase chain reaction (Björneholm et al., 2002). Information about eating habits, oral hygiene habits, and social background factors were collected through a questionnaire. In addition, data on dental caries were also collected from Public Dental Health Service records at 3 and 6 years of age.

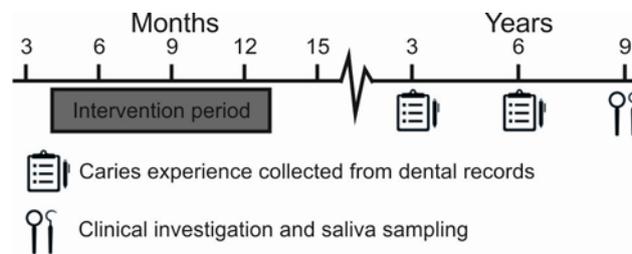


Figure 4. Overview of the study protocol **Study V**.

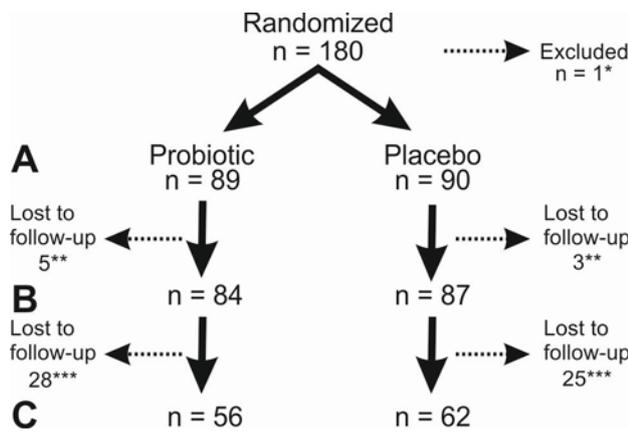


Figure 5. Participant flow chart of **Study V**. **A.** Randomized to the probiotic or placebo group. **B.** Completed the intervention phase. **C.** Participated in the follow-up study.

*Delivered by caesarean section. **Withdrawal of consent by parents. ***Had moved from the region/did not consent.

Ethical considerations

The RCT studies were reviewed by the ethics committee of Umeå University and the University of Copenhagen. Informed consent was obtained from the students and in case of children, their parents.

Study IV included students screened for high levels of MS and subjects with the highest values were included. Because the probiotic lactobacilli that were tested had been demonstrated to be safe, side effects were not discussed. The participants obtained both written and oral information about the study and were not students at the Departments of Odontology in Umeå. This strategy was considered as important to secure that no student felt forced to participate. The ethical considerations about **Study V** were more difficult because the participants were 9 years old at the time of the investigation. When performing research with children, the power imbalance between the adult researcher and the child has to be considered. Factors that need to be considered include language use, the setting, confidentiality, information provided to children and parents. An important question is how we make sure that the child is willing to participate in a study. We wrote a special information letter to the children and let them actively participate in the investigation process. The parents were aware of the procedures that would be followed because the study was a follow-up of an earlier intervention and many investigations were repetitions from the initial study, except the oral examination. The examinations were performed on two separate days to make it easier for the children. The oral examination included bitewing radiographs that were taken after an individual assessment. All radiographs were kept in

the child's dental record, so that they are available at the next regular dental appointment. At 9 years of age, most children are comfortable at the dentist office and no child refused to cooperate at the dental examination. All participating children were given individual information about the results of the oral examination. Most of the families appreciated the extra dental check-up associated with the study.

Statistical analysis

Data were analysed using the software Statistica (version 7.1; Statsoft, Inc., Tulsa, OK, USA) and SPSS (version 17.0 to 19.0, Chicago, IL, USA). A p-value of <0.05 was considered significant. In **Study I**, only descriptive data were presented. In **Study II**, growth inhibition scores were subjected to Pearson's chi-squared test and in **Study III**, the relative co-aggregation ratios were subjected to one-way analysis of variance (ANOVA). In **Studies IV** and **V**, bacterial counts were logarithmically transformed to improve normality. Pearson's chi-squared test was used for group comparisons of categorical data. One-way ANOVA or the non-parametric Mann-Whitney U-test was used for continuous data. Linear regression analysis was used to test the association between selected variables and dental caries.

Results and Discussion

In the present thesis, some properties that are important for an oral application of probiotic lactobacilli were studied *in vitro*. Further, the impact of three selected strains on the oral microbiota and of one strain on dental caries were studied. The *in vitro* properties investigated were the ability of the *Lactobacillus* strains to ferment sugars and sugar alcohols, the ability to inhibit growth of MS and *C. albicans*, and their capacity to co-aggregate with MS. These *in vitro* properties were hypothesized to be important for possible probiotic effects in the oral cavity. The chosen probiotic lactobacilli were already commercially available and mainly intended for positive effects on gut health. The strategy of choosing already available strains for further investigation for possible new applications has both advantages and disadvantages. Advantages are that they are approved and considered safe. Disadvantages may be that the strains had been tested for use in the GIT and, thus, might not have traits necessary for oral colonization and oral benefits. A different approach would be to identify and isolate lactobacilli from the oral cavity and test them for probiotic use.

Acidogenicity assessed by sugar fermentation assay (Study I)

In an attempt to study if the acidogenic potential differed between some commercially available probiotic *Lactobacillus* strains, **Study I** aimed to assess if the sugar fermentation capacity differed between the strains and the null hypothesis was rejected. Under the given conditions, the six tested probiotic *Lactobacillus* strains showed differences in their metabolic capacity. *L. paracasei* F19 and *L. reuteri* PTA 5289 were least active, followed by the two *L. rhamnosus* strains and the two *L. plantarum* strains. *L. plantarum* 931 and *L. plantarum* 299v fermented all sugars, except melibiose, raffinose, and xylitol, under both anaerobic and aerobic conditions.

Acid production of oral bacteria has conflicting effects on the oral ecology and oral health. The production of acids by microorganisms in the oral cavity is the direct causative factor for the demineralization of the tooth (Takahashi and Nyvad, 2011). On the other hand, the production of acids from lactobacilli is thought to be important for their ability to compete and affect other bacteria (Servin, 2004). In addition, the potential for the production of antimicrobial substances in lactobacilli are known to be affected by the pH (Sookkhee et al., 2001, Simark-Mattsson et al., 2009). The acid production of lactobacilli could be considered a double-edged sword. The interaction between lactobacilli and other bacteria might depend on a low pH, but a low pH might also affect the re- and demineralization process. Theoretically, one side effect could be that the acidogenicity in the dental plaque increases with a daily intake of probiotic

lactobacilli. Although acid production alone does not make a bacterial strain cariogenic, questions have been raised about the potential risks in adding lactobacilli to the oral cavity (Haukioja et al., 2008b). The experimental setting in **Study I** was very basic and, therefore, the limitations are considerable, although, a different pattern of fermentation by the strains was demonstrated. The translation of our results to a clinical setting is, however, far-fetched. We tested different strains in monocultures and the impact of each strain in the oral cavity could be very different. In another *in vitro* study, sugar fermentation of lactobacilli was studied in a more advanced model (Haukioja et al., 2008b). pH changes caused by 14 probiotic and diary bacterial strains were followed over 30 minutes using glucose, lactose, sucrose, sorbitol, and xylitol as substrates in the assays. It was concluded that the decrease in the pH was fast with glucose as substrate and that all tested strains could be considered as acidogenic. Because the settings between the two studies were different, a comparison of the results is not justified. In a study using suspensions of plaque and probiotic lactobacilli (*L. reuteri* DSM 17938 and *L. plantarum* 299v), acid production was shown to be strain-dependent and significantly less lactic acid was produced with *L. reuteri* DSM 17938 compared to *L. plantarum* 299v (Keller and Twetman, 2012).

However, the testing in monocultures *in vitro* is just the first step in evaluating possible effects on the oral biofilm and oral health. Two *in vivo* studies have been conducted since our study was reported. A RCT with crossover design showed that consumption of *L. rhamnosus* GG and *L. reuteri* ATCC 55730 and PTA 5289 twice a day had no effect on the acidogenicity of supragingival plaques (Marttinen et al., 2012). Likewise, a double-blinded RCT showed that consumption of *L. reuteri* DSM 17938 and ATCC PTA 5289 did not increase plaque acidity (Keller and Twetman, 2012). The few clinical studies on the impact of daily consumption of probiotic lactobacilli on plaque acidogenicity have, thus, not been able to show any negative effect.

Growth inhibition assay (Studies II and III)

The growth inhibition experiment showed that the different *Lactobacillus* strains inhibited growth of both MS and *C. albicans in vitro*. The capacity to inhibit growth differed between the investigated strains and the null hypothesis was rejected. The inhibitory effect was dependent on the cell concentration of lactobacilli. *L. plantarum* strains 299v and 931 exhibited the highest inhibition of MS and were the only lactobacilli that inhibited growth at a cell concentration of 10^3 .

The inhibitory capacity on *C. albicans* was weaker. The two *L. plantarum* strains and the *L. reuteri* ATCC 55730 strain displayed the strongest inhibition of *C. albicans*. The lowest pH was observed in the plates with *L.*

plantarum 299 and 931 and the highest with *L. acidophilus* La5. The inhibitory effect on both MS and *C. albicans* was dependent on the surface pH of the plates.

The growth inhibition assay with MS strains isolated from caries-free and caries-susceptible individuals showed growth inhibition of MS with all *Lactobacillus* strains, similar to the findings of **Study II**. At cell concentrations of $\geq 10^7$ CFU/mL there was complete growth inhibition mediated by all *Lactobacillus* strains. There were differences at lower concentrations, in which case the two *L. plantarum* strains were most effective. No differences were found between the different MS strains isolated from caries-free or caries-susceptible subjects.

Bacterial interference such as antagonism has an important role in maintaining the balance of the microbial ecology. Bacteriocins and organic acids, which can hamper the growth of pathogens, have been considered important for the microbial balance (McFarland, 2000, Teanpaisan et al., 2011). The growth inhibition capacity of probiotic bacteria are one of the selection criteria when screening for probiotics that are suitable for an oral application. Bacteriocins are ribosomally synthesized. Bacteriocins that are produced by gram-positive bacteria have a narrow killing spectrum to inhibit strains of closely related species (Lee and Salminen, 2008). The optimal pH for bacteriocin production has been found to be between pH 4.5–5.5 (Simark-Mattsson et al., 2009). In addition, organic acids (lactic acid and acetic acid) that are produced by lactobacilli from carbohydrate fermentation render a low pH, which can hamper the growth of neighbouring microorganisms. The levels and types of organic acids formed depend on the species and growth conditions (Lee and Salminen, 2008).

A simple interaction test is to study growth inhibition. We used the agar-overlay technique (Simark-Mattsson et al., 2007), which is convenient because multiple strains can be studied on the same agar plate. Another technique is the deferred antagonism methods, where an inhibitory zone is measured (Koll et al., 2008) and which is a more exact method. Efforts were made to optimize our experiment with careful calibration by an experienced researcher and the experiment was repeated three times. It was clear that the pH affected the inhibitory effect of lactobacilli on MS and *C. albicans*. The results were consistent with those reported by Simark Mattsson (Simark-Mattsson et al., 2007, Simark-Mattsson et al., 2009), who demonstrated pH-dependent growth inhibition of lactobacilli isolated from subjects with contrasting levels of caries. In addition, naturally occurring lactobacilli isolated from caries-free subjects had a greater inhibitory potential on MS

compared to lactobacilli isolated from subjects with caries (Simark-Mattsson et al., 2007).

Co-aggregation assay (Study III)

In the formation of a biofilm, co-aggregation and co-adhesion are fundamental. Bacteria that are able to co-aggregate with other bacteria may have advantages over bacteria that cannot co-aggregate, which are easier removed (Collado et al., 2007a). Co-aggregation patterns have been estimated for different species of bacteria and it is apparent that some species are more prone to co-aggregate than other species. Co-aggregation tests have been proposed to be useful as a preliminary screening of probiotic strains as this ability is a desirable property (Collado et al., 2007a, Lee and Salminen, 2008). It has been proposed that selective interaction between MS and probiotic strains could present a way to remove MS from the oral microbiota without interfering with the other, normal microflora (Lang et al., 2010).

The co-aggregation assay tested the ability of eight probiotic lactobacilli to co-aggregate with strains of MS isolated from caries-free or caries-susceptible subjects. All tested probiotic lactobacilli co-aggregated with different strains of MS. There were small but statistically significant differences in the co-aggregation ratio between the strains and the null hypothesis was rejected. There were, however, no clear systematic patterns between MS from caries-free and caries-active subjects. *L. acidophilus* La5 was most prone to co-aggregate with all eight clinical isolates of MS. *L. rhamnosus* GG and *L. rhamnosus* LB21 was least prone to co-aggregate. Collado et al. studied different probiotic strains in their ability to inhibit the adhesion of pathogens. They demonstrated that the ability was dependent on both the specific probiotic strain and the pathogen tested, indicating a very high specificity (Collado et al., 2007b). This was in line with our results.

The findings on growth inhibition and co-aggregation are summarized in Table 4. The two *L. plantarum* strains had the best capacity to inhibit the growth of MS and *C. albicans*, while *L. acidophilus* was the most effective strain to co-aggregate with MS.

Table 4. Summary of the *in vitro* tests of growth inhibition and co-aggregation, **Study II and III.**

Strain	Inhibition MS	Inhibition <i>C. albicans</i>	Co-aggregation MS (120 min)
<i>L. plantarum</i> 299v	Excellent	Excellent	Medium
<i>L. plantarum</i> 931	Excellent	Excellent	Medium
<i>L. rhamnosus</i> LB21	Medium	Medium	Poor
<i>L. rhamnosus</i> GG	Medium	Medium	Medium
<i>L. paracasei</i> F19	Medium	Medium	Poor
<i>L. reuteri</i> PTA 5289	Medium	Poor	Medium
<i>L. reuteri</i> 55730	Medium	Excellent	Medium
<i>L. acidophilus</i> La5	Poor	Poor	Excellent

The possible effects of probiotic bacteria can be studied *in vitro*, *in situ*, in experimental animal models, and in clinical trials. The discipline of oral microbiology has traditionally studied planktonic cells in monospecies cultures. The *in vitro* inhibition test studied the interaction between two strains. It could be argued that studies in the context of dental caries, as a biofilm-mediated disease, should be more oriented toward biofilm studies. Bacteria in a biofilm express different genes and respond differently to antimicrobial substances. In the natural setting, there are also many other factors that may play a role for the effect of probiotic bacteria in the oral cavity, e.g. components from the saliva, availability of substrates, other bacteria, and food ingredients. Therefore, any result from *in vitro* studies with planktonic cells must be interpreted carefully. Before clinical application, such findings need to be verified in more complex settings. However, *in vitro* assays may be the first step in the process of finding good candidates for application of probiotics in new target places.

Re-colonization (Study IV)

The two *L. reuteri* strains were chosen to be evaluated in a clinical trial. The choice was a combination of the good results in the *in vitro* testing and practical reasons. The treatment with CHX resulted in reduced MS levels in both groups.

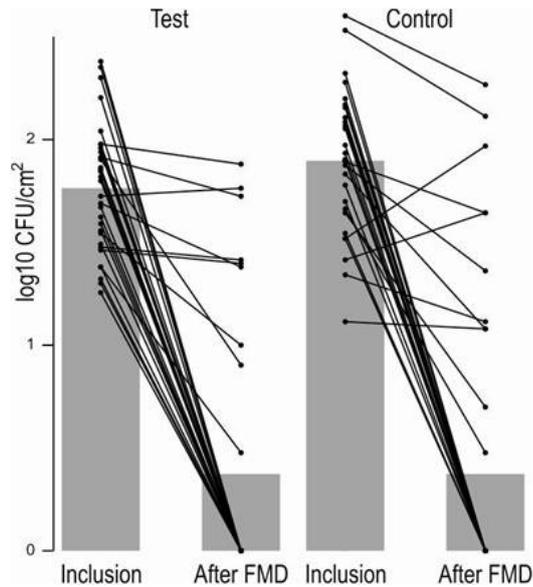


Figure 6. Individual levels of salivary MS (\log^{10} CFU/cm²) after full mouth disinfection with CHX in **Study IV**. FMD= full mouth disinfection.

The terminal goal of an oral application of antimicrobial therapy with CHX is to achieve a shift from an ecologically unfavourable to an ecologically stable biofilm (Twetman, 2004). Several studies have shown that treatment with CHX could selectively reduce the number of MS in the dental microbiota. However, this effect is usually short-termed and re-colonization usually occurs. Several RCT studies with *L. reuteri* strains showed a reduction in MS (Nikawa et al., 2004, Caglar et al., 2006, Caglar et al., 2007, Caglar et al., 2009), but that was not always the case (Cildir et al., 2012). We thought to investigate if the expected re-colonization of MS could be hampered by daily intake of *L. reuteri* DSM 17938 and ATCC PTA 5289. The full mouth disinfection regime with CHX reduced the salivary MS levels significantly ($p < 0.05$) in both the test and control groups. Notably, seven subjects did not respond to the treatment with CHX.

Evaluated with both chair side kits (Dentocult®) and the DNA-DNA checkerboard technique, it was demonstrated that the suppression of MS levels lasted less than 6 weeks in both groups and there were no statistical significant differences between the test and control groups at any of the testing occasions. The DNA-DNA checkerboard displayed no considerable differences in the microbial re-growth pattern with regard to the microorganisms in the checkerboard panel, except for *L. rhamnosus*, which was increased in the test group. *L. reuteri* was not included in the test panel.

Nigatu reported that *L. reuteri* and *L. rhamnosus* could not be distinguished by biochemical analysis only (Nigatu, 2000).

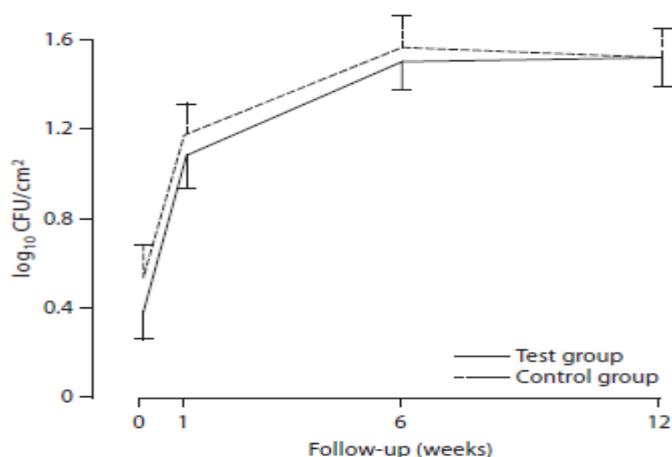


Figure 7. Mean salivary log₁₀ MS (CFU/cm²) at baseline and at the follow-ups in the intervention and control groups in **Study IV**. The vertical bars denote the standard deviation.

Only subjects with high levels of MS were included in this study as compared to previous studies, where an effect of *L. reuteri* DSM 17938 and ATCC PTA 5289 was demonstrated. It has been discussed whether the success of probiotic intervention is dependent of the stage of the disease and on the bacteria that are present at the diseased site (Devine and Marsh, 2009). It is important, however, to test the strains that are under consideration for caries prevention in subjects that have a clinical problem. Therefore, we decided to evaluate the effect in highly colonized (salivary MS counts $\geq 10^5$ CFU/mL) and demonstrated that supplementation with *L. reuteri* did not affect the re-growth of MS. The null hypothesis was, thus, firmly confirmed. The different inclusion criteria could be an explanation for the different results obtained here compared to those of most previous studies. It has later been demonstrated, however, that subjects in the test group that had *L. reuteri*, which was detected with molecular methods, seemed to have a slower re-growth of MS (Romani Vestman et al., 2013). It is an open question why the response to a probiotic intervention differs between individuals.

The reduction of MS is regarded as a surrogate measure for a caries-preventive effect. As dental caries usually develops over a long period, it is handy to measure MS fluctuations. It is easy to obtain a saliva or plaque sample and it

takes a short time to evaluate changes in MS counts. However, there are several drawbacks when using MS as a surrogate measure for dental caries. According to 'the ecological plaque hypothesis', MS is important but not the only microorganism in the pathogenesis of dental caries. In addition, surrogate endpoints such as levels of MS or plaque reduction may not always correlate with caries reduction (Caufield et al., 2001). One criticism of the replacement strategy and probiotic approaches is that they do not aim at other pathogens that may be involved in the disease (Anderson and Shi, 2006).

Concerning the 'replacement strategy' in the prevention of dental caries, there are other approaches than using probiotic lactobacilli and bifidobacteria. Hillman and colleagues introduced a non-acid-producing *S. mutans* strain that was developed to replace disease-causing strains. *In vitro* and animals studies with this strain have been promising, but evaluations in human RCT studies are still lacking (Hillman, 2002).

Long-term effects of intervention early in life (Study V)

A particular rationale for the follow-up study of the early intervention with LF19 was that it was a unique opportunity. At the time of the start of the study, no long-term studies had been reported on the possible effect of a probiotic intervention, so early in life on the development of the oral microbiota and dental caries. The rationale for introducing LF19 during weaning was to maintain the presence of the probiotic strain in the gut and the positive health outcomes associated with that (West et al., 2008). Because an effect of reduced eczema was demonstrated in the probiotic group at 1 year of age, it was crucial to investigate if that difference remained during childhood. It has also been questioned whether the use probiotic lactobacilli in young children for long periods may result in altered gastrointestinal microbiota with undesirable effects in the intestine (Benno et al., 2010). The few studies on probiotics that have examined caries as an outcome suggest that probiotics are beneficial rather than hazardous to the dental health in children (Nase et al., 2001, Stecksén-Blicks et al., 2009) and adults (Petersson et al., 2011). Therefore, it was considered important to evaluate possible effects of this intervention.

The two groups were similar with regard to background factors such as duration of breastfeeding, general health and socioeconomic background. The education level of the parents of the participating children was high and it is known that a lower socioeconomic status is associated with more caries during pre-school ages (Stecksén-Blicks et al., 2008). The incidence of caries was low in both groups of children, which limited the statistical power in the analysis of the main outcome measure.

There were no statistically significant differences for any of the outcomes and the null hypothesis was confirmed. MS was detected in 76% of children in both the probiotic and control groups. Lactobacilli colonies were detected in 84% of subjects in the probiotic group and 77% in the placebo group. The MS-total viable count ratio did not differ between the groups. MS was correlated to caries in the control group but not in the probiotic group. It is difficult to speculate what this interaction stands for. The prevalence of MS was correlated to parental smoking in both groups.

At 3 years of age, the mean caries score was 0 in the probiotic group and 0.2 ± 1.0 in the placebo group. At 6 years of age, it was 0.6 ± 1.7 and 0.7 ± 2.4 , respectively. At 9 years of age, 41% of participants in the probiotic group and 37% of participants in the placebo group had experienced caries.

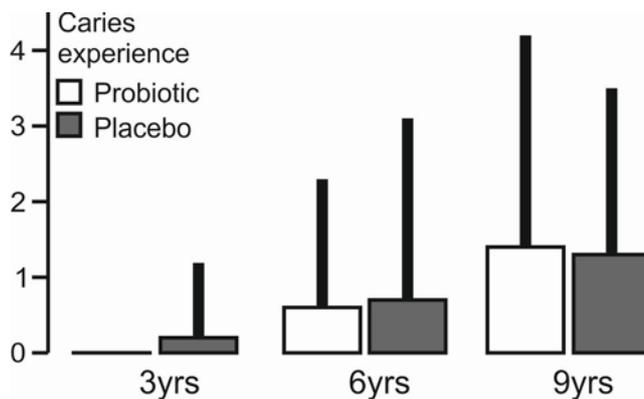


Figure 8. Total caries experience (mean dmfs + DMFS) in the probiotic and placebo groups at 3, 6, and 9 years of age in **Study V**. Black lines denote standard deviation.

The findings on dental caries were in line with those published by Taipale, who investigated if early intervention with *B. animalis* ssp. *lactis* BB-12 from 2 to 24 months of age had an effect on dental caries at 4 years of age. In that study, no differences were seen between the probiotic and placebo groups (Taipale, 2012).

It has been questioned whether exposure to probiotic bacteria early in life may facilitate a permanent incorporation into the oral microbiota (Meurman, 2005). In the *in vitro* studies of this thesis, LF19 was shown to have low acid production from sugars and to inhibit growth of MS. Further, LF19 was able to co-aggregate with different MS strains. These properties of LF19 were hypothesized to have potential to counteract early acquisition of MS, which is associated with increased caries prevalence in the primary and permanent dentition (Kohler et al., 1988, Thibodeau and O'Sullivan, 1999). In addition, it

was known that among certain naturally occurring lactobacilli that inhibit the growth of the patient's autologous MS *in vitro*, *L. paracasei* exhibits maximal interference activity against *S. mutans*, in particular in caries-free subjects (Simark-Mattsson et al., 2007). One interesting hypothesis from these findings is that naturally occurring lactobacilli may interfere with the colonization of caries-associated MS (Simark-Mattsson et al., 2007).

The lack of an effect on MS is in contrast to studies in children and young adults that have shown that the ingestion of various strains of probiotic lactobacilli can reduce levels of MS, although with a somewhat differing results. This inconsistency may be due to differences in the study designs/study populations and the different strains investigated (Nase et al., 2001, Stecksén-Blicks et al., 2009, Cildir et al., 2009, Lexner et al., 2010, Singh et al., 2011, Jindal et al., 2011, Cildir et al., 2012, Taipale et al., 2012, Juneja and Kakade, 2012, Sudhir et al., 2012). Because the follow-up was conducted eight years after the intervention, the composition of the oral microbiota at 13 months when the intervention was terminated remains an open question.

Probiotic lactobacilli can be recovered in the oral cavity during an intervention; however, they act as transient colonizers (Yli-Knuuttila et al., 2006, Saxelin et al., 2010, Caglar et al., 2009, Ravn et al., 2012). These earlier clinical studies on a possible installation of probiotic lactobacilli in the oral cavity were performed in subjects with mature microbiota. A recent study in infants showed that early exposure (from 2 to 24 months) to *B. animalis* ssp. *lactis* BB-12 did not result in permanent colonization by probiotic bacteria (Taipale et al., 2012). The findings in **Study V** support the view that probiotic lactobacilli are transient colonizers, even when administered early in life. The short contact time of the extrinsically administered probiotics to the oral cavity may have been a limiting factor. To be able to colonize the oral cavity, microorganisms have to attach to saliva proteins (the acquired pellicle), attach to epithelial cells, or co-aggregate with other bacteria (Haukioja et al., 2006). In an *in vitro* study in which hydroxyapatite assays were carried out, it was shown that the capacity of different probiotic strains to adhere varied. It was further demonstrated that the presence of *F. nucleatum* altered the capacity to adhere, showing the importance of other oral bacteria in modulating the colonization potential of probiotic strains (Haukioja et al., 2006). Using fluorescence *in situ* hybridization (FISH) and confocal laser scanning microscopy it was assessed if *B. animalis* ssp. *lactis* BB-12, *L. acidophilus* La-5, and LF19 were present in saliva, on oral mucosal and dental surfaces after frequent exposures for three days (Ravn et al., 2012). It was shown that the bacteria were present sporadically on oral mucosal surfaces and in saliva but not on dental surfaces. The authors discuss if the negative outcome may

depend on that probiotic bacteria are less competitive during weak acidic circumstances such as in young dental biofilms. This may apply to the children in **Study V**. Although *in vitro* studies and short term intervention studies has shown promising outcomes, the role of probiotic bacteria in oral biofilm mediated diseases still remains not satisfactory explored.

The two clinical studies, **Study IV** and **Study V**, failed to show any effect on caries-associated MS and no effect on caries prevalence was seen in **Study V**. It is important to emphasize that no probiotic strains have similar properties and, therefore, reproducible results could not be expected from investigations that use different species or strains, variable formulations, and diverse dosing schedules (Minocha, 2009). The participants' characteristics in a clinical study are also very important for the outcome. We tried to optimize the inclusion criteria for **Study IV** by excluding people with systemic diseases or those under medication. However, the oral microbiota of the participants is highly individual and compositional differences might define who is a responder and who is not (Reid et al., 2010). The International Scientific Association for Probiotics and Prebiotics (ISAPP) concludes that clinical trials investigating probiotics show that some patients benefit from the treatment while others do not (Reid et al., 2010).

A positive effect of *L. reuteri* ATCC 55730 and ATCC PTA 5289 was shown in an *in vitro* oral biofilm assay with alterations in both nascent and developed plaque ecosystems (Madhwani and McBain, 2011). These results was not the case in our *in vivo* study which is an good example of how difficult it is to translate results of *in vitro* testing to the clinical setting. To verify the effects of a certain probiotic strain or mixture of strains, it has to be evaluated in RCT studies.

One philosophy for the selection of certain strains is whether they are naturally occurring in the target site (Reid et al., 2010). One of the strains used in **Study IV** was originally isolated from the oral cavity of a Japanese woman and has been shown to exhibit potent immunomodulatory activity against the human inflammatory cytokine tumour necrosis factor (Egervarn et al., 2007, Jones and Versalovic, 2009). So far, however, no probiotic has been selected on the basis of its activity in a dynamic multi-species biofilm (Reid et al., 2010).

Strengths and weakness of the studies

Study I, II, and III: The evaluation of probiotic lactobacilli *in vitro* is an important step, to be followed by studies in more complex settings. Results from *in vitro* testing must be regarded as one piece in a puzzle and a good way of generating hypotheses that need to be further tested in the complex oral

environment. To increase the validity of *in vitro* testing between strains of probiotic bacteria and other strains, a fermentor in which biofilms could grow could have been used.

Study IV was a double-blinded RCT with two parallel arms. The RCT design is regarded as a golden standard for the evaluation of interventions. **Study IV** was a two-centre RCT, which enhances the external validity of the study results. The dropout rate was zero and the compliance to the study protocol was regarded as excellent. Another strength of the study was that we used both cultivation and molecular biology techniques to study the re-colonization of MS. A weakness of this study was the poor effect of the CHX-treatment in seven study participants. It is possible that the strategy of a short but intensive CHX treatment was not sufficient to reduce MS in all participants.

Study V was a follow-up of an earlier RCT. The original study had a low dropout rate and very good compliance. In the follow-up eight years after the intervention, almost 70% of the original study population agreed to participate, which is a good number for these kinds of studies. A weakness of this study was that the caries data at the age of 3 and 6 years were collected retrospectively from the participants' dental records. A drawback with such data is that many clinicians were involved in the registrations, which limits the reliability. Ideally, the same examiner would have examined all children in both groups to remove the effects of variation in caries diagnosis between different examiners. A strength, however, was that the data collectors were blinded to the group allocation. When bitewing radiographs were available, recordings on posterior approximal caries were performed by the data collector, which increased the level of evidence. The caries prevalence was low and, therefore, the results should be interpreted carefully. The long time between exposure and follow-up is a weakness. It is an open question whether LF19 could have been recovered from oral samples during the intervention and at earlier follow-ups.

Future perspectives

The projects within this thesis have been tremendously interesting to be a part of and they have provided some answers to the research questions; however, more questions have arisen. There is so much to be studied in the chain of reactions, which bacteria colonize the child, the complex interactions of bacteria in the biofilm, and the development of an aciduric biofilm, which is able to cause disease. One interesting approach would be to study if and how different probiotic lactobacilli could affect biofilm formation *in vitro* and *in vivo*. Another interesting project in the future would be to investigate if an intervention with a selected probiotic strain given to mother and child during the end of pregnancy and during a few years of childhood would affect the colonization of MS and the risk of other diseases (for example allergic diseases).

Conclusions

The capacity to produce acid from dietary sugars and sugar alcohols differed between the investigated probiotic strains.

The probiotic strains were able to inhibit growth of MS and *C. albicans in vitro*. The inhibitory capacity was strain- and dose-dependent and depended on the pH in the medium. No apparent pattern was seen, regardless of whether the MS strain was a clinical isolate or not, or if it was isolated from subjects with or without caries.

The probiotic *Lactobacillus* strains displayed co-aggregation activity with all MS strains at varying degrees and the ratio varied between strains from different subjects.

Intervention with two *L. reuteri* strains (DSM 17938 and PTA 5289) after treatment with CHX did not affect or delay the re-colonization pattern of MS. Furthermore, the intervention did not alter the re-colonization pattern of other oral species.

Feeding LF19 during weaning (between 4 and 13 months of age) did not affect the presence of MS or lactobacilli at 9 years of age. The prevalence of caries was not affected at 3, 6, or 9 years of age. LF19 was not incorporated into the oral microbiota of the children.

The quality and quantity of evidence regarding the effectiveness of the application of specific probiotics in the prevention of dental caries are limited and do not justify conclusions concerning the use of probiotics in caries prevention.

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