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PROBIOTICS AND ORAL HEALTH: *IN VITRO* AND CLINICAL STUDIES

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ABSTRACT

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Probiotics and oral health: *in vitro* and clinical studies

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Probiotics are used, for example, to prevent and treat diarrhea, allergies and respiratory infections, and there is an increasing interest to use probiotics also for oral health purposes. The most commonly used probiotic bacteria are lactobacilli and bifidobacteria, which are acidogenic and aciduric. From the oral point of view, use of these probiotics may, at least in theory, mean an increased risk of caries.

In this thesis, the effects of probiotics on oral microbial composition, acid production of dental plaque and gingival health were studied through *in vitro* studies and two clinical studies. In a randomized, double-blind and crossover study, 13 healthy adults were allocated into two groups. Half of the subjects first consumed *Lactobacillus rhamnosus* GG tablets twice a day for two weeks, and after the washout period, *L. reuteri* tablets twice a day for two weeks. The other half of the subjects used the tablets in reverse order. In another controlled, randomized and double-blind study, 62 healthy adults were allocated into two groups. One group used the test tablets containing *L. rhamnosus* GG and *B. lactis* BB-12 and the other group used control tablets without probiotics. The recommendation for the use of the tablets was 4 per day for 4 weeks.

Probiotic lactobacilli interfered with *S. mutans* biofilm formation and the adhesion of *S. mutans* to saliva-coated hydroxyapatite *in vitro*. No effect was found in *S. mutans* levels in the three-species biofilms. In clinical studies, the studied probiotics had no effect on the acid production of plaque. The counts of mutans streptococci and the oral microbial composition remained the same. Tablets containing *L. rhamnosus* GG and *Bifidobacterium animalis* subsp. *lactis* BB-12 did decrease the amount of plaque and gingival bleeding. According to our results, it seems that probiotics have beneficial effects on gingival health.

The present results confirmed that probiotics are safe and have beneficial effects on oral health. Since the consumption of probiotics by the general population is steadily increasing, an understanding of the functions of probiotics in the oral cavity has become more important.

Keywords: lactobacilli, bifidobacteria, caries, periodontal disease, mutans streptococci, probiotics

TIIVISTELMÄ

Aino Toiviainen

Probiootit ja suun terveys: *in vitro* ja kliiniset tutkimukset

Turun yliopisto, Lääketieteellinen tiedekunta, Hammaslääketieteen laitos, Parodontologia, Suun terveystieteiden tohtoriohjelma (FINDOS-Turku), Turku, Suomi

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Probiootteja käytetään esimerkiksi ripulin, allergioiden ja hengitystieinfektioiden ehkäisyssä ja hoidossa. Kiinnostus niiden käyttöön myös suun terveyden edistämiseksi lisääntyy. Yleisimmin käytetyt probiootit kuuluvat laktobasilleihin tai bifidobakteereihin, joten ne ovat hyviä hapontuottajia ja viihtyvät happamissa olosuhteissa. Suun terveyden kannalta tämä voisi teoriassa tarkoittaa lisääntynyttä riskiä hampaiden reikiintymiselle.

Väitöskirjatutkimuksessa selvitettiin probioottien vaikutusta suun mikrobistoon, plakin hapontuottoon ja ienterveyteen laboratoriokokeiden ja kahden kliinisen tutkimuksen avulla. Kliiniset tutkimukset olivat satunnaistettuja kaksoissokkotutkimuksia, joista ensimmäisessä 13 tervettä aikuista jaettiin kahteen ryhmään. Puolet koehenkilöistä käytti ensin *Lactobacillus rhamnosus* GG -tabletteja kahdesti päivässä kahden viikon ajan ja puhdistusjakson jälkeen *L. reuteri* -tabletteja kahdesti päivässä kahden viikon ajan. Toinen puoli koehenkilöistä käytti tabletteja päinvastaisessa järjestyksessä. Toisessa kliinisessä tutkimuksessa 62 tervettä aikuista jaettiin kahteen ryhmään. Puolet koehenkilöistä käytti testitabletteja, jotka sisälsivät *L. rhamnosus* GG- ja *B. lactis* BB-12 -probiootteja, ja toinen puoli käytti kontrollitabletteja, joista probiootti puuttui. Käyttösuositus oli 4 tablettia päivässä 4 viikon ajan.

Laktobasillit vaikuttivat *S. mutans*in biofilmin muodostukseen ja tarttumiseen syljellä päällystetylle hydroksiapatiitille *in vitro*. Vaikutusta ei havaittu *S. mutans*in määrässä kolmen lajin biofilmissä. Kliinisissä tutkimuksissa tutkituilla probiooteilla ei ollut vaikutusta plakin hapontuottoon. Myöskään mutans streptokokkien määrässä tai suun mikrobikoostumuksessa ei havaittu eroja. *L. rhamnosus* GG- ja *Bifidobacterium animalis* subsp. *lactis* BB-12 -probiootteja sisältänyt yhdistelmätabletti vähensi plakin määrää ja ienverenvuotoa. Probiooteilla on tulostemme perusteella ienterveyttä parantava vaikutus.

Saadut tulokset tukevat käsitystä siitä, että probiooteilla olisi edullisia vaikutuksia suun terveyteen. Probioottien käytön yleistyessä väestössä on yhä tärkeämpää ymmärtää probioottien toimintaa suuontelossa.

Avainsanat: laktobasillit, bifidobakteerit, karies, iensairaus, mutans streptokokit, probiootit

TABLE OF CONTENTS

ABBREVIATIONS

BHI	Brain-heart infusion medium
BSA	Bovine serum albumin
CFU	Colony-forming unit
DNA	Deoxyribonucleic acid
FAO	Food and Agriculture Organization of the United Nations
FMM	Fermentation minimal medium
FUM	Fluid universal medium
HA	Hydroxyapatite
HOMIM	Human oral microbe identification microarray
LB	Lactobacilli
LGG	*Lactobacillus rhamnosus* GG
MRS	de Man, Rogosa and Sharpe medium
MS	Mutans streptococci
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
rRNA	Ribosomal ribonucleic acid
sFUM	Fluid universal medium supplemented with 0.3 % sucrose
SHA	Saliva-coated hydroxyapatite
TSB	Tryptic soy broth
WHO	World Health Organization

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications, which will be referred to in the text by the Roman numerals I–IV:

I. Söderling E, **Marttinen A**, Haukioja A. Probiotic lactobacilli interfere with *Streptococcus mutans* biofilm formation in vitro. Curr Microbiol 2011: 62:618-622.

II. **Marttinen A**, Haukioja A, Keskin M, Söderling E. Effects of *Lactobacillus reuteri* PTA 5289 and *L. paracasei* DSMZ16671 on the adhesion and biofilm formation of *Streptococcus mutans*. Curr Microbiol 2013: 67:193-199.

III. **Marttinen A**, Haukioja A, Karjalainen S, Nylund L, Satokari R, Öhman C, Holgerson P, Twetman S, Söderling E. Short-term consumption of probiotic lactobacilli has no effect on acid production of supragingival plaque. Clin Oral Investig 2012: 16:797-803.

IV. **Toiviainen A**, Jalasvuori H, Lahti E, Gürsoy U, Salminen S, Fontana M, Flannagan S, Eckert G, Kokaras A, Paster B, Söderling E. Impact of orally administered lozenges with *Lactobacillus rhamnosus* GG and *Bifidobacterium animalis* subsp. *lactis* BB-12 on the number of salivary mutans streptococci, amount of plaque, gingival inflammation and the oral microbiome in healthy adults. Clin Oral Investig 2015: 19:77-83.

1. INTRODUCTION

Lilly and Stillwell (1965) introduced the word 'probiotics' as 'substances produced by microorganisms which promote the growth of other microorganisms'. Currently the word 'probiotic', meaning 'for life', is used when referring to bacteria associated with beneficial effects on humans and animals, and by the definition probiotics are live micro-organisms that, when administered in adequate amounts, confer a health benefit on the host (WHO/FAO 2002, Hill et al. 2014). Existing evidence suggests that probiotic health effects are strain-specific. The most commonly used and most widely studied probiotic bacteria are lactobacilli and bifidobacteria, and they are used for example in yoghurts and other dairy products, juices and tablets. The primary target for probiotics is the gastrointestinal tract, but when used orally, also the mouth is exposed to probiotic bacteria. Furthermore, the effects of probiotics on oral health are studied extensively at the moment, and new studies are published at a growing rate. To be effective in the mouth probiotic microorganisms should resist the oral environmental conditions and defense mechanisms, be able to adhere to saliva-coated surfaces, colonize and grow in the mouth, and inhibit oral pathogens (Stamatova and Meurman 2009a). They should also be safe for the host. The use of probiotic bacteria is 'generally regarded as safe' (GRAS) by the United States Food and Drug Administration (FDA).

2. REVIEW OF LITERATURE

2.1 Oral microbiota and dental plaque

2.1.1 Bacterial colonization

Contact with microbes begins *in utero* and continues during birth and early infancy (for review see Rautava et al. 2012). The mode of delivery affects the composition of the intestinal and oral microbiota in infants. Infants get their first bacteria from the birth canal of the mother when delivered vaginally. Infants delivered by Caesarean section (C-section) are exposed to bacteria originating from the hospital environment and health-care workers. Studies have shown that there is a difference in intestinal microbial composition between infants delivered vaginally or by C-section (Biasucci et al. 2008, Adlerberth and Wold 2009). Infants delivered by C-section have a less diverse microbial composition and fewer health-promoting strains of lactobacilli and bifidobacteria in the gut, compared to vaginally born infants (Penders et al. 2006). Also the oral microbiota is less diverse in infants delivered by C-section (Lif Holgerson et al. 2011), and in the oral microbial profile, common oral streptococci and lactobacilli are more prevalent in infants delivered vaginally compared with those delivered by C-section (Nelun Barfod et al. 2011). Mode of delivery affects also *Streptococcus mutans* colonization, and it seems that infants delivered by C-section acquire *S. mutans* 11 months earlier than infants delivered vaginally (Li et al. 2005). Colonization is also influenced by medical, cultural and other environmental factors such as diet, familial environment, diseases and therapies (Matamoros et al. 2013).

In the oral cavity, the first bacterial colonization takes place on oral surfaces including the mucosal surfaces of lips, cheeks, palate and tongue. Plonka et al. 2012 showed that mutans streptococci and lactobacilli can colonize an infant's oral cavity even before the eruption of the first tooth. The first streptococci to colonize the infant's oral cavity include *S. oralis, S. mitis* and *S. salivarius* (Smith et al. 1993, Pearce et al. 1995). The mother is considered to be the principal source of an infant's mutans streptococci (Berkowitz and Jones 1985, Li and Caufield 1995). After tooth eruption, the oral microbiota changes and *S. sanguinis* and *S. mutans* colonize the oral cavity (Caufield et al. 1993). The teeth provide the bacteria with a good colonization habitat because they form a non-shedding surface. Microorganisms that do not adhere to oral surfaces are washed away with saliva.

Tanner et al. (2002) found that a wide range of species, including *S. mutans* and putative periodontal pathogens, can be detected in oral samples from children under 3 years old. *S. mutans* and the periodontal pathogens *Porphyromonas gingivalis* and *Bacteroides forsythus* (now known as *Tannerella forsythia*) were detected even in the youngest subjects. Species

detection from tooth and tongue samples was highly associated, with most species detected more frequently from tongue than tooth samples in the younger children, suggesting that the tongue serves as a reservoir for tooth-associated species.

In adults, approximately 700 bacterial species have been detected in the oral cavity (Aas et al. 2005). There is a distinctive, predominant bacterial flora of the healthy oral cavity that is highly diverse and site and subject specific. Most sites harbor 20–30 different predominant species, and the number of species varies from 34 to 70 in an individual mouth (Aas et al. 2005). Mager et al. (2003) showed that in healthy subjects, the proportions of 40 oral bacterial species significantly differ on different intraoral surfaces. They also found that the microbiota of the soft tissues were more similar to each other than the microbiota of supragingival and subgingival plaques.

2.1.2 Biofilm formation

The term 'biofilm' is used to describe communities of microorganisms attached to a surface, and microbial community associated with teeth is referred to as dental plaque. Dental plaque is a form of biofilm found naturally on health, but it is also associated with dental caries and periodontal disease. Dental plaque is formed via an ordered sequence of events, resulting in a structurally and functionally organized species-rich microbial biofilm (Marsh et al. 2011).

Biofilm formation has different stages. First, the conditioning film (the acquired pellicle) is formed immediately after tooth eruption or cleaning. Then, weak, long-range, physico-chemical interactions form between the microbial surface and the conditioning film, resulting in a reversible adhesion, which leads eventually to irreversible adhesion between the adhesins on the microbial cell surface and the receptors present in the acquired pellicle. After the early colonizers have attached to the conditioning film, secondary and later colonizers start co-adhesion to already attached early colonizers, which leads to an increase in microbial diversity within the developing biofilm. Multiplication of the attached cells leads to confluent growth and a three-dimensional, spatially and functionally organized, mixed-culture biofilm, which also has a complex extracellular matrix made up of, for example, soluble and insoluble glucans, fructans and heteropolymers. The matrix makes a significant contribution to the known structural integrity and general resistance of biofilms. After the mature and quite stable biofilm has formed, active detachment can occur, enabling cells to colonize elsewhere (Marsh 2004, Marsh et al. 2011).

The term 'coaggregation' is used if the binding between bacteria occurs in suspension. If the adherence of microbial cells occurs in already attached bacteria in a biofilm, the term used is 'coadhesion'. The mechanisms behind coaggregation and coadhesion are supposed to be identical (for review see Kolenbrander et al. 2010). The predominant initial colonizers include streptococci such as *S. gordonii, S. mitis, S. oralis* and *S. sanguinis* but also genus *Actinomyces*. *Fucobacterium nucleatum* is essential in the formation of

biofilm because it can coaggregate with initial, early and late colonizers and it acts as a bridge between organisms in developing dental plaque (for review see Kolenbrander et al. 2010).

Plaque bacteria have broader habitat range, more efficient metabolism, increased tolerance to inhibitory agents and host defenses, and enhanced virulence when in the form of a biofilm (Marsh et al. 2011). In health, there is a symbiosis between oral microbiota and the host. The composition of the oral microbiota is influenced by temperature, pH, nutrients as well as host genetics and defenses. When changes occur in oral environmental conditions, the balance between the host and the oral microbiota can shift and result in an increased risk of disease (Marsh and Devine 2011).

2.2 Dental caries

Several hypotheses concerning the role of plaque bacteria in the etiology of dental caries have been proposed. Miller (1889) linked microbial acid production from dietary substrates to the etiology of dental caries. According to Miller, caries is a bacteriologically non-specific process, which means that there is no single acidogenic species that can be associated with caries. Instead, caries is an outcome of increased amounts of acid formed by the bacteria on tooth surface. In the non-specific plaque hypothesis the disease is considered to be the outcome of the overall activity of the total plaque microflora. Loesche (1976) introduced the specific plaque hypothesis, which proposed that only a few species out of the diverse collection of organisms comprising the resident plaque microflora are actively involved in disease. The ecological plaque hypothesis combined the two earlier hypotheses and proposed that the organisms associated with disease may also be present at sound sites, but at levels too low to be clinically relevant. Disease is a result of a shift in the balance of the resident microflora due to a response to a change in local environmental conditions (Marsh 1994, 2004).

The most recent hypothesis is the extended caries ecological hypothesis (Takahashi and Nyvad 2011). According to this hypothesis, the caries process consists of 3 reversible stages. The first stage is called the dynamic stability stage, and the microflora on clinically sound enamel surface contains mainly non-mutans streptococci and *Actinomyces*. The acidification is mild and demineralization is in equilibrium with remineralization. In the second stage, which is called the acidogenic stage, acidification becomes more frequent due to increased sugar consumption. This leads to an increase in counts of aciduric strains. The initiation of dental caries then occurs due to demineralization. The final stage is called the aciduric stage. More aciduric bacteria become dominant if the acidic conditions continue. Mutans streptococci and lactobacilli as well as aciduric strains of non-mutans streptococci, *Actinomyces*, bifidobacteria and yeasts may become dominant. Many acidogenic and aciduric bacteria are involved in the caries process.

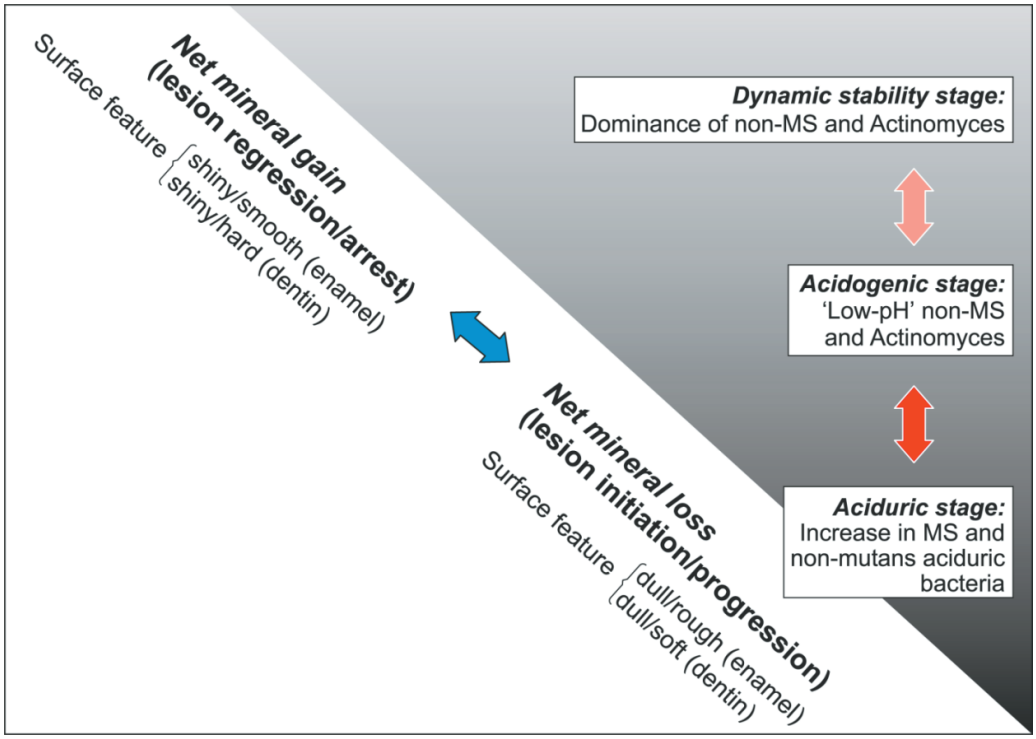

Figure 1. An extended caries ecological hypothesis according to Takahashi and Nyvad (2008; with permission).

Early mutans streptococci colonization is a risk factor for caries (Köhler et al. 1988, for review see Thenisch et al. 2006, Meurman and Pienihäkkinen 2010). Although high mutans streptococci counts do not necessarily mean increased caries risk in adults, decreasing mutans streptococci levels without affecting the healthy microbiota should make the plaque less virulent. Mutans streptococci are frequently isolated from cavitated caries lesions, can induce caries formation in animals fed a sucrose-rich diet and are highly acidogenic and aciduric (Hamada and Slade 1980, Loesche 1986). Mutans streptococci have a central role in the initiation of dental caries on enamel and root surfaces (for review see Tanzer et al. 2001). Lactobacilli and bifidobacteria have also been isolated from carious lesions together with mutans streptococci (Becker et al. 2002).

2.3 Periodontal disease

Gingivitis is a bacterial infection-induced inflammation of the gingival tissues surrounding the tooth. Bacterial attachment on the tooth surface and coadhesion of initial colonizers with other species initiate the formation of biofilm. Mature biofilm, which is also called bacterial dental plaque, stimulates a cascade of events that leads to gingival inflammation. Gingivitis, either at an acute or chronic state, is a reversible disease, since there is no loss of hard tissues that support the teeth. The gingival tissues return to their healthy state when the bacterial biofilm is removed.

The extension of inflammation to the connective tissue, periodontal ligament and alveolar bone initiates an irreversible tissue loss around the tooth, which is called periodontitis. The microbial composition of periodontitis differs from healthy gingiva and gingivitis (Darveau et al. 1997). *P. gingivalis, Treponema denticola* and *T. forsythia* (formerly known as *B. forsythus*) are significantly more prevalent in both supra- and subgingival plaque samples of periodontitis subjects compared to those of healthy subjects (Ximénez-Fyvie et al. 2000) and are also related to clinical measures of periodontal disease, including pocket depth and bleeding on probing (Socransky et al. 1998). The main etiological factor for a plaque-related periodontal inflammation is the presence of pathogenic bacteria, and, in connection, the absence of so-called 'health-related beneficial' bacteria; however, environmental factors and the susceptibility of the host also play major roles (Slots and Rams 1991, Socransky and Haffajee 1992, Wolff et al. 1994). Altogether, these four main factors determine whether a subject develops periodontitis, or whether the inflammation is limited within the gingiva.

2.4 Probiotic lactobacilli and bifidobacteria

2.4.1 Probiotics and general health

Recent studies have demonstrated a beneficial health impact of specific probiotic bacteria in humans, leading to several new recommendations for probiotic use (for review see Zhang et al. 2015 and Zajac et al. 2015).

Probiotic bacteria have beneficial health effects especially when used to prevent or treat gastrointestinal infections (Hatakka and Saxelin 2008) and in the outcome of respiratory tract infections (for review see Lehtoranta et al. 2014). *Lactobacillus rhamnosus* GG seems to be effective in preventing and treating rotavirus diarrhea, atopic eczema and upper respiratory infections (Isolauri and Salminen 2008, Floch et al. 2011, Kumpu et al. 2012). It has also decreased symptoms associated with crying and fussing in preterm infants (Pärtty et al. 2013). Similarly to *L. rhamnosus* GG, also *Bifidobacterium animalis* subsp. *lactis* BB-12 (*B. lactis* BB-12) has been used to prevent and treat diarrhea and respiratory infections (Weizman et al. 2005, Taipale et al. 2011). It seems that together, *L. rhamnosus* GG and *B. lactis* BB-12 have additional efficacy in the prevention and treatment of allergic disorders, acute respiratory infections and acute otitis media (Isolauri et al. 2000, Rautava et al. 2009, Smith et al. 2013).

The combination of *L. rhamnosus* GG and *B. lactis* BB-12 has also reduced the frequency of gestational diabetes mellitus in normal-weight women when combined with dietary counseling during pregnancy (Luoto et al. 2010). A multicenter RCT (randomized controlled trial) study, SPRING, focusing on the combination of the two probiotics in prevention of gestational diabetes mellitus in overweight and obese women, was begun recently (Nitert et al. 2013).

2.4.2 Probiotics and oral health

2.4.2.1 *In vitro* studies

In oral health, important mechanisms of probiotics include the capability to adhere and persist on the oral mucosa and teeth. Probiotic lactobacilli have shown different degrees of adhesion to saliva-coated hydroxyapatite surfaces in *in vitro* studies, and it seems that *L. rhamnosus* strains can adhere better than *L. reuteri* or *L. delbrueckii* subsp. *bulgaricus* strains to saliva-coated hydroxyapatite (Haukioja et al. 2006, Stamatova et al. 2009, Samot et al. 2011). Probiotics form also biofilms, as demonstrated for example with *L. rhamnosus* GG and *L. reuteri* (Lebeer et al. 2009, Jalasvuori et al. 2012).

One factor that can influence adhesion is the interaction between oral microorganisms and probiotics. Haukioja et al. (2006) showed that *Fusobacterium nucleatum* -coating of hydroxyapatite modified the binding of probiotics *in vitro.* Lactobacilli have also been shown to be able to coaggregate with oral streptococci (Twetman et al. 2009, Keller et al. 2011). Heat-killed *L. paracasei* DSMZ16671 can also coaggregate with mutans streptococci (Lang et al. 2010).

Both lactobacilli and bifidobacteria are acidogenic and aciduric. Some lactobacilli and bifidobacteria, for example *L. rhamnosus* GG and *B. lactis* BB-12, are not able to ferment sucrose (Silva et al. 1987, Haukioja et al. 2008a). There are similar results with *L. reuteri* and *L. paracasei* (Hedberg et al. 2008). Sucrose fermentation by probiotics can be considered a virulence factor for dental caries. Probiotic lactobacilli may inhibit caries-associated microorganisms via antimicrobial substances active at a low pH (Meurman et al. 1995, Hasslöf et al. 2010, Keller at al. 2011). The growth inhibition of *S. mutans in vitro* has been attributed to the generation of a low pH either via organic acid production and/or production of bacteriocins or metabolites active at a low pH (Simark-Mattsson et al. 2009).

In biofilm experiments, heat-inactivated *B. lactis* BB-12 have reduced the cariogenicity of *S. mutans* in dentinal cavities (Schwendicke et al. 2014), and different lactobacilli have inhibited the growth and biofilm formation of mutans streptococci (Lee and Kim 2014, Lin et al. 2015, Wu et al. 2015). *L. rhamnosus* GG inhibited the growth of *S. mutans* both in dual species biofilms and in complex saliva-derived biofilms without affecting the pH (Pham et al. 2011).

Simark-Mattsson et al. (2007) isolated lactobacilli from the saliva and plaque of young subjects with different caries experience to study if naturally occurring oral lactobacilli have probiotic properties. The results showed that naturally occurring lactobacilli inhibited the growth of mutans streptococci *in vitro*, and the effect was most efficient with lactobacilli from subjects without caries or *S. mutans*.

Early *in vitro* studies demonstrated that lactobacilli possess antimicrobial activities against periodontopathogens including *A. actinomycetemcomitans, P. gingivalis* and *P. intermedia* (Sookkhee et al. 2001, Kõll-Klais et al. 2005). Baca-Castañón et al. (2015) tested the

antimicrobial activity of *L. reuteri* on pathogenic bacteria involved in the formation of dental caries and periodontal disease. According to the results, *L. reuteri* was shown to have an inhibitory effect against *S. mutans*, followed by *T. forsythia* and *S. gordonii,* and a less significant effect against *Actinomyces naeslundii*. Kang et al. (2011) found that different *L. reuteri* strains had significant inhibitory effects on the growth of periodontopathic bacteria, including *Aggregatibacter actinomycetemcomitans, F. nucleatum, P. gingivalis* and *T. forsythia*, and the formation of *S. mutans* biofilms. Stamatova et al. (2007) found that *A. actinomycetemcomitans* strains were inhibited by different lactobacilli strains, and *L. rhamnosus* GG strains were able to inhibit *P. gingivalis* and *F. nucleatum*.

2.4.2.2 Colonization of the oral cavity

Clinical studies demonstrate that probiotic lactobacilli and bifidobacteria are not able to permanently colonize the oral cavity of adults. The presence of *L. reuteri* in saliva is only temporary and, after the consumption stops, *L. reuteri* is gradually eliminated from the saliva within a few weeks (Caglar et al. 2009, Sinkiewicz et al. 2010). There are similar results for *L. rhamnosus* GG (Yli-Knuuttila et al. 2006, Saxelin et al. 2010), although in the study by Yli-Knuuttila et al. (2006), there was one female subject who harbored *L. rhamnosus* GG even 5 months after the probiotic intervention period had stopped. She had received *L. rhamnosus* GG milk at the age of 10 for 1 year as a treatment for atopic dermatitis.

Saxelin et al. (2010) studied administration of a combination of *L. rhamnosus* GG and LC705, *Propionibacterium freudenreichii* subsp. *shermanii* JS and *B. lactis* BB-12 as capsules, yoghurt or cheese. The study included a 4-week run-in, 2-week intervention and 3-week follow-up period. The amount of probiotics consumed was 10^{10} CFU/day. Saliva and faecal samples were collected before, during and after the intervention. *L. rhamnosus* GG was the only probiotic strain regularly recovered in saliva samples. During the intervention period, *L. rhamnosus* GG was recovered in 88 % of the volunteers at least once. There were no differences between the yoghurt and the cheese group. At the end of the follow-up period, three participants had *L. rhamnosus* GG in their saliva but they were carrying it already at the end of the run-in period. During the intervention period, *B. lactis* BB-12 could be detected only in one participant in the yoghurt and in one participant in the cheese group. Taipale et al. (2012) showed that the administration of *B. lactis* BB-12 in early childhood did not result in permanent oral colonization of the probiotic. Also Hasslöf et al. (2013) found no oral colonization of the probiotic after early administration of *L. paracasei* F19.

2.4.2.3 Probiotics and dental caries

Most studies with probiotics focus on measuring the effects of probiotics on mutans streptococci, since they are easy to study in short-term trials and can be considered to be a risk factor of caries (for review see Stamatova and Meurman 2009b). Several probiotics have in clinical studies demonstrated the capacity to reduce mutans streptococci counts in saliva and plaque (for review see Cagetti et al. 2013). However, only a few trials on probiotics and caries occurrence have been published (for review see Laleman et al. 2014).

Several studies demonstrate that probiotics in different products can reduce the salivary or dental plaque levels of mutans streptococci. *L. reuteri* has decreased the counts of mutans streptococci in saliva when used in gums, straws, tablets or yoghurt (Nikawa et al. 2004, Caglar et al. 2006, 2007). In a recent study, the persistence of *L. reuteri* DSM 17938 and ATCC PTA 5289 in saliva could delay the regrowth of mutans streptococci after full-mouth disinfection with chlorhexidine (Romani Vestman et al. 2013). The consumption of *L. rhamnosus* GG has shown a trend of decreasing mutans streptococci counts when consumed for 3 weeks in the form of cheese or for 7 months in milk (Näse et al. 2001, Ahola et al. 2002).

Short-term consumption of *L. paracasei* seemed to enhance its inhibitory effect against *S. mutans* after the intervention (Chuang et al. 2011). Heat-killed *L. paracasei* DSMZ16671 significantly reduced salivary mutans streptococci when used as suckable candies (Holz et al. 2013). *L. paracasei* DSMZ16671 has also decreased caries occurrence in animal experiments (Tanzer et al. 2010). Probiotic milk containing *L. paracasei* SD1 reduced salivary mutans streptococci counts (Ritthagol et al. 2014, Teanpaisan and Piwat 2014). *L. casei* Shirota in milk has also reduced salivary mutans streptococci (Yadav et al. 2014).

Probiotic ice-cream containing *B. lactis* BB-12 and *L. acidophilus* LA-5 reduced salivary mutans streptococci levels (Singh et al. 2011). Daily consumption of a dairy probiotic product, Espar, decreased mutans streptococci counts of saliva (Poureslami et al. 2013). There are also contradictory results. The consumption of *L. rhamnosus* LB21 or lozenges with *L. reuteri* DSM 17938 and ATCC PTA 5289 did not affect mutans streptococci counts (Stecksén-Blicks et al. 2009, Keller and Twetman 2012). The early administration of *B. lactis* BB-12 did not affect mutans streptococci colonization in children (Taipale et al. 2012, Taipale et al. 2013). Yoghurt containing *B. lactis* DN-173010 did not affect either salivary or dental plaque levels of mutans streptococci in patients undergoing orthodontic treatment (Pinto et al. 2014).

Both lactobacilli and bifidobacteria are acidogenic and aciduric. From an oral health point of view, this could mean an increase in caries risk since the exposure to probiotic lactobacilli could lead to increased production of organic acids in dental plaque. Clinical studies, however, do not support this. Keller and Twetman (2012) studied the effect of lozenges with *L. reuteri* DSM 17938 and ATCC PTA 5289 on the concentration of lactic acid in supragingival plaque. Subjects consumed either a probiotic lozenge or a placebo for 2 weeks, and the concentration of lactic acid in supragingival plaque samples was determined at baseline and after the consumption. The results showed that there were no significant differences in lactic acid production between the baseline and the 2-week samples or between the test and placebo. In a recent study, consumption of *L. salivarius* did not affect salivary pH, but salivary buffering capacity significantly increased in the *L. salivarius* group compared to the xylitol group (Nishihara et al. 2014).

The few studies on the effects of probiotics on caries occurrence suggest that probiotics could be beneficial rather than harmful to oral health. In a Finnish study (Näse et al. 2001), 594 children aged 1–6 in day-care centers were given either milk containing *L. rhamnosus*

GG (5–10 x 10^5 CFU/ml) or a placebo milk 5 days a week for 7 months. The results showed that the *L. rhamnosus*-milk reduced caries occurrence, especially in the group of 3- to 4-year-old children. In a Swedish study (Stecksén-Blicks et al. 2009), 248 children aged 1–5 years in day-care centers were given either 150 ml milk supplemented with *L. rhamnosus* LB21 (10^7 CFU/ml) and 2.5 mg fluoride per liter or standard milk on weekdays for 21 months. The results showed that the daily consumption of milk containing probiotic bacteria and fluoride reduced caries in the preschool children. In this study, fluoride was a confounding factor, and it is therefore difficult to estimate the effect of the probiotics. In a recent study, older adults with primary root caries lesions were given milk supplemented with fluoride and/or probiotic lactobacilli, which seemed to reverse the soft and leathery texture of primary root caries lesions better than milk alone (Petersson et al. 2011). Stensson et al. (2014) compared caries prevalence in the primary dentition of 9-year-old children when the mothers during the last month of gestation and the children during the first year of life had received daily oral *L. reuteri* ATCC 55730 supplementation. The prevalence of approximal caries lesions was lower in the probiotic group compared to the placebo group.

The study of Taipale et al. (2013) found no effect for *B. lactis* BB-12 on caries prevalence in 4-year-old children who had been given BB-12 tablets with a slow-release pacifier. Nor did Hasslöf et al. (2013) find any effects on caries occurrence in 9-year-old children who had eaten *L. paracasei* F19 in their cereal for the first 4–13 months of their life.

Interestingly, a cheese containing a mixture of *L. rhamnosus* GG, *L. rhamnosus* LC705 and *P. freudenreichii* ssp. *shermanii* JS has been reported to decrease oral *Candida* infections in the elderly (Hatakka et al. 2007). Even though *Candida* is not a risk factor of caries occurrence, it is an oral pathogen when present in the oral cavity in high numbers. In addition, *C. albicans* is suggested as a caries pathogen due to its high acidogenicity and prevalence in caries lesions (Beighton et al. 2004, Klinke et al. 2008).

2.4.2.4 Probiotics and periodontal disease

Traditionally, anti-infective treatment of periodontal diseases aimed to reduce the number of periodontitis-associated bacteria in the oral cavity, either by mechanical or chemical therapies. However, in periodontal diseases, not only do pathogenic bacteria numbers get higher, but the number of 'beneficial' bacteria gets lower. Therefore, mainly during the last decade, a new hypothesis has been proposed, where the aim is for probiotics to regain the healthy beneficial/pathogenic bacteria ratio in the oral cavity (for review see Teughels et al. 2011).

In a recent clinical study (Karuppaiah et al. 2013), 216 school children, aged 14–17 years, either included or excluded curd in their daily diet for 30 days. The plaque index and gingival index were recorded before and after the intervention. The results showed that a short-term daily ingestion of probiotics delivered via curd in the diet reduced the levels of plaque in healthy school children, although no significant improvements were found in gingival health. On the other hand, daily supplementation with *L. reuteri* from birth and during the

first year of life was associated with reduced gingivitis in the primary dentition at 9 years of age (Stensson et al. 2014).

Krasse et al. (2005) studied the effect of *L. reuteri* on gingivitis. Fifty-nine patients with moderate to severe gingivitis were included and given one of two different *L. reuteri* formulations at a dose of 2 x 10^8 CFU per day, or a corresponding placebo, for 2 weeks. Gingival index and plaque index were measured at baseline and after the intervention. The results showed that *L. reuteri* was efficacious in reducing both gingivitis and plaque in patients with moderate to severe gingivitis. *L. reuteri* has also reduced the number of periodontal pathogens in the subgingival microbiota in patients with gingivitis (Iniesta et al. 2012). There are also contradictory results. In a recent study *L. reuteri* did not significantly affect the plaque accumulation, inflammatory reaction or the composition of the biofilm during experimental gingivitis (Hallström et al. 2013).

In studies of subjects with periodontitis, significantly higher attachment gains and reduced plaque index, bleeding on probing, pocket probing depth scores and decreased number of *P. gingivalis* were observed after *L. reuteri* consumption together with scaling and root planning, in comparison to scaling and root planning alone (Vicario et al. 2012, Teughels et al. 2013). The consumption of *L. reuteri*-containing lozenges combined with scaling and root planning provided a better clinical improvement when compared to placebo lozenges combined with scaling and root planning in chronic periodontitis patients (Ince et al. 2015).

Shimauchi et al. (2008) studied the effect of *L. salivarius* WB21 on periodontal health. Sixty-six subjects received either tablets containing *L. salivarius* WB21 (6.7 x 10^8 CFU) with xylitol or xylitol alone three times a day for 8 weeks. The results showed that *L. salivarius* WB21 improved the plaque index and probing pocket depth of current smokers. *L. salivarius* WB21 has also reduced the numbers of periodontal bacteria, including *A. actinomycetemcomitans, P. intermedia, P. gingivalis, T. denticola* and *T. forsythia*, in subgingival plaque (Mayanagi et al. 2009). *L. salivarius* WB21 showed beneficial effects on bleeding on probing from the periodontal pocket (Iwamoto et al. 2010).

3. AIMS OF THE STUDY

The aim of this study was to evaluate the effects of the commonly used probiotics on oral microbiota, dental plaque and periodontal status, with special reference to mutans streptococci.

The specific aims were:

To compare the effects of four probiotic lactobacilli on the ability of *S. mutans* to adhere to and grow on a glass surface *in vitro.*

To investigate the *in vitro* effects of two lactobacilli on the adhesion of *S. mutans* to glass and saliva-coated hydroxyapatite, and the counts of *S. mutans* in a three-species biofilm.

To expose dental plaque in a clinical study to *L. rhamnosus* GG and *L. reuteri*, and to study the changes in the acid production of the plaque, the counts of mutans streptococci and lactobacilli, as well as the retention of the probiotics to the plaque.

To expose dental plaque in a clinical study to a combination of *L. rhamnosus* GG and *B. lactis* BB-12, and to study the effects of the exposure on periodontal status, mutans streptococci counts and salivary microbiota.

4. MATERIALS AND METHODS

A summary of materials and methods is described below. More information can be found in studies I–IV.

4.1 *In vitro* studies (studies I and II)

4.1.1 Microorganisms

The lactobacilli used in studies I and II were *L. rhamnosus* GG (ATCC 53103), *L. reuteri* SD2112 (ATCC 55730), *L. reuteri* ATCC PTA 5289, *L. plantarum* 299v (DSM 9843) and *L. paracasei* DSMZ16671. The *L. paracasei* strain is not a probiotic since it is heat-killed (Lang et al. 2010, Tanzer et al. 2010). The freeze-dried strain was a kind gift from BASF Future Business GmbH, Ludwigshafen am Rhein, Germany.

The following reference strains of mutans streptococci were used in the adhesion and biofilm experiments: *S. mutans* NCTC 10449 and *S. mutans* Ingbritt. Of the two clinical *S. mutans* isolates used, *S. mutans* 2366 represented higher and *S. mutans* 195 lower production of water-insoluble polysaccharides. The origin, isolation and identification of the clinical isolates have been described in detail earlier (Söderling and Hietala-Lenkkeri 2010).

In the three-species biofilm experiments, *S. sanguinis* NCTC 10904 and *A. naeslundii* ATCC 12104 were used in addition to the mutans streptococci.

4.1.2 Saliva

Paraffin-stimulated whole saliva used in study II was collected from 5 healthy subjects after one-minute pre-stimulation. The salivas were pooled and cleared by centrifugation. The supernatant was pasteurized at +60 ˚C for 30 min, recentrifuged and stored at -70 ˚C before use.

4.1.3 Adhesion of *S. mutans* to a smooth glass surface

In studies I and II, the adhesion of *S. mutans* to glass was assessed using the method of Mattos-Graner et al. (2000), which measures the polysaccharide-mediated ability of *S. mutans* to adhere to a smooth glass surface and to form biofilm on it.

The cells of *S. mutans* were grown overnight in Brain-Heart Infusion Medium (BHI; Difco, MI, USA) at +37 ˚C. The lactobacilli (LB) were grown in MRS medium (Chemie S.A., Barcelona, Spain). The following morning, they both were transferred into fresh media. The LB were washed after being cultured for 4 h. The pellets containing LB were dissolved to produce a bacterial solution with an absorbance of 0.2–0.3 at A550 (4–8 x 10^8 CFU/ml). The *S. mutans* cells were grown for 6 h and subsequently transferred to fresh BHI, supplemented with 2 %

sucrose w/v to make a suspension with the final absorbance of 0.2–0.3 at A550 (4–8 x 10^8 CFU/ml). Next, 950 µl of LB suspension was combined with 1 ml of *S. mutans* suspension in a glass tube, and 50 µl of MES (Sigma, St. Louis, MO, USA) was added. The final sucrose concentration was 1 %. The glass tubes were incubated anaerobically at +37 ˚C, at a 30˚ angle, for 18 h. At that point, the glass tubes were vortexed, and unattached cells poured into another tube. The cells that adhered to the glass tubes were then washed once with 2 ml K-phosphate buffer (0.05 M, pH 7). The content was transferred into the tube with the unattached cells. The cells that remained were suspended in 2 ml K-phosphate buffer and gently sonicated to disperse the cells; absorbance was measured at A550. Next, 100 µl samples of the suspensions were pipetted into 900 µl Tryptic Soy Broth (TSB, Difco, MI, USA) with 10 % glycerol. They were stored at -70 ˚C prior to microbial analyses with plate culturing. Centrifugation was used to collect the unattached cells, which were treated in the manner described above, for cells attached to the glass surface.

4.1.4 Adhesion of *S. mutans* to saliva-coated hydroxyapatite

In study II, the adhesion tests examining the effects of the LB on streptococcal adhesion *in vitro* were performed as described earlier (Haukioja et al. 2008b). The *S. mutans* cells were labelled by adding 5 µl (50 µCi) of ^{35}S-labelled methionine (Amersham Biosciences, Little Chalfont, UK) to 5 ml of growth medium (BHI; Becton–Dickinson and Company, Sparks, MD, USA) and grown overnight at +37 ˚C. The LB were grown in MRS medium (Scharlau Chemie S.A., Barcelona, Spain). After overnight growth, the bacteria were washed and suspended in buffered KCl (50.0 mM KCl, 0.35 mM K_2HPO_4, 0.65 mM KH_2PO_4, 1.0 mM $CaCl_2$, 0.1 mM $MgCl_2$ at pH 6.5) with 0.5 % bovine serum albumin (BSA, Sigma Chemicals Co., St Louis, MO). Three different experiments were performed. First, the LB were allowed to adhere before the streptococci. After coating HA with parotid saliva, the HA beads were coated with bacterial suspension of LB, or with 0.5 % BSA alone for 30 min. After washing, the labelled streptococci were allowed to adhere for 60 min. In the second set of experiments, the streptococci and LB were allowed to adhere simultaneously for 60 min. Finally, the labelled streptococci were allowed to adhere first for 60 min on SHA and then the LB, or buffered KCl (control), were added for 60 min.

4.1.5 Biofilm experiments

To perform the biofilm experiments, the method of Guggenheim et al. (2001), used in study II, was performed. *S. mutans* and *S. sanguinis* were grown overnight at +37 ˚C in TSB and *A. naeslundii* for 2 days at +37 ˚C in filter-sterilized fluid universal medium (FUM) that contained 0.3 % glucose w/v. All of the cultures were initially centrifuged and washed with PBS, and later suspended in FUM that, in addition to glucose, also contained 0.3 % sucrose (sFUM). The seed cultures of the bacteria were adjusted to A550 = 1.0, corresponding to approximately 10^7 CFU/ml. Because of differences in the way the microorganisms grew in the biofilm model, the absorbance of each bacterial suspension used at the initial adhesion stage was adjusted accordingly. *S. mutans* was adjusted to A550 = 0.05, and the suspension further diluted to 1:50 with sFUM. *S. sanguinis* was adjusted to A550 = 0.05, and the

suspension further diluted 1:10 with sFUM. *A. naeslundii* was adjusted to A550 = 1.0 in sFUM (this suspension was undiluted).

The biofilms were grown on hydroxyapatite (HA) discs (5 mm in diameter, Clarkson Chromatography Products Inc., South Williamsport, PA, USA). The discs were placed in holders made of orthodontic wire. The holders with the discs were positioned in the wells of 24-well cell culture plates (Corning Inc., Corning, NY, USA), three discs in one well. The holder allowed the discs to be incubated at a 45˚ angle. The discs were first incubated with a mixture of 1 ml PBS and 0.5 ml pasteurized saliva for 30 min at +37 ˚C and then rinsed with 1.5 ml PBS. In the initial adhesion step, 1 ml of a 1:1:1 mixture of the three microbial suspensions and 500 µl of pasteurized saliva was added to each well. The incubation was performed in a Whitley A35 anaerobic chamber (Don Whitley Scientific Ltd., Shipley, UK) at +37 ˚C under 80 % N_2, 10 % CO_2 and 10 % H_2 atmosphere for one hour. Following this step, fresh media – 1 ml sFUM and 500 µl saliva – were added to the wells. In the first set of experiments, the discs were exposed to the LB solutions (1.5 ml of *L. reuteri* in sFUM: A550 = 0.3 corresponding 10^8 CFU/ml or 1.5 ml of 1 mg/ml of *L. paracasei* corresponding 2 x 10^8 cells/ml) for 2 x 30 min or 3 x 15 min. Both of these suspensions retained their original turbidity for more than 30 min. In the second set of experiments, 150 µl of the lactobacilli suspensions were added already at the initial adhesion step (to the control 150 µ sFUM). After 5 h of incubation at +37 ˚C under 80 % N_2, 10 % CO_2 and 10 % H_2, 1 ml of fresh sFUM containing Sörensen buffer and 500 µl of pasteurized saliva was added to the wells. After a total incubation time of 24 h, each disc was rinsed in 10 ml PBS, and the biofilm was collected in a standardized manner using two microbrushes (Quick-Stick®, Dentsol AB, Saltsjö-Boo, Sweden), the ends of which were cut with sterile scissors to a transport tube containing 1 ml TSB with 10 % glycerol. The transport tubes were stored at -70 ˚C until plate cultured. At the end of the 24-h experiments pH was measured from each culture medium.

4.1.6 Antimicrobial properties of the culture media

Testing the antimicrobial effects of the culture media of study I's LB was done by growing the MS and LB at +37 ˚C, overnight (18 h), in THYE medium (Todd-Hewitt medium, supplemented with 0.3 % yeast extract; Chemie S.A.) and MRS medium. In the morning, the MS were transferred to fresh medium, and allowed to grow until the absorbance (A550) had doubled. Then, the MS were centrifuged and suspended in saline so that they formed a suspension whose absorbance was 0.7 (A550). The LB that had grown overnight were centrifuged. The media were filtered (Minisart High-Flow, Sartorius AG, Göttingen, Germany) and the LB media's end-pH was recorded. In the final step, 500 µl of the *S. mutans* suspension and 500 µl of the filtered LB supernatant were combined and then incubated at +37 ˚C for 60 min using a shaking water-bath. Samples (100 µl) were taken before and after incubation and placed in TSB tubes, and stored at -70 ˚C before conducting a microbial analysis.

Media with a pH adjusted to 4.8 with NaOH was also used in carrying out the experiment, with the incubation time increased to 2 h.

The two strains of *L. reuteri* were incubated using glycerol so as to produce the kind of reuterin-containing solutions described by Spinler et al. (2008). The antimicrobial activities of the solutions thus produced were tested by incubating. Of each reuterin-containing solution, 500 µl was incubated with 500 µl of each *S. mutans* saline-suspension (A550 = 0.7), and prepared as described above. Collection of samples to assess viability, using plate culturing, was done at the beginning as well as following 2 h of incubation at +37 ˚C.

4.1.7 Plate culturing of bacteria

The tubes of TSB were thawed, then vortexed for 1 min, and the content pumped back and forth using a disposable pipette. The resulting suspension was mildly sonicated. Following serial, tenfold dilutions, the bacteria were plated on Difco™ Mitis salivarius agar (Becton Dickinson & Company, Sparks, MD, USA) that contained bacitracin (MSB). The plates were then incubated at +37 ˚C in a 7 % CO_2 atmosphere for 2 days. As described earlier (Söderling et al. 2000), MS were identified; their number was identified based on colony morphology and counted using a stereomicroscope. The identification of *S. mutans* was based on consistent findings of 'rough' colony morphology, positive fermentation with sorbitol, mannitol, raffinose and melibiose, and negative dextran agglutination. The identification of *S. sobrinus* was based on 'smooth' colonies, positive fermentation with mannitol, but negative fermentation with raffinose and melibiose, and positive dextran agglutination. Then, the LB were grown anaerobically (80 % N_2, 10 % CO_2, 10 % H_2) for 2 days at +37 ˚C, on Difco™ Rogosa SL agar (Becton Dickinson & Company). In biofilm experiments, the total facultative bacteria (*S. mutans*, *S. sanguinis*, *A. naeslundii*) were cultivated for 3 days at +37 ˚C anaerobically on blood agar (Orion Diagnostica, Espoo, Finland). It was possible to detect *A. naeslundii* in the biofilm samples on the basis of colony morphology that differed from that of the streptococci. The results were enumerated as colony-forming units per milliliter; the plate culturing detection limit was 100 CFU/ml.

4.1.8 Determination of the polysaccharide:protein ratio of the biofilms

For determining the polysaccharide:protein ratio in study II, the 24-h biofilms were heated for one hour in 700 µl 1 N NaOH. Thereafter, polysaccharides (Dubois et al. 1956) and proteins (Lowry et al. 1951) were determined from the solution.

4.2 Clinical studies (studies III and IV)

4.2.1 Ethical considerations

The clinical studies were both approved by the ethics committee of the Hospital District of Southwest Finland. Written informed consent was obtained from the study participants. All data were treated confidentially.

4.2.2 Subjects

Thirteen healthy students of the University of Turku, Finland, showing salivary MS counts of ≥10^4 CFU/ml (Dentocult SM Strip Mutans, Orion Diagnostica, Espoo, Finland) volunteered for study III. The inclusion criteria were good general health, willingness to participate and salivary MS counts ≥10^4 CFU/ml.

Altogether, 77 students of the University of Turku, Finland, were screened for the presence of salivary MS (Dentocult SM Strip Mutans, Orion Diagnostica). The 62 students showing salivary MS counts of ≥10^3 CFU/ml were invited to participate and they volunteered for study IV. The inclusion criteria were good general health, willingness to participate and salivary MS counts ≥10^3 CFU/ml. Two subjects dropped out during the study.

4.2.3 Test products

In study III, the commercially available test tablets containing *L. reuteri* ATCC 55730 and PTA 5289 (Reladent®, Biogaia AB, Sweden) were bought from a Finnish pharmacy. According to the manufacturer there were 2 x 10^8 CFU of *L. reuteri* per tablet. In our quality control by plate culturing, we found 1.8 x 10^8 CFU/tablet. Tablets containing *L. rhamnosus* GG were specially manufactured free of charge by Karl Fazer AB (Vantaa, Finland). The *L. rhamnosus* GG used in the LGG tablets was from Probiotical S.p.A., Novara, Italy. During plate culturing we found 1.96 x 10^8 CFU of *L. rhamnosus* GG per tablet.

In study IV, a run-in gum was used: a noncommercial chewing gum manufactured free of charge for the study by Karl Fazer AB. The gum pieces weighed 1.2 g and contained 42 % xylitol, 18 % sorbitol and 5 % maltitol. Fazer also manufactured the 1 g tablets for the test period. They were compressed from 50 % xylitol and 46 % sorbitol. The *L. rhamnosus* GG used in the probiotic tablets was from Probiotical S.p.A. The *B. lactis* BB-12 was from Chr. Hansen A/S, Hoersholm, Denmark. Each probiotic tablet contained 4.4 x 10^8 CFU of LGG and 4.8 x 10^8 CFU of BB-12.

4.2.4 Study design

Study III had a randomized, double-blind and crossover design. The study started with 3-week run-in period. During the run-in period and the whole study the subjects were advised not to use products containing LGG or *L. reuteri* but otherwise to continue normal oral hygiene and dietary habits. Compliance with instructions was checked throughout the study. The subjects were randomly allocated into two groups. Half of the subjects consumed first LGG tablets twice a day for two weeks and after that *L. reuteri* tablets twice a day for two weeks. Between the two tablet periods was a 5-week washout period. The other half of the subjects used the tablets in reverse order.

Study IV was controlled, randomized and double-blind study. The study started with 4-week run-in period. During the run-in period and the whole study the subjects were advised not to use products containing LGG or *B. lactis* BB-12 but otherwise to continue normal oral hygiene and dietary habits. The subjects were instructed not to use their regular xylitol

products during the study. Compliance with instructions was checked throughout the study. The subjects were randomly allocated into two groups. Both groups used the same run-in chewing gum for four weeks before the test period started. After the run-in period one group used the test tablets containing LGG and *B. lactis* BB-12 and the other group used control tablets without probiotics. The recommendation for the use of the run-in gum and also for the tablets was 4 per day.

4.2.5 Outcome measure

In study III, the primary outcome measure was the lactic acid production of plaque and the secondary outcome was the MS level of plaque. In study IV, the primary outcome measure was the MS level of saliva and the secondary outcome measure was the plaque index.

4.2.6 Determination of plaque and gingival index

In study IV, plaque accumulation was allowed to continue for 24 hours without any oral hygiene at baseline and at the end of the tablet-taking period. On plaque collection morning, the subjects were told not to use the run-in chewing gum/test tablet. In the afternoon, the Silness-Löe plaque index (PI; Silness and Löe, 1964) and the Löe-Silness gingival index (GI; Löe and Silness, 1963) were determined using a periodontal probe, with special care taken to avoid bleeding.

4.2.7 Collection of plaque and saliva

In study III, plaque accumulation was allowed to continue for 24 h without any oral hygiene at baseline and at the end of the tablet periods. On plaque collection morning, subjects were told to use the probiotic tablet, and plaque samples were taken that afternoon. All of the available supragingival plaque was collected using dental curettes, with a standard volume of 8 µl of plaque collected in a surgical spoon, transferred into a test tube that contained 1 ml of cold fermentation minimal medium (FMM), with the rest transferred to 1 ml of cold saline.

In study IV, following determination of PI and GI, 4 ml paraffin-stimulated saliva was collected. The collection time was recorded. In the HOMIM analyses, the salivas of 15 randomly chosen subjects per group were selected. The 1 ml saliva samples were pipetted onto 10 µl TE-buffer (Sigma-Aldrich, St. Louis, MO, USA), stored at -70 °C, and then shipped on dry ice to the Forsyth Institute, Cambridge, MA, USA. For plate culturing, samples (100 µl) of the salivas were added into 900 µl Tryptic Soy Broth with 10 % glycerol (TSB; Scharlau Chemie S.A., Barcelona, Spain) and stored at -70°C before microbial analysis. After the collection of saliva, all of the available supragingival plaque was collected, using dental curettes, from the left half of the mouth. The plaque was suspended in tubes containing 0.5 ml 1 N NaOH, and the samples stored at -70 °C before analysis.

4.2.8 Acid production of plaque

Immediately after plaque collection in study III, the FMM-tube was vortexed for 5 s and 50 µl of 200 mM glucose was added. The tube was vortexed again for 5 s and incubated

at +37°C for 30 min. The cells were separated by centrifugation and the supernatant was stored at -70°C until lactic acid determination (L-lactic acid kit, R-biopharm AG, Darmstadt, Germany). The method measures NADH, the amount of which is stoichiometric to the amount of L-lactic acid in the sample. The increase in NADH was determined by means of its light absorbance at 340 nm.

4.2.9 Microbiological analyses

Plate culturing of bacteria was done as described in 4.1.7.

In study III, quantitative PCR detection of *L. rhamnosus*, strain-specific PCR of *L. rhamnosus* GG, and species-specific PCR of *L. reuteri* were done.

In study IV, the HOMIM analyses were performed at the Forsyth Institute. The microbial profiles were generated using image files of scanned HOMIM microarrays. The concentration levels of approximately 300 oral taxa were determined by means of microarray hybridization and using fluorescent readout reverse-capture method (Colombo et al. 2009). Fluorescent-labelled sample microbial DNA was captured by 16S rRNA-based probes that were attached to glass slides. Each probe's fluorescent intensity was scanned, normalized and scaled as previously reported (Colombo et al. 2009). Signals of < 2x background were deemed negative and assigned a 0 HOMIM level score. Positive hybridization signals were categorized into 5 levels. Level 1 indicated a just detectable signal and 5 indicated maximum signal intensity. All of the test group samples yielded enough PRC product for HOMIM, but one of the control group samples was not valid for analysis.

4.2.10 Determination of polysaccharide:protein ratio of the plaque samples

For determining the polysaccharide:protein ratio in study IV, the test tubes with the plaque samples in NaOH were heated for 1 hour in a boiling water bath. Thereafter, polysaccharide (Dubois et al. 1956) and protein (Lowry et al. 1951) concentrations were determined and their ratio was calculated.

4.3 Statistical analyses

Statistical analyses were performed using SPSS versions 14.0–19.0 (IBM Inc., New York, USA). The level of significance was set at $p < 0.05$ in each study.

In studies I and II, all experiments were carried out at least in triplicate and repeated at least twice. Dunnett's two-sided t-test was used to compare tests with control in both studies. In study II, ANOVA was also used.

In study III, the tests used to study the differences in lactic acid and the microbiological results of plaque between baseline and end of each tablet period were the *t* test for paired samples and the Wilcoxon signed ranks test. The same tests were also used to compare baseline values and those that preceded the experimental periods. The associations between

individual intervention-induced changes in the acidogenicity of plaque, and changes in the levels of LB and MS, were studied using Spearman's rank correlation test.

In study IV, *t* test for paired samples and Wilcoxon signed ranks test were used to study differences in plaque index, gingival index and salivary MS and LB counts between baseline and the end of the test period. Independent samples *t* test was used to compare values between the two groups at baseline and after the test period.

5. RESULTS

5.1 *In vitro* studies (studies I and II)

5.1.1 Biofilm formation of *S. mutans* on a smooth glass surface (study I)

We compared the effects of probiotic lactobacilli, *L. rhamnosus* GG, *L. plantarum* 299v, *L. reuteri* PTA 5289 and SD2112 on biofilm formation of *S. mutans.* Each of the four lactobacilli strongly inhibited the biofilm formation of the two clinical isolates of *S. mutans* ($p < 0.001$). In the case of the two reference strains of *S. mutans*, the inhibition pattern was different. *L. reuteri* SD2112 and *L. rhamnosus* GG were more efficient ($p < 0.001$) in inhibiting the biofilm formation of the reference strains than *L. reuteri* PTA 5289 and *L. plantarum* 299v ($p < 0.05$).

The lactobacilli also differed in their effects on the viability of *S. mutans* in the biofilms. Experiments with *L. rhamnosus* GG and *L. plantarum* 299v showed the biggest viability losses for mutans streptococci. Viable *S. mutans* cells could only be found in the experiments with *L. reuteri* PTA 5289 and SD2112. However, *S. mutans* Ingbritt lost its viability in every experiment.

In the antimicrobial tests, the four mutans streptococci lost their viability completely when tested with over-night culture supernatants of *L. rhamnosus* GG and *L. plantarum* 299v ($p < 0.001$). Weaker effects were observed with *L. reuteri* PTA 5289 and SD2112 culture supernatants ($p < 0.001$). The end pHs of the culture supernatants of *L. rhamnosus* GG and *L. plantarum* 299v were pH 4.0 and of the two *L. reuteri* strains pH 4.3. When the pH of the culture supernatants was adjusted to 4.8, none of the lactobacilli showed any antimicrobial activity against *S. mutans*.

5.1.2 Three-species biofilm on SHA (study II)

We compared the effects of probiotic lactobacilli, *L. reuteri* PTA 5289 and *L. paracasei* DSMZ16671, on the adhesion of *S. mutans* and on the counts of mutans streptococci in a biofilm. Both of the lactobacilli inhibited adhesion of *S. mutans* when the streptococci and lactobacilli were allowed to adhere simultaneously to saliva-coated hydroxyapatite, or when the streptococci were allowed to adhere before the lactobacilli ($p < 0.001$). *L. paracasei* showed stronger inhibition of adhesion of the reference strain and clinical isolate of *S. mutans*, compared to *L. reuteri*. When the saliva-coated hydroxyapatite was first exposed to the lactobacilli and then to the mutans streptococci, *L. reuteri* PTA 5289 significantly inhibited binding of both mutans streptococci ($p < 0.01$) but *L. paracasei* DSMZ16671 did not.

Much weaker, but statistically significant, inhibitions were detected when the effect of the lactobacilli on the adhesion of the mutans streptococci to a smooth glass surface was studied. *L. reuteri* inhibited the adhesion of *S. mutans* Ingbritt, and *L. paracasei* the adhesion of *S. mutans* 2366, but only when the streptococci were allowed to adhere before the lactobacilli.

Exposure of the three-species biofilm to *L. reuteri* and *L. paracasei* suspensions had no effect on the counts of either the reference strain or the clinical isolate of *S. mutans*, or the levels of other bacteria in the biofilm. The two lactobacilli had no effect on the biofilm formed. All three facultative bacteria (*S. mutans*, *S. sanguinis* and *A. naeslundii*) could be cultured from the 24-h biofilm. The clinical isolate *S. mutans* 2366, however, was inferior to the reference strain in biofilm-forming ability. *L. reuteri* could also be cultured from the 24-h biofilms. In all experiments, the pH of the media remained above pH 6.1.

5.2 Clinical studies (studies III and IV)

5.2.1 Effect of probiotics on acid production and mutans streptococci counts of plaque (study III)

We studied the acidogenicity and the levels of mutans streptococci in dental plaque after the consumption of *L. rhamnosus* GG and *L. reuteri*.

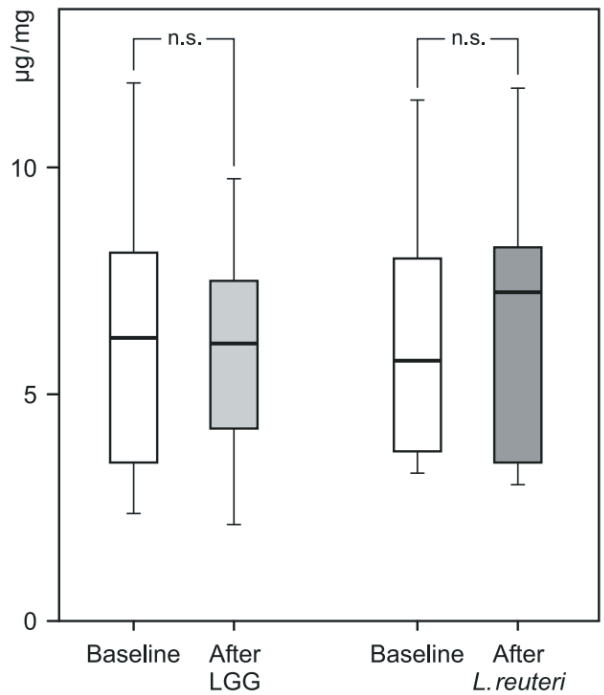

Figure 2. Lactic acid production of plaque suspension (in micrograms per milligram plaque) at baseline and after 2-week consumption of tablets containing *L. rhamnosus* GG or *L. reuteri.* The boxplots show medians, first and third quartiles and the minimum and maximum of the lactic acid concentrations in the cell-free supernatants after 30-min incubation. *N.s.* = not significant. (Marttinen et al. 2012; with permission).

In the acid production tests, all of the plaque samples from subjects produced detectable amounts of lactic acid. We detected differences between different subjects in acid production potentials of plaque, but the differences were quite small per individual subject when compared to the baseline plaque sample and the plaque sample after probiotic intervention. The consumption of *L. rhamnosus* GG or *L. reuteri* had no effect on lactic acid production (Figure 2). Intervention-induced changes in the acidogenicity of plaque showed no association with changes in the lactobacilli and mutans streptococci levels.

At baseline, one subject had a combination of *S. mutans* and *S. sobrinus* in the plaque while others had *S. mutans* in the plaque. We did not detect any differences in plaque mutans streptococci counts after the probiotic intervention compared to baseline. There were also no differences in the counts of total streptococci or facultatives.

At baseline, most of the subjects did not have detectable amounts of lactobacilli in the plaque. Only one subject had high amounts of lactobacilli (log CFU > 4) and three subjects had such a low amount of lactobacilli that it was barely detectable (log CFU < 4). The consumption of *L. rhamnosus* GG did not affect the lactobacilli counts. After consumption of the *L. rhamnosus* GG, seven subjects had *L. rhamnosus* in the plaque when studied with quantitative, species-specific PCR. The qualitative PCR showed that in fact only four of the seven samples actually contained *L. rhamnosus* GG. Contrary to the *L. rhamnosus* GG consumption, the amount of lactobacilli was significantly higher after the consumption of *L. reuteri* (p=0.011). Two subjects had an increase from low amounts to high amounts of lactobacilli. The increase in the amount of lactobacilli was barely detected in three subjects. Five subjects had no lactobacilli in their plaque during the study. In the 16S rRNA gene sequence analysis of lactobacilli-like colonies, collected from the eight lactobacilli-positive subjects, six cases presented with *L. reuteri*.

5.2.2 Impact of probiotics on gingival health-related endpoints (study IV)

We studied the effects of consumption of *L. rhamnosus* GG and *B. lactis* BB-12 on the amount of plaque, gingival inflammation, oral microbiota as well as mutans streptococci counts in saliva.

5.2.2.1 Plaque and gingival index

We detected a significant reduction in the mean plaque and gingival index values in the probiotic group (p=0.016 for plaque index and p=0.012 for gingival index), while no change was detected in the control group. Figure 3 shows the individual gingival index values at baseline and at the end of the study for both the probiotic group and the control group. The number of cases with decreased plaque and gingival index values in the probiotic group showed a positive correlation (r=0.389; p=0.037), and they were also significantly associated (OR 5.8, 95 % CI 1.0-30). This connection was not found in the control group.

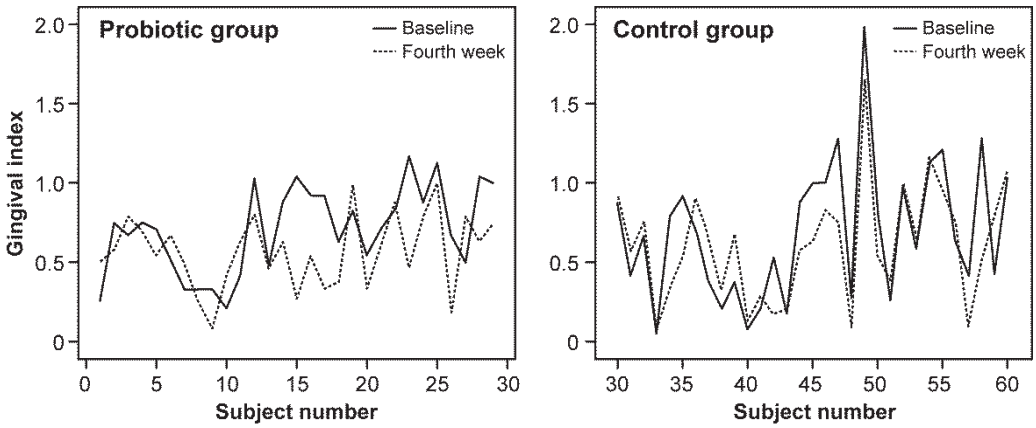

Figure 3. Gingival index values at baseline and after the 4-week study period in the probiotic and control group.

5.2.2.2 Microbial findings

At baseline, the saliva of one subject had both *S. mutans* and *S. sobrinus* while others had *S. mutans* in their saliva. Altogether 33 subjects had high mutans streptococci counts in their saliva (> 10^5 CFU/ml), and they were equally represented in the probiotic and control group. We did not detect any differences in mutans streptococci counts in either group.

At baseline, 29 subjects had salivary lactobacilli, and they were equally represented in the probiotic and control group. We did not detect any differences in the counts of lactobacilli after the probiotic consumption compared to baseline.

With the HOMIM analysis we could detect about 300 predominant oral bacterial taxa. The probiotic and control group had similar microbiota at baseline and none of the subjects had *Porphyromonas gingivalis*, *Prevotella intermedia* or *Aggregatibacter actinomycetemcomitans* (Figure 4). Only three subjects had salivary lactobacilli. No statistically significant differences were found in the microbiota after the probiotic consumption compared to baseline values.

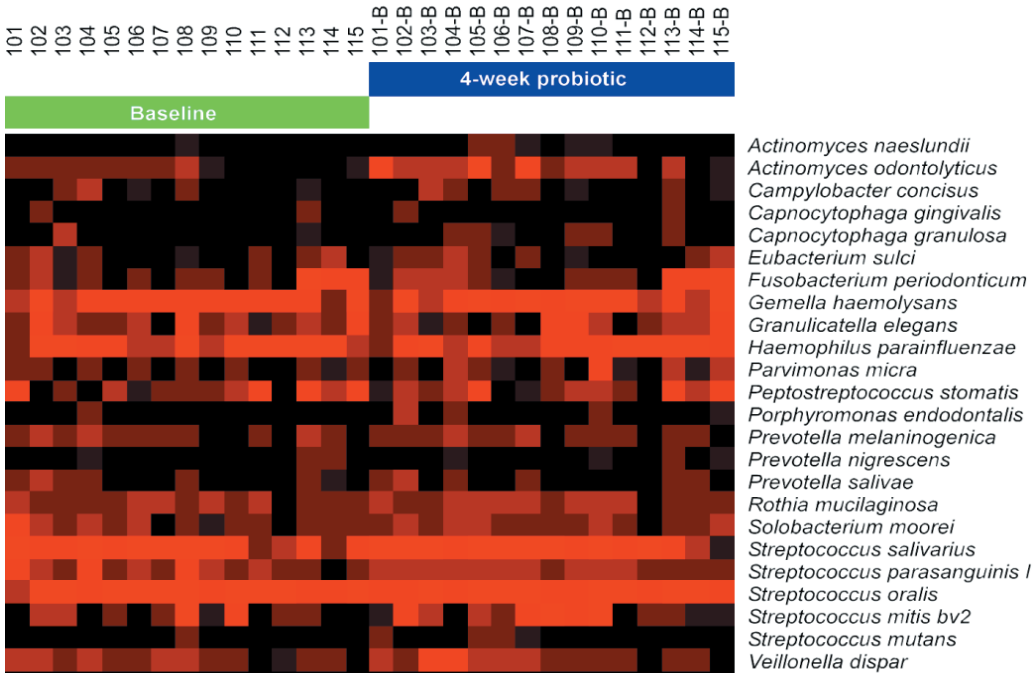

Figure 4. Individual HOMIM profiles for 27 selected predominant microbes representing the oral microbiota of the 15 subjects of the probiotic group at baseline and after 4-week use of the probiotic lozenge. The color intensity reflects relative proportions of the species present in the sample. (Toiviainen et al. 2015; with permission).

6. DISCUSSION

6.1 Probiotic lactobacilli and *S. mutans* biofilm formation *in vitro* (studies I and II)

The aim of study I was to compare the effects of probiotic lactobacilli, *L. rhamnosus* GG, *L. plantarum* 299v, *L. reuteri* PTA 5289 and SD2112 on the biofilm formation of *S. mutans.* In study II, we compared the effects of probiotic lactobacilli, *L. reuteri* PTA 5289 and *L. paracasei* DSMZ16671 on the adhesion of *S. mutans* and on the counts of mutans streptococci in a biofilm.

The viability of the mutans streptococci was significantly reduced in the biofilms in study I after exposure to lactobacilli. Studies with *L. rhamnosus* GG and *L. plantarum* showed the biggest viability losses for mutans streptococci, and also their culture supernatants were efficient in reducing the viability of the tested *S. mutans* strains. The two *L. reuteri* strains showed weaker effects against *S. mutans*. The two *L. reuteri* strains had significantly higher end-pHs in their culture supernatants, compared to *L. rhamnosus* GG and *L. plantarum*. It seems that the antimicrobial activity against *S. mutans* was pH-dependent. In an earlier *in vitro* study the growth inhibition of *S. mutans* has been attributed to the generation of a low pH either via organic acid production and/or production of bacteriocins or metabolites active at a low pH (Simark-Mattsson et al. 2009). In study I, after the exposure to glycerol both *L. reuteri* PTA 5289 and SD2112 decreased the counts of *S. mutans*, which may be due to the production of reuterin. *L. reuteri* has previously been shown to produce reuterin from glycerol, and reuterin has been attributed to the antimicrobial effects of *L. reuteri* against gut microorganisms (Spinler et al. 2008). Glycerol is present in the gut, and therefore it is possible that *L. reuteri* can produce reuterin. However, in the mouth the amount of glycerol in plaque is rather small (Runnel et al. 2013). When considering oral health, the importance of the production of reuterin can be questioned for this reason.

In study II, both *L. reuteri* PTA 5289 and *L. paracasei* DSMZ16671 interfered with adhesion of *S. mutans* to saliva-coated hydroxyapatite. Earlier results suggest that only those probiotic *Lactobacillus* strains which bind to saliva-coated hydroxyapatite can directly interfere with *S. mutans* binding by competing for common binding sites (Haukioja et al. 2008b). *L. reuteri* PTA 5289 can bind to saliva-coated hydroxyapatite and even form biofilm on it (Jalasvuori et al. 2012) and thus, with regard to *L. reuteri* PTA 5289, the results from this study are in line with earlier observations. On the other hand, the *L. paracasei* strain does not bind to hydroxyapatite (Lang et al. 2010) and apparently also not to saliva-coated hydroxyapatite, since in our experiments, in which saliva-coated hydroxyapatite was first exposed to the *L. paracasei* and then to the mutans streptococci, no inhibition of binding was detected. Still, the effect of *L. paracasei* was even stronger than that of *L. reuteri* PTA 5289. *L. reuteri* PTA 5289 and *L. paracasei* DSMZ16671 both coaggregate with mutans streptococci (Twetman

et al. 2009, Lang et al. 2010, Keller et al. 2011). Thus, it is likely that the observed inhibition of binding was due to coaggregation. This is in accordance with previous findings with a strongly aggregating strain of *L. casei* ATCC 11578 (Haukioja et al. 2008b).

All of the four probiotic lactobacilli in study I strongly and similarly inhibited the biofilm formation of the clinical isolates of *S. mutans.* With the two reference strains of *S. mutans*, the inhibition showed a different pattern. It is possible that these reference strains may have altered virulence properties compared to the clinical isolates.

In study II, *L. reuteri* PTA 5289 and *L. paracasei* DSMZ16671 did not affect the *S. mutans* levels in the three-species biofilms. In other biofilm experiments different lactobacilli have inhibited the growth and biofilm formation of mutans streptococci (Lee and Kim 2014, Lin et al. 2015, Wu et al. 2015). *L. rhamnosus* GG inhibits the growth of *S. mutans* both in dual-species biofilms and in complex, saliva-derived biofilms without affecting pH (Pham et al. 2011). Surprisingly, even though both *L. reuteri* PTA 5289 and *L. paracasei* DSMZ16671 have decreased counts of mutans streptococci in clinical studies (for review see Twetman and Keller 2012, Holz et al. 2013), no effects on the levels of these bacteria in the biofilm experiments were detected in this study. On the other hand, the results from clinical study III are in line with results obtained with *L. reuteri* PTA 5289 in study II. Although *L. paracasei* has the ability to coaggregate *S. mutans* (Lang et al. 2010), it could not affect the *S. mutans* levels in the three-species biofilms. In the exposures, we used high concentrations of the two lactobacilli, so a low amount of bacteria does not explain the result. The pH value could be one of the explaining factors for the results. In study I, we found that biofilms of *S. mutans*, formed on smooth glass surfaces were efficiently inhibited by probiotics, including *L. reuteri* PTA 5289. The inhibition was attributed to a low pH generated by the growing lactobacilli. Naturally, this does not apply to *L. paracasei* DSMZ16671, which was used in study II in a non-viable form. Also the pH of the medium remained above pH 6.1 in all experiments, so the pH was higher in study II than in study I. The saliva used to coat the enamel could have affected the results. In the adhesion experiments we used parotid saliva, but in the biofilm experiments we used whole saliva.

6.2 Effects of probiotics on dental plaque, levels of mutans streptococci, oral microbiome and gingival inflammation (studies III and IV)

The aim of study III was to study the acidogenicity and the levels of mutans streptococci in dental plaque after the use of *L. rhamnosus* GG and *L. reuteri*. In study IV, we studied the effects of orally administered *L. rhamnosus* GG and *B. lactis* BB-12 on the number of salivary mutans streptococci, amount of plaque, gingival inflammation and the oral microbiota.

Plaque acidogenicity is a risk factor of dental caries (Bradshaw and Lynch 2013). Lactobacilli are both aciduric and acidogenic and could in theory increase the acid-production potential of plaque. In study III, the consumption of *L. reuteri* or *L. rhamnosus* GG had no effect on the acidogenicity of plaque. The results are similar with the findings of Keller and Twetman

(2012) who studied the effect of lozenges with *L. reuteri* DSM 17938 and ATCC PTA 5289 on the concentration of lactic acid in supragingival plaque. Their results showed no significant differences in lactic acid production between baseline and 2-week samples and the test and the placebo. One factor affecting the acidogenicity of plaque may be the retention of the probiotics to plaque. Our results are in accordance with earlier studies showing poor retention for both *L. reuteri* and *L. rhamnosus* GG to the oral cavity (Yli-Knuuttila et al. 2006, Caglar et al. 2009, Saxelin et al. 2010). The existing studies have, as a rule, looked at saliva, while we studied plaque. According to our results, plaque appears not be an oral reservoir for ingested probiotics. Since *L. reuteri* and *L. rhamnosus* GG show poor retention to the oral cavity and have no effect on plaque acidogenicity, it seems that these probiotic lactobacilli are safe from an oral perspective.

In study III, the consumption of *L. reuteri* or *L. rhamnosus* had no effect on plaque mutans streptococci counts. In clinical studies, *L. reuteri* ATCC 55730 itself or combined with *L. reuteri* ATCC PTA 5289 has decreased the counts of mutans streptococci in saliva when used as gums, straws or tablets (Caglar et al. 2006, 2007). *L. reuteri* has decreased the counts of mutans streptococci in saliva also when used in yoghurt (Nikawa et al. 2004). The consumption of *L. rhamnosus* GG has showed a trend of decreasing mutans streptococci counts when consumed for 3 weeks in the form of cheese or for 7 months in milk (Näse et al. 2001, Ahola et al. 2002). In our study, compliance was good, the subjects were healthy young adults and the daily *L. reuteri* doses and consumption frequencies were similar to the earlier studies. The differences in those findings and our results may be a consequence of the fact that we studied the levels of mutans streptococci from plaque when most of the studies have focused studying mutans streptococci counts from saliva.

In study IV, no effects on salivary mutans streptococci counts were found after consumption of *L. rhamnosus* GG and *B. lactis* BB-12 lozenges. The effect of *L. rhamnosus* GG on mutans streptococci counts has been discussed above. Short-term *B. lactis* consumption has been shown to decrease mutans streptococci counts (for review see Twetman and Keller 2012). Clearly, combining these two probiotics did not add to their effects on mutans streptococci. Although high mutans streptococci counts do not necessarily mean increased caries risk in adults, decreasing mutans streptococci levels without affecting the healthy microbiota should make the plaque less virulent. According to a recent review, probiotics decrease mutans streptococci counts, and probiotics may have a positive effect in the prevention of caries (for review see Laleman et al. 2014, for review see Laleman and Teughels 2015). Unfortunately, in our three-species biofilm experiments in study II and in clinical studies III and IV, this beneficial effect of probiotics on mutans streptococci could not be detected.

The effect of probiotic bacteria on periodontal health is a relatively new research area. Normally, the focus of the treatment of periodontal diseases has been in reducing, periopathogens, because in disease there is an increase in pathogenic bacteria. However, in periodontal diseases also the number of 'beneficial' bacteria is lower, and therefore the use of probiotics might be beneficial (for review see Teughels et al. 2011). *L. reuteri* has

reduced the number of periodontal pathogens in the subgingival microbiota in patients with gingivitis (Iniesta et al. 2012). There are no studies that could have connected *L. rhamnosus* GG and/or *B. lactis* BB-12 consumption with decreases in counts of periodontopathogens. In study IV, our subjects possessed a microbiota typical of healthy subjects (Aas et al. 2005). The subjects did not harbor any major periodontopathogens at baseline. There were no changes in the oral microbiota after the consumption of probiotics.

In study IV, short-term administration of *L. rhamnosus* GG- and *B. lactis* BB-12-containing lozenges decreased the plaque index and also the gingival index of healthy subjects. To our knowledge, this is the first study to show this positive effect of the probiotic combination in question on gingival health. There are several studies on the effects of probiotics on subjects with disease. *L. reuteri* has been efficacious in reducing both gingivitis and plaque in patients with moderate to severe gingivitis (Krasse et al. 2005). On the other hand, in a recent study, *L. reuteri* did not significantly affect plaque accumulation, inflammatory reaction or the composition of the biofilm during experimental gingivitis (Hallström et al. 2013). However, it is important to obtain results also from healthy subjects. Because the subjects had no major periodontopathogens at baseline, the improvement in their plaque index and gingival index cannot be connected to these microorganisms. One possible explanation for the improved periodontal status in the probiotic group could be immune modulation of the host, since no change in the oral microbiota or the adhesion properties of plaque was detected. *L. rhamnosus* GG and *B. lactis* were both evaluated in a consensus opinion to have the effectiveness of grade A in improving the immune response of the host (Floch et al. 2011). Based on the results of a recent review, probiotics have shown small benefits on plaque and gingival inflammation but no effects on probing pocket depth (for review see Yanine et al. 2013). In study IV, the short-term consumption of *L. rhamnosus* GG- and *B. lactis* BB-12-containing lozenges improved periodontal status without affecting the oral microbiota, and it seems that these two probiotics used together have beneficial effects on gingival health. Further studies are needed to clearly understand the mechanisms behind these probiotic actions.

6.3 Summary

The primary target for probiotics is the gastrointestinal tract, but when used orally also the mouth is exposed to probiotic bacteria. It is important that probiotics that are used to improve general health are also safe from an oral point of view. Optimally, they could have a beneficial impact on oral health. Probiotic bacteria are generally considered to be both acidogenic and aciduric, which could in theory mean an increased caries risk.

Although the results from our *in vitro* studies do not fully reflect the real situation in the mouth, it is important to first test the properties of probiotics with *in vitro* studies before conducting a clinical study. The results from *in vitro* studies can show us the direction in which to continue. For example, our results with *L. reuteri* PTA 5289, in the biofilm experiments in study II, are in accordance with the results from clinical study III. *L. reuteri*

PTA 5289 had no effect on mutans streptococci counts in the biofilm experiments, and also none in the clinical study.

According to our results, it seems that *L. rhamnosus* GG and *B. lactis* BB-12 have beneficial effects on gingival health. One possible target for probiotic use could thus be prevention or even the treatment of periodontal disease. Probiotic bacteria, including *L. reuteri* and *L. salivarius* WB21, have been shown in other clinical studies to improve gingival health in subjects with pre-existing gingivitis and periodontal disease. Our results were achieved in healthy, young adults and therefore, these results help us to better understand how probiotics work.

In study IV, the recommended frequency for probiotic use was 4 times per day, and this administration showed beneficial effects on gingival health. There is also evidence that when the consumption of probiotics stops, the probiotic bacteria do not permanently stay in the mouth and are gradually eliminated. For this reason, in order to get the beneficial effects from probiotics, probiotic bacteria need to be consumed regularly. One possible strategy for probiotic use, for example in the case of periodontal disease, could be the combination of probiotic therapy and traditional methods of treatment, including, for example, root scaling and planning.

There are probiotic products that are meant mainly to improve oral health. Is there really a need for probiotic products that are primarily intended to improve oral health but that have no impact on general health? The best-case scenario would be for probiotics that are used to improve general health to also have beneficial – or at least non-harmful – effects on oral health. Since the beneficial health effects of probiotics are strain-specific, we cannot give accurate, overall recommendations for probiotic use. Each promising probiotic strain and combination needs to be tested by itself.

The use of probiotic bacteria is ‘generally regarded as safe’ (GRAS) by the United States Food and Drug Administration (FDA). It seems that dietary probiotics do not confer a major risk for oral health and have beneficial rather than harmful effects on oral health. But, since the consumption of probiotics is increasing, and even infants are given probiotics, more studies in this field are needed.

7. CONCLUSIONS

On the basis of the results presented in this thesis, the following conclusions can be drawn:

1. *L. rhamnosus* GG, *L. plantarum* 299v, *L. reuteri* PTA 5289 and SD2112 interfered with *S. mutans* biofilm formation, and the antimicrobial activity against *S. mutans* was pH-dependent.

2. *L. reuteri* PTA 5289 and *L. paracasei* DSMZ16671 both interfered with adhesion of *S. mutans* to SHA, the effect of *L. paracasei* being stronger. These lactobacilli did not affect adhesion to glass or the *S. mutans* levels in the three-species biofilms.

3. The short-term administration of *L. rhamnosus* GG or *L. reuteri* ATCC 55730 and PTA 5289 did not affect plaque acidogenicity or mutans streptococci counts of plaque. Plaque appears not to be a reservoir for ingested probiotics.

4. Short-term administration of *L. rhamnosus* GG and *B. lactis* BB-12 improved the periodontal status without affecting the oral microbiota.

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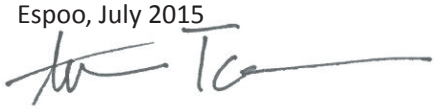

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