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PROBIOTIC BIFIDOBACTERIA AND LACTOBACILLI IN ORAL HEALTH – INTERACTIONS WITH BIOFILMS AND THE HOST

Heli Jäsberg

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University of Turku

Faculty of Medicine

Institute of Dentistry

Cariology and Restorative Dentistry

Finnish doctoral programme in Oral Sciences (FINDOS-Turku)

Supervised by

Anna Haukioja, PhD
Institute of Dentistry
University of Turku, Finland

Professor Leo Tjäderhane, DDS, PhD
Department of Oral and Maxillofacial Diseases
University of Helsinki, Finland

Reviewed by

Professor Christina Stecksén-Blicks, DDS, PhD
Department of Odontology
Umeå University, Sweden

Professor Georgios Belibasakis, DDS, MSc, PhD
Department of Dental Medicine
Karolinska Institutet, Sweden

Opponent

Professor Svante Twetman, DDS, PhD
Deparment of Odontology
University of Copenhagen, Denmark

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ABSTRACT

Heli Jäsberg, née Jalasvuori

Probiotic bifidobacteria and lactobacilli in oral health – interactions with biofilms and the host

University of Turku, Faculty of Medicine, Institute of Dentistry, Cariology and Restorative Dentistry, Finnish Doctoral Programme in Oral Sciences (FINDOS-Turku), Turku, Finland

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Probiotic bacteria confer a health benefit to the host when administered in adequate amounts. Usually probiotics belong to the genera of *Lactobacillus* and *Bifidobacterium*. Dental caries and periodontal diseases are common oral microbial diseases characterized by a dysbiosis in oral microbiota. Introducing probiotics into the oral cavity is suggested as supporting therapy or prevention also in caries and periodontal disease. Probiotics may enhance gingival health, but their acidogenic properties might be harmful from the cariological point of view.

The aim of this thesis was to study the following aspects of probiotics: their cariogenic properties, integration and actions in oral biofilms, and effects on health-related oral microbiota and host response. The work included *in vitro* and clinical studies with probiotic bacterial strains: *L. reuteri* ATCC 55730 and ATCC PTA 5289, *L. rhamnosus* GG (LGG), *B. animalis* subsp. *lactis* BB-12 (BB-12), and oral *Bifidobacterium* isolates.

Probiotic *L. reuteri* exerted cariogenic properties, when the environment was favorable, and the studied strains differed in their adhesion and capacity for biofilm formation. BB-12 and oral bifidobacteria integrated into oral biofilms *in vitro.* They inhibited the growth of *P. gingivalis* and were inhibited by *S. mutans*. In a clinical study, BB-12 and LGG had no effect on the oral microbiota in young healthy adults, but they had a positive effect on gingival health by reducing plaque and gingival indices, and by affecting salivary MMP-9 and TIMP-1 levels.

In conclusion, probiotic effects are strain specific and dependent on environmental factors. The effect on *S. mutans* or total oral microbiota seems to be limited. However, probiotics may have promising effects on gingival health by direct microbial interactions and immunomodulation.

Keywords: *Lactobacillus*, *Bifidobacterium*, probiotic, dental caries, gingivitis, periodontitis, mutans streptococci, oral microbiota, biofilm, matrix metalloproteinases

TIIVISTELMÄ

Heli Jäsberg, o.s. Jalasvuori

Probioottiset bifidobakteerit ja laktobasillit suussa – vuorovaikutuksista biofilmien ja isännän kanssa

Turun yliopisto, Lääketieteellinen tiedekunta, Hammaslääketieteen laitos, Kariologia ja korjaava hammashoito, Suun terveystieteiden tohtoriohjelma (FINDOS), Turku, Suomi

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Probiootit ovat bakteereita, jotka edistävät riittävinä määrinä nautittuna terveyttä. Hammaskaries sekä iensairaudet, gingiviitti ja parodontiitti, ovat seurausta suun mikrobiston tasapainon järkkymisestä. Suussa probiootit saattavat edistää ienterveyttä, mutta ne voivat olla haitallisia hampaiston terveyden näkökulmasta. Tässä väitöskirjatutkimuksessa selvitettiin probioottien ominaisuuksia hammaskarieksen näkökulmasta, niiden integraatiota ja vaikutuksia suun biofilmeihin *in vitro*, sekä kliinisessä tutkimuksessa vaikutusta terveiden koehenkilöiden syljen mikrobiston koostumukseen ja mutans-streptokokkien määrään, ienterveyteen ja isännän immuunivasteeseen. Tutkimuksissa käytettiin probiootteja *Lactobacillus reuteri* ATCC 55730 ja ATCC PTA 5289, *L. rhamnosus* GG, *Bifidobacterium animalis* subsp. *lactis* BB-12, sekä suusta eristettyjä bifidobakteereita.

Tutkitut *L. reuteri* -kannat erosivat toisistaan sylkivälitteisessä kiinnittymisessä ja biofilmin muodostuksessa hydroksiapatiitin pinnalle. Ympäristötekijät vaikuttivat niiden hapontuottoon. BB-12 ja suun bifidobakteerit inhiboivat parodontopatogeeni *P. gingivaliksen* kasvua, ja kariespatogeeni *S. mutans* inhiboi bifidobakteerien kasvua suun biofilmimallissa. Kliinisessä tutkimuksessa BB-12 ja LGG -probiootteja sisältävän tabletin käyttö ei vaikuttanut syljen mutans-streptokokkien määrään tai kokonaismikrobistoon. Probiootteja käyttäneiden koehenkilöiden plakin määrä oli kuitenkin vähentynyt ja ienterveys kohentunut ja heidän sylkensä matriksimetallo-proteinaasi (MMP)-9:n ja MMP-estäjän, TIMP-1:n, pitoisuudet erosivat alkutilanteesta.

Probioottibakteerien vaikutukset suussa riippuvat käytetystä bakteerikannasta ja ympäristötekijöistä. Vaikutus mutans-streptokokkeihin ja suun kokonaismikro-bistoon on vähäinen. Isännän immuunivasteen tehostaminen saattaa olla probioottien olleellinen vaikutusmekanismi suussa. Niillä voi olla edullisia vaikutuksia ienterveyteen.

Avainsanat: probiootti, *Lactobacillus*, *Bifidobacterium*, hammaskaries, ientulehdus, parodontiitti, mutans-streptokokki, suun mikrobisto, biofilmi, matriksimetalloproteinaasi

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ABBREVIATIONS

B.	*Bifidobacterium*
BB-12	*Bifidobacterium animalis* subsp. *lactis* BB-12
BD	Becton Dickinson Company
BHI	brain-heart infusion medium
BOP	bleeding on probing
BSA	bovine serum albumin
CFU	colony forming unit
eDNA	extracellular deoxyribonucleic acid
EPS	extracellular polysaccharide
FMM	fermentation minimal medium
FUM	fluid universal medium
GCF	gingival crevicular fluid
GI	gingival index
HA	hydroxyapatite
HOMIM	human oral microbe identification microarray
L.	*Lactobacillus*
LAB	lactic acid bacteria
LB	lactobacilli
LGG	*Lactobacillus rhamnosus* GG
MMP	matrix metalloproteinase
MRS	deMan, Rogosa et Sharpe
MS	mutans streptococci
PBS	phosphate-buffered saline
PI	plaque index
PPD	probing pocket depth
SRP	scaling and root planing
TIMP	tissue inhibitor of matrix metalloproteinases
TSBY	tryptic soy broth with 0.5 % yeast extract
wk	week
s-HA	saliva coated hydroxyapatite

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the original publications listed below. Below, they will be referred by the Roman numerals I-IV.

I **Jalasvuori H**, Haukioja A, Tenovuo J. Probiotic *Lactobacillus reuteri* strains ATCC PTA 5289 and ATCC 55730 differ in their cariogenic properties *in vitro*. Arch Oral Biol 2012: Dec; 57(12): 1633-1638.

II **Jäsberg H**, Söderling E, Endo A, Beighton D, Haukioja A. Bifidobacteria inhibit the growth of *Porphyromonas gingivalis* but not of *Streptococcus mutans* in an *in vitro* biofilm model. Eur J Oral Sci 2016: Jun;124(3):251-8.

III Toiviainen A, **Jalasvuori H**, Lahti E, Gursoy 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: Jan; 19(1): 77-83.

IV **Jäsberg H**, Sorsa T, Tervahartiala T, Söderling E, Haukioja A. Probiotic intervention influences the salivary levels of Matrix Metalloproteinase (MMP)-9 and Tissue Inhibitor of metalloproteinases (TIMP)-1 in healthy adults. (Submitted manuscript)

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1. INTRODUCTION

Microbial colonization of human body begins already in the uterus and an enrichment of microbiota continues in the birth and during life (Collado et al. 2016). There are over 2000 prokaryotic species identified in the human body; the human microbiota and associated genome, microbiome, is considered as an organ, living symbiotically with the host (Hugon et al. 2015). When the equilibrium in the microbiome is disturbed, diseases, *e.g.*, inflammatory bowel disease and atopic dermatitis can occur (Muszer et al. 2015).

The tradition of lactic acid bacteria (LAB) fermented milk products has a long history, and LAB are used for food safety and health benefits (Hammes and Tichaczek 1994). Already in the early 1900s, Elie Methnikoff (in Meurman, 2005) claimed, that the intake of yogurt containing lactic acid bacteria increased the longevity of the host. In the modern world, probiotic products are also widely consumed for their health benefits (Schrezenmeir and de Vrese 2001). WHO has defined probiotics as live bacteria which, when administered in adequate amounts, confer a health benefit to the host (Hill et al. 2014). The most commonly used and studied genera fulfilling these criteria are *Lactobacillus* and *Bifidobacterium.* The idea behind probiotic consumption is to expose indigenous microbiota and the host's immune system to viable micro-organisms for health benefits, as well as to strengthen the host's colonization resistance against potential pathogens. Requirements that probiotic bacteria are expected to fulfil are scientifically proven beneficial physiological effects, human origin, safety for human use, survival in gastrointestinal tract and adhesion ability (Isolauri 2001; Meurman and Stamatova 2007). The use of probiotics has been added even to the national dietary recommendations in several countries (Ebner et al. 2014; Smug et al. 2014). Probiotics have health effects at least in acute gastroenteritis, antibiotic-associated diarrhoea, infantile colic, and atopic eczema (Szajewska 2016).

Microbiota colonizing the oral cavity is relatively diverse and the equilibrium between host and microbiome is important for oral health (Marsh et al. 2015). Despite their known aetiology, plaque induced infectious diseases, dental caries and periodontal disease, are common and still a huge health problem all over the world (Jin et al. 2016). Since both diseases begin with a shift of microbial composition from the healthy to the pathogenic, introducing beneficial bacteria is suggested as a method of prevention, treatment or supporting therapy in both (Devine and Marsh 2009; Laleman and Teughels 2015).

Some promising results in the use of probiotics in periodontal disease have been reported, but their role in the treatment of dental caries is contradictory (Gruner et al. 2016). Information on probiotic interactions with oral microbiota and their immunomodulatory effects in the oral cavity is scarce. In the present study, the characteristics of commonly used probiotic strains and their effects on oral microbiota and host response were investigated.

2. LITERATURE REVIEW

2.1. Dental biofilm and oral microbiota

2.1.1. Dental biofilm

Salivary pellicle

Biofilm formation on a freshly cleaned tooth surface begins with the formation of dental salivary pellicle, a selective adsorption process, where proteins from whole saliva adsorb on dental surfaces. Electrostatic interactions play a role in the formation of an initial pellicle and the first adhering macromolecules include phosphoproteins such as acidic proline-rich proteins, statherins and histatins, as well as high-molecular-weight glycoproteins, amylase, cystatins, lysozyme, and lactoferrin. The formation of pellicle is followed by maturation, where protein-protein interactions take place. The pellicle has a biphasic structure, where the inner layer consists of firmly attached proteins with only a small amount of water, while the outer layer has more loosely attached proteins and more water between the proteins/molecules (Lindh et al. 2014). The formation of salivary pellicle is a rapid process. Most of the proteins found in the pellicle are attached already in a 10-second period after the cleaning of the surface, and a maturated pellicle is formed in under 20 minutes (Vacca Smith and Bowen 2000).

Biofilm formation

Biofilm is a natural habitat for bacteria. In the human body, tooth surfaces are unique non-shedding surfaces. They have a distinct nature in bacterial adhesion and biofilm formation when compared with mucosal surfaces anywhere else in the body. However, the biofilm formation on tooth surfaces and the maturation of dental plaque follows the same basic rules of biofilm formation as anywhere else in natural biofilms (Barraud et al. 2015; Kolenbrander et al. 2010).

Bacterial adhesion on the dental salivary pellicle is mediated by non-specific and specific interactions. Non-specific adhesion mechanisms include Van der Waals, electrostatic and acid-base-interactions and specific adhesion mechanisms are ligand-receptor interactions. Planktonic bacteria recognize the binding proteins, such as α-amylase and proline-rich-proteins, and adhere to the pellicle (Ellen et al. 1997; van der Mei et al. 2008).

Main early colonizing bacteria attaching to the tooth surface after pellicle formation belong to the genera *Actinomyces*, *Streptococcus*, *Haemophilus*, *Capnocytophaga*, *Veillonella,* and *Neisseria* (Diaz et al. 2006; Dige et al. 2009; Huang et al. 2011; Kolenbrander et al. 2010; Li et al. 2004). *Actinomyces* and streptococci are in the inner clusters of early multi-species biofilm (Dige et al. 2009; Kolenbrander et al. 2010). Co-aggregation of other bacterial species takes place during biofilm maturation. For example, *Fusobacterium nucleatum* is an important 'bridge' organism between initial, early and late colonizers (Kolenbrander et al. 2010). Later colonizing bacteria include periodontal pathogens, such as *Tannerella forsythia*, *Treponema* spp., *Porphyromonas gingivalis,* and *Aggregatibacter actinomycetemcomitans* (Li et al. 2004). These bacteria recognize specific binding sites and they attach to the pioneer organisms either directly with polysaccharide or protein receptors, or through salivary glycoproteins. Microbiota in the biofilm is subject specific and it reaches high diversity rapidly (Diaz et al. 2006).

Biofilm structure

In addition to the bacteria, biofilm has also extracellular components, *e.g.*, an extracellular polysaccharide network (EPS) and extracellular DNA (eDNA). These polymeric molecules are produced by bacteria in the biofilm and they form the biofilm matrix. This matrix is involved in bacterial adhesion and provides structure and stability to the biofilm. It acts as a communicating medium between bacteria in the biofilm, provides shelter, blocks harmful agents, and traps nutrients from the environment (Fanning et al. 2012). A mature biofilm has a porous structure with water channels providing essential nutrients for the bacteria (Huang et al. 2011). The EPS produced by commensal bacteria might protect the host from colonization by pathogens, but especially in the cariogenic biofilms, EPS plays a critical role in determining the virulence of dental plaque (Koo et al. 2013). For example, the availability of the sugar substituents, *e.g.*, sucrose and starch, affects the EPS production (Duarte et al. 2008). eDNA carries genetic information between bacterial cells and is important in the early stages of biofilm formation (Jakubovics and Burgess 2015; Rostami et al. 2016).

The biofilm community provides bacteria with many benefits. Individual bacterial taxa are localized in their own niche and together they build a biofilm consortium. Bacterial mutualism is used also with metabolites, such as lactate; the consumers and producers tend to be near each other. Anaerobic species lie in the inner parts of biofilm, while facultative and obligate aerobes reside at the periphery of the

community. Intermicrobial communication is mediated, *e.g.*, by quorum-sensing and bacteriocin production. Since biofilms are multispecies communities, bacteria also compete for nutrients, binding sites, and survival (Flemming et al. 2016).

2.1.2. Microbiota associated with oral health

Oral cavity has a high biodiversity of microbial species and approx. 700 microbial species or phylotypes have been identified (Mark Welch et al. 2016; Xie et al. 2010). The microbial diversity and composition in the oral health is being researched intensively to understand the role of microbiome in normal oral physiology, and this research has lately been focused on microbial clusters associated with specific conditions, rather than individual species. Microbial composition varies between different sites, niches, in oral cavity. Dental plaque has richer microbial structure, when compared with other environments, such as saliva and buccal mucosa. Microbial composition also varies during ageing and the development of dentition (Xu et al. 2015). Bik *et al*. (2010) studied the microbiome associated with oral health and found that 15 bacterial genera were present in all tested individuals. These genera belonged to the phyla *Proteobacteria* (including *Neisseria*, *Cardiobacterium*, *Haemophilus,* and *Campylobacter*), *Firmicutes* (*Streptococcus*, *Granulicatella*, and *Veillonella*), *Fusobacteria* (*Fusobacterium*), *Actinobacteria* (*Rothia*, *Actinomyces*, *Corynebacterium,* and *Atopobium*), and *Bacteroidetes* (*Prevotella*, *Caphocytophaga,* and *Bergeyella*). However, inter-individual variation in the oral microbiome was large (Bik et al. 2010). Species classified as oral commensals include, *e.g.*, *Streptococcus mitis*, *Streptococcus oralis*, *Actinomyces naeslundii*, *Fusobacterium nucleatum*, *Haemophilus parainfluenzae*, *Eikenella corrodens,* and *Prevotella* spp. (Kilian et al. 2006).

2.1.3. Colonization resistance

The stability of the microbial communities and the equilibrium between microbiota and the host, are important for the protective role of intrinsic commensal microbiota. Resident oral microbiota offers a colonization resistance, which protects the host from microbial invasion (He et al. 2014; Zaura et al. 2014). Proposed mechanisms of colonization resistance include the stimulation of host immune response, competition for substrates or adhesion sites, generation of microenvironment inhibitory potential, and production of antibiotic substances (Corthésy et al. 2007). He et al. (2014) has shown, that microbial community at a specific site inhibited the growth of microbes of foreign origin *in vitro*. The idea of

the health benefits of colonization resistance is based on the findings, where pathogen colonization increases after the disturbance of intrinsic microbiota by antimicrobial treatment. Furthermore, the resident microbiota is regained after probiotic consumption or faecal microbiota transplant (Kumar and Mason 2015). In oral microbial diseases, exogenous bacteria have only a limited role in the disease process (Kuramitsu et al. 2007; Zaura et al. 2014).

2.2. Oral microbial diseases

2.2.1. Dental caries

Definition and aetiology of dental caries

Dental caries is defined as an infectious disease leading to the local demineralization of enamel and dentin and the destruction of tooth organic tissues. Plaque bacteria produce acids which dissolve the minerals on tooth surface (Takahashi and Nyvad 2016). In cavitated caries lesions, dentinal enzymes, mainly matrix metalloproteinases (MMPs) activated by acid pulses from bacterial metabolism, destroy the organic dentin matrix (Mazzoni et al. 2015).

Plaque associated bacteria have a role in the disease process, and several hypotheses have been introduced to explain the role of microbiota in dental caries. W.D. Miller (1889) introduced a chemico-parasitic theory in 1889, when he recognized, that caries process included actions of acids and germs. In the chemico-parasitic and non-specific plaque theories, caries outcome is a result of the overall activity of total microbiota in dental plaque (Theilade 1986). According to the specific plaque hypothesis by Loesche (1976), specific bacteria in dental plaque are responsible for the acid production and tooth demineralization. Mutans-streptococci (MS, mainly *S. mutans* and *S. sobrinus*) were identified as the main caries pathogens (Loesche 1986).

The relationship between MS and caries is strong. MS are frequently isolated from caries lesions and, together with a high-sugar-diet, they can induce the caries process in animals (Tanzer et al. 2001). However, MS are found also on clinically sound tooth surfaces, and caries lesions may develop also in the absence of MS (Aas et al. 2008; Nyvad and Kilian 1990). Therefore, in 1994, Marsh introduced an ecological plaque hypothesis to explain the complicated role of microbiota in the caries process. This theory relies on the equilibrium of dental plaque; the plaque bacterial composition does not significantly change during minor changes in the

environment, and the dynamic stability of the microbiota is due to a balance between microbial interactions. However, if dental plaque is exposed to the changes in environmental factors, homeostasis might break down and predispose these sites to caries. Such an environmental change could be, for example, an increased amount or frequency of sugar intake leading to the acid-production, microbial adaptation, and selection of aciduric bacteria (*e.g.*, MS and lactobacilli) and the inhibition of acid-sensitive species (Marsh 1994; Takahashi 2008).

The most recent hypothesis of the caries etiology is extended ecological plaque hypothesis, by Takahashi and Nyvad (2011), in which the caries process consists of three reversible stages: dynamic stability stage, acidogenic stage, and aciduric stage. In the dynamic stability stage, the microbiota on dental surfaces represents one typical to sound enamel surfaces: *Actinomyces* and non-mutans streptococci (Dige et al. 2009; Li et al. 2004). These bacteria can produce acids, but when the sugar intake is infrequent, *e.g*., in people having regular meal-times the balance between de- and remineralization phases remains in equilibrium and tooth surfaces remain sound. Environmental changes, such as an increased sugar intake frequency, leads to the acidogenic stage: the plaque bacteria respond to the environmental changes by increased acidogenity and acidurance, and the acidification of dental plaque leads to the selection of more aciduric bacterial strains. Acid-induced microbial adaptation and selection disturb the equilibrium in the demineralization and remineralization cycles initiating a caries process. In the aciduric stage, rapid pH decreases in plaque lead to the replacement of *Actinomyces* and non-mutans streptococci by more aciduric bacteria, *e.g.*, MS, lactobacilli and bifidobacteria. Acid production by these bacteria leads to mineral loss and lesion development (Takahashi and Nyvad 2011).

Microbiota associated with dental caries

The microbiota in dental caries is versatile and varies between the different stages of the lesion development. Mutans streptococci are responsible for the initiation of the caries process, while in the cavitated lesions other bacterial species, *e.g.*, bifidobacteria, lactobacilli, and *Actinomyces* spp. are dominant (Alcaraz et al. 2012; Corby et al. 2005; Lapirattanakul and Nakano 2014; Mantzourani et al. 2009a; Mantzourani et al. 2009b). In a study of occlusal caries in its various stages, the microbiota on enamel surfaces, and in fissures and carious dentin was studied by FISH and confocal microscopy (Dige et al. 2014). Sound and non-cavitated carious enamel surfaces were colonized by streptococci, including *S. mitis*, as well as

Veillonella and *Fusobacterium*. Mutans streptococci were found on active and non-active carious sites, but not on clinically sound enamel surfaces. *Actinomyces* and scattered streptococci were observed in shallow fissures, whereas *Lactobacillus* spp., *Bifidobacterium* spp., and *S. mutans* were found on cavitated enamel and dentin (Dige et al. 2014). The deepest parts of dentin caries lesions are not as acidic as more superficial parts, and the microbial diversity in the most acidic parts is significantly reduced, when compared to neutral sites. Species found in the most acidic conditions in dentin caries lesions include *L. rhamnosus* and *L. fermentum* (Kianoush et al. 2014). *A. naeslundii* is an early colonizer associated with microbiota on healthy tooth surfaces (Bik et al. 2010; Marchant et al. 2001). However, in dental caries, it is found in the initial fissure caries lesions (Dige et al. 2014). The presence of *F. nucleatum* in carious dentin microbiota has been reported (Corby et al. 2005), but in another study, caries lesions lacked *F. nucleatum* (Dige et al. 2014).

Dental caries and arginolytic bacteria

Acidogenic bacteria in dental biofilms are responsible for the acidification of dental plaque. Consequently, other, mostly non-acidogenic, bacteria need to find a way to survive in the acidic conditions. Some bacteria produce alkaline substances resulting in a rise in intracellular and environmental pH (Takahashi and Nyvad 2011). Non-acidogenic bacteria can utilize arginine or arginine-containing peptides using arginine-deiminase-pathway (ADP) to produce ammonia, carbon dioxide, and ATP (Burne and Marquis 2000; Wijeyeweera and Kleinberg 1989). The overall arginolytic activity may affect the cariogenic potential of dental plaque, thus decreasing caries incidence. When the arginolytic activity of the plaque samples from children was studied, the activity of ADP was found to be higher in the samples from caries-lesion-free subjects than in those from subjects with enamel or dentin caries (Nascimento et al. 2013). Bacterial arginolytic activity seems to become synergistic as a response to environmental changes in dental plaque (Huang et al. 2015).

2.2.2. Periodontal disease

Definition and aetiology of periodontal disease

Periodontal diseases, mainly gingivitis and periodontitis, are multifactorial infectious diseases of the periodontium. Gingivitis is a reversible inflammatory state in the gingiva, developing as a response to local plaque accumulation. Periodontitis is an extended inflammation resulting in the loss of connective tissue around the tooth (Pihlstrom et al. 2005). In periodontal diseases, the equilibrium between the

host and microbiota changes and certain microbial complexes associated with these diseases form. During the disease process, microbial species in the gingival sulcus change from gram-positive organisms to predominantly gram-negative, anaerobic, chemo-organotrophic, and proteolytic organisms (How et al. 2016). However, bacteria associated with periodontal diseases are frequently found also in periodontally healthy subjects and, therefore, the crucial point in the disease process is the host response. In clinically healthy periodontium, normal oral microbiota lines the junctional epithelium and stimulates a mild inflammatory response. In comparison, clinically diseased periodontal tissues express high levels of inflammatory molecules and host inflammatory reactions contribute to the tissue destruction in periodontitis (Berezow and Darveau 2011).

Microbiota associated with periodontal disease

Bacterial clusters associated with periodontal diseases, such as orange complex (with, *e.g.*, *F. nucleatum* and *Prevotella intermedia*) and red complex (with *P. gingivalis*, *Tannerella forsythia*, and *Treponema denticola*) have been recognized (Socransky et al. 1998). Furthermore, the total bacterial count in periodontal diseases is greater than in periodontally healthy subjects (Ximénez Fyvie et al. 2000).

P. gingivalis is a gram-negative late-colonizing organism, and it is considered as the major agent in periodontitis with subgingival sulcus as the main habitat. The count of *P. gingivalis* correlates with pocket depth and bleeding on probing (Oliveira et al. 2016; Socransky et al. 1998). *P. gingivalis* requires amino acids and iron for energy production. It produces several virulence factors, defined as constituents or metabolites essential to the various stages of the life cycle. External environment of the periodontopathogen often regulates the expression of virulence factors. In a susceptible host, virulence factors cause the destruction of periodontal tissues, bone resorption, inhibition of host protective mechanisms, as well as induction of host responses (cytokine production) (How et al. 2016). *P. gingivalis* is a keystone pathogen in periodontitis (Hajishengallis et al. 2012).

In periodontitis, *A. naeslundii* is present in subgingival or sulcular microbiota, but it is also considered to be important in maintaining periodontal health. The count of *A. naeslundii* decreases with increasing pocket depth in periodontitis subjects (Kamma et al. 2000; Liu et al. 2012; Vielkind et al. 2015; Ximénez Fyvie et al. 2000). In an *in vitro* biofilm study, the presence of *A. naeslundii* was important to the growth of *F. nucleatum* (Periasamy et al. 2009). *F. nucleatum* is a member of mature dental biofilm. It is the linking organism between co-aggregating initial and late

colonizers (Kolenbrander et al. 2010). *F. nucleatum* is found in periodontally healthy subjects, but also in periodontal disease (Kamma et al. 2000). Gram-negative *F. nucleatum* is a part of the orange complex associated with the development of periodontal disease (Socransky et al. 1998). It plays a central role in the maturation of biofilm, from an aerobic one, consisting mainly of gram-positive species, to a gram-negative dominant anaerobic one. It also promotes the growth of *P. gingivalis* by providing it with a capnophilic environment (Huang et al. 2011).

2.2.3. Matrix metalloproteinases in dental caries and periodontal disease

Matrix metalloproteinases (MMPs) are a multigene family of enzymes responsible for extracellular matrix degradation. They are involved in physiological development, tissue remodelling, and pathogenic inflammatory and malignant tissue destruction. They are mainly expressed in polymorphonuclear leukocytes and monocytes (Hannas et al. 2007). The tissue inhibitors of matrix metalloproteinases (TIMPs) control and inhibit MMPs. Due to the multiple role of MMPs in anti- and pro-inflammatory processes, their levels can be utilized as indicators of tissue destruction, but also as a sign of physiological and anti-inflammatory defence (Sorsa et al. 2016).

At least MMP-2, MMP-3, MMP-8, and MMP-20, produced by odontoblasts, are present in human dentin (Mazzoni et al. 2015; Palosaari et al. 2003). MMPs are suggested to have an active role in the caries process through dentin matrix degradation. While the acids of bacterial origin are responsible for the mineral loss in caries lesion formation process, MMPs (MMP-8, MMP-9, and MMP-2), activated by acidic pH, degrade the exposed collagen matrix in dentine. MMP preforms are activated in low pH, but they function best in neutral environment. Thus, periodic environmental acidic-neutral shifts, typical of caries process, result in MMP activity and finally in cavity formation (Tjäderhane et al. 1998). In animal models, MMP inhibition resulted in a significant reduction in dentinal caries (Sulkala et al. 2001; Tjäderhane et al. 1999), whereas elevated salivary MMP-8 levels correlated with manifested caries (Hedenbjörk Lager et al. 2015).

In periodontal disease, host MMPs are involved in the destruction of periodontal tissue. They are secreted as a response to the bacterial challenge and in periodontal disease, relative over-expression of MMPs in relation to TIMPs is involved (Gursoy et al. 2010; Sorsa et al. 2016). The major MMPs in gingival crevicular fluid (GCF) are MMP-8 and MMP-9 (Sorsa et al. 1988; Sorsa et al. 2016). The GCF concentrations of these inflammatory markers reflect the salivary concentrations, and the salivary

levels of these inflammatory markers can be used to determine the persistence or activity of the disease (Sorsa et al. 2016). In several studies, elevated salivary levels of MMP-8 and MMP-9 and their elevated ratio to TIMP-1 have been associated with gingivitis and periodontal disease (Nedzi Góra et al. 2014; Nizam et al. 2014; Rathnayake et al. 2013; Salminen et al. 2014). When gingivitis is appropriately treated, salivary MMP-8 levels decrease close to the levels in healthy subjects (Ebersole et al. 2015; Syndergaard et al. 2014).

2.3. Probiotics and oral health

2.3.1. Probiotic mechanisms of actions in oral cavity

Probiotic treatment has been suggested as a method for the prevention or supporting therapy of dental caries and periodontal disease, common oral diseases. The potential action mechanisms of probiotics in the oral cavity can be divided in three categories: the modulation of the host inflammatory response, direct effects against pathogenic bacteria, and indirect effects against pathogenic bacteria (Laleman and Teughels 2015). Some potential action mechanisms are summarized in Table 1.

Table 1. Suggested probiotic actions in oral cavity

Adhesion on oral surfaces, at least temporary colonization in oral cavity, integration into biofilm (Stamatova and Meurman 2009)
Competition on adhesion sites, effect on attachment of other bacteria, aggregation with other bacteria (Devine and Marsh 2009; Haukioja 2010)
Competition for nutrients and growth factors (Haukioja 2010)
Production of antimicrobial substances, e.g.*, bacteriocins, to inhibit oral microbiota* (Haukioja 2010, Stamatova and Meurman 2010)
Local and systemic immunomodulation, reduction of pro-inflammatory cytokine production, increase in anti-inflammatory cytokines production (Devine and Marsh 2009; Haukioja 2010)

2.3.2. Probiotics in oral health

Numerous randomized controlled trials of varying quality have been performed to find out the effects of probiotics on dental caries and periodontal disease (Dhingra et al. 2012). With respect to dental caries, the measured outcome has most often been MS/LB counts in saliva and plaque. The effects on caries incidence and plaque acidogenity have also been studied. The effect on periodontal health has been studied by measuring plaque and gingival index (PI, GI), gingival inflammation, bleeding on probing (BOP), probing pocket depth (PPD), clinical attachment level (CAL), and periodontal pathogen count. Recently, four systematic reviews summarizing the effects of probiotics on oral health have been published. Probiotics exert no severe adverse effects, and they do not increase the risk of caries or periodontal disease. Current evidence is insufficient to recommend probiotics for the management of dental caries, and further studies should be performed to ascertain their efficacy and safety for teeth. However, gingival health seems to be positively affected by probiotic therapy (Cagetti et al. 2013; Gruner et al. 2016; Martin Cabezas et al. 2016; Yanine et al. 2013).

2.3.3. Oral colonization of probiotic bacteria

Adhesion on oral surfaces or integration into plaque biofilm are necessary for at least a temporarily successful oral cavity colonization by probiotics. Probiotic bacteria may adhere on dental surfaces via acquired salivary pellicle or by co-aggregation with other bacteria. In an *in vitro* study, probiotic bacterial strains showed significant differences in adhesion on saliva-coated hydroxyapatite (s-HA). Some lactobacilli (*e.g.*, *Lactobacillus rhamnosus* GG (LGG)) adhered well on s-HA, while only a low number of *Bifidobacterium* strains (*e.g.*, *Bifidobacterium animalis* subsp. *lactis* BB-12 (BB-12)) adhered successfully. When *Fusobacterium nucleatum* was added to the test arrangement, the adhesion of bifidobacteria increased significantly and the adhesion of high-binding lactobacilli decreased significantly, when compared to the adhesion on s-HA alone (Haukioja et al. 2006). Probiotic LGG can also integrate into the oral multispecies biofilms *in vitro* and some probiotic lactobacilli have co-aggregation properties with MS (Jiang et al. 2016; Twetman et al. 2009).

Probiotic bacteria seem to colonize oral cavity only temporarily. Caglar et al. (2009a) studied the oral colonization of probiotic *L. reuteri* ATCC 55730 in healthy young adults. Probiotic-containing tablets were consumed for a 2-week intervention period. During the intervention, probiotics were detected in all saliva samples. After

the intervention, only 48 % of the subjects carried *L. reuteri* in their saliva on day 1, and after a five-week post-treatment period, none of the subjects had detectable amounts of *L. reuteri* (Caglar et al. 2009a). Also, LGG has been found to colonize only temporarily after probiotics consumption (Yli-Knuuttila et al. 2006). In a 2-week intervention with *L. reuteri* (strains ATCC 55730 and ATCC PTA 5289) and LGG, four of thirteen subjects were found with LGG and six with *L. reuteri* in their plaque (Marttinen et al. 2012). With other probiotic bacterial strains (BB-12, *Lactobacillus acidophilus* LA-5, and *L. paracasei* subsp. *paracasei* F19), no colonization of probiotics on dental surfaces after probiotic consumption has been observed (Ravn et al. 2012).

Hypothetically, the more persistent colonization may occur, when probiotics are introduced to the still developing oral microbiota in children. In one study, infants received probiotic BB-12 twice a day from the age of 1-2 months to the age of 18 months. Only three subjects had barely detectable amounts of BB-12 in their oral samples (from dental plaque and oral mucosa) at the age of 8 months and none at the age of two years (Taipale et al. 2012). However, chlorhexidine seems to result in a higher level of probiotic LGG in the saliva of children when administered as a pre-treatment before probiotics consumption (Aminabadi et al. 2011).

2.3.4. Probiotics in dental caries

Probiotics, caries risk factors, and caries incidence

Salivary or plaque MS/LB counts are easily accessible risk factors of dental caries. Thus, numerous studies of the effects probiotics have on dental caries utilize them as indicators. Probiotic interventions have reduced MS count in saliva or plaque (Aminabadi et al. 2011; Campus et al. 2014; Jindal et al. 2011; Näse et al. 2001; Singh et al. 2011). Some studies, which have reported decreased MS count, have found no evidence of effects on or even an increase in LB count (Cildir et al. 2009; Singh et al. 2011; Taipale et al. 2012). Probiotics may also cause an increase in salivary LB count with no effect on MS count (Marttinen et al. 2012; Montalto et al. 2004). However, several studies report no effects on the caries-associated organisms (MS/LB in plaque/saliva) after probiotics consumption (Ahola et al. 2002; Keller et al. 2012; Petersson et al. 2011; Stecksén-Blicks et al. 2009; Taipale et al. 2013).

Probiotic studies focusing of caries incidence, show contrasting results with each other. LGG-containing milk reduced caries in subjects who were consuming probiotics, especially in 3-4-year-old children, when caries incidence was studied in

1-6-year-old children (Näse et al. 2001). Root caries lesions were more remineralized after intervention with milk containing fluoride and probiotic *L. rhamnosus* LB21. However, the effect of fluoride cannot be underestimated, since the greatest mineral gain was received in the groups with fluoride (Petersson et al. 2011). In adolescents, probiotic *Lactobacillus reuteri* ATCC PTA 5289 and DSM 17938 may restrict early caries lesion development (Keller et al. 2014). Taipale et al. (2013) observed no reduction in caries at 4 years of age, despite the consumption of BB-12 at the age of 2-18 months.

According to a systematic review by Gruner et al. (2016), probiotic therapy significantly increases the chance of reducing MS count in ordinal counts, but when the used probiotic genera (lactobacilli and bifidobacteria) are analysed separately, only bifidobacteria contributed to this beneficial effect significantly. When MS reduction after probiotic consumption is compared to the control groups, both bifidobacteria and lactobacilli caused MS reduction. However, the heterogeneity of the studies is high and publication bias is suspected.

Cariogenic properties of probiotic bacteria

Potential cariogenity is considered as a safety issue with probiotics. Resistance to acidic conditions is one of the inclusion criteria of probiotic bacteria in the intestines and the production of lactic acid is a natural characteristic of bifidobacteria and lactobacilli (Isolauri 2001; Meurman 2005). However, cariogenic organisms generate acidic conditions and survive in them (Kianoush et al. 2014). The most commonly used probiotics, lactobacilli and bifidobacteria, inhabit also the oral cavity and caries lesions (Dal Bello and Hertel 2006; Kaur et al. 2013; Kianoush et al. 2014). Probiotic lactobacilli and bifidobacteria produce acids rapidly from glucose, some also from sucrose, fructose, and lactose and some even from sugar alcohols mannitol and sorbitol. Acidogenity differs between strains (Haukioja et al. 2008; Hedberg et al. 2008). *L. salivarius* W24 integrates in salivary microcosms *in vitro* and results in more acidic and less diverse microbiota (Pham et al. 2009). Furthermore, *L. salivarius* LS1952R has been shown to induce caries in rats alone and together with *S. mutans* (Matsumoto et al. 2005). However, when the cariogenic potential of LGG was studied *in vitro*, other bacteria were responsible for the increased cariogenity of the multispecies microcosm. LGG did not lower the pH but it survived in a highly cariogenic microcosm (Pham et al. 2011). *L. reuteri* ATCC 55730 and ATCC PTA 5289 also integrate in oral biofilms *in vitro* with no effect on biofilm pH (Madhwani and McBain 2011) and *L. reuteri* strains DSM 17938 and PTA 5289, LGG,

or *L. plantarum* 299v do not increase the plaque acidogenity either (Keller and Twetman 2012; Marttinen et al. 2012). *L. reuteri* produces EPS from sucrose, which hypothetically increases the amount and cariogenity of dental plaque (Arsköld et al. 2007). When environmental factors are favourable, organisms with cariogenic characteristics may disturb the balance in dental biofilms and increase plaque virulence.

2.3.5. Probiotics in periodontal disease

There are numerous clinical studies of probiotics in periodontal disease, and the heterogeneity between different studies and measured parameters is large. Patients with gingivitis, experimental gingivitis, and periodontitis have been used as subjects and study parameters include salivary and plaque levels of periodontal pathogens; probing pocket depth (PPD) and clinical attachment level (CAL); and BOP, GI, and PI. Some studies report also immunological effects. Generally, probiotic interventions have been short with periods lasting only 2-3 weeks. In gingivitis patients and subjects with experimental gingivitis, probiotics have been shown to decrease the amount of plaque and enhance gingival health (reduced GI and/or BOP) (Campus et al. 2014; Krasse et al. 2006; Slawik et al. 2011; Twetman et al. 2009). In periodontitis patients, the use of probiotics has also decreased PPD and CAL) (Shimauchi et al. 2008; Tekce et al. 2015; Teughels et al. 2013; Vivekananda et al. 2010).

Probiotics reduce the numbers of periodontal pathogens (*e.g.*, *P. gingivalis*, *P. intermedia*, *T. forsythia*, and *A. actinomycetemcomitans*) in saliva and supra/subgingival plaque (Iniesta et al. 2012; Mayanagi et al. 2009; Shah et al. 2013; Teughels et al. 2013; Vivekananda et al. 2010). Probiotic effects in gingival health may also be mediated by enhanced oral immunity through reduced amounts of GCF, elastase, MMP-3, IL-8, and TNF-α in GCF; and lowered salivary lactoferrin levels (Shimauchi et al. 2008; Slawik et al. 2011; Staab et al. 2009; Twetman et al. 2009).

2.3.6. Probiotics and matrix metalloproteinases

Since MMPs play a role in both caries and periodontitis, the possible immunomodulatory actions of probiotics in these diseases may be mediated by their effects on MMPs. Probiotic milk with *L. casei* Shirota have been shown to decrease the levels of MMP-3 in gingival crevicular fluid in healthy subjects with experimental gingivitis (Staab et al. 2009). In chronic periodontitis patients, GCF MMP-9 levels were shown to decrease and TIMP-1 levels to increase significantly

more when probiotic *L. reuteri* was added to traditional SRP therapy (Ince et al. 2015). The effect of probiotic bacteria on the MMP levels in caries patients is unknown. However, probiotics have been found to affect MMP levels in other conditions: in a mouse asthma model, LGG has been shown to decrease MMP-9 expression in lung tissue (Wu et al. 2014); cell-free supernatant of *L. rhamnosus* and *L. casei* have been found to decrease the MMP-9 levels in cancer cells; and *L. rhamnosus* has been discovered to inhibit activity against MMP-9 (Escamilla et al. 2012). Furthermore, it has been found that *L. bulgaricus* does not convert proMMP-9 into an active form (Stamatova et al. 2009).

2.3.7. Probiotics used in this study

Lactobacillus reuteri

L. reuteri is frequently found in the gut and it has numerous health effects, *e.g.*, in infants (Urbanska et al. 2016; Valeur et al. 2004). *L. reuteri* strain ATCC 55730 has originally been isolated in the breast milk and ATCC PTA 5289 in the oral cavity (Spinler et al. 2008). Both strains are widely used in probiotic products meant, for example, for infants, or designed to improve gastrointestinal health or gingival health. Probiotic studies of *L. reuteri* strains ATCC PTA 5289, ATCC 55730 and its daughter strain DSM 17938, and other *L. reuteri* strains in the mouth are reviewed in this chapter.

L. reuteri ATCC 55730 has been found to bind to s-HA with a low intensity (Haukioja et al. 2006), but the adhesion of ATCC PTA 5289 on s-HA has not been studied. However, they seem to have differences in their adhesion and biofilm formation tendency. In biofilm on a polystyrene plate, *L. reuteri* ATCC PTA 5289 has been show to produce bacterial densities that are 10 times higher than those produced by ATCC 55730 (Jones and Versalovic 2009). *In vivo* oral cavity colonization by both strains seems to be temporary (Caglar et al. 2009a; Twetman et al. 2009).

The antimicrobial activity of *L. reuteri* against other bacteria includes growth and adhesion inhibition. *L. reuteri* strains ATCC 55730, KCTC 3594, and KCTC 3678 have wide antimicrobial activity against oral pathogens (*e.g.*, *S. mutans*, *S. gordonii*, and *T. forsythia*) and they also weakly inhibit *A. naeslundii* (Baca Castañón et al. 2015; Kang et al. 2011). In an experiment using the agar-overlay method, *L. reuteri* ATCC PTA 5289 and ATCC 55730 were found to inhibit the growth of MS, and especially the strain ATCC 55730 was discovered to be efficient also in inhibiting the growth of *Candida albicans* (Hasslöf et al. 2010; Keller et al. 2011). The growth of MS in *L.*

reuteri (strain not indicated) supplemented milk formula was found to be lower when compared to non-supplemented formula *in vitro* (Duse et al. 2014).

L. reuteri ATCC PTA 5289 inhibits the adhesion of *S. mutans* on s-HA (Söderling et al. 2011). Also, the biofilm formation of *S. mutans* is inhibited by both ATCC PTA 5289 and ATCC 55730, and the latter strain shows stronger inhibition. This effect is pH dependent (Marttinen et al. 2013). In addition to the effects on adhesion and biofilm formation, possible mechanisms include high co-aggregation between *L. reuteri* ATCC PTA 5289 and *S. mutans* (Keller et al. 2011). *L. reuteri* may also inhibit the adhesion of *S. mutans* by affecting its gene-expression. *L. reuteri* DSM20016-derived biosurfactants have resulted in the down-regulation of the gene-expression of *S. mutans* glucosyltransferases (gtfs) and that of fructosyltransferase (ftf) (Salehi et al. 2014).

Some of *L. reuteri*'s antimicrobial activity may be mediated by reuterin, a wide-spectrum antimicrobial substance, which *L. reuteri* produces from glycerol (Talarico et al. 1988; Talarico and Dobrogosz 1989). In dental plaque, glycerol concentrations vary between 100 and 400 µg per g (Runnel et al. 2013). Glycerol supplementation increases reuterin production and, consequently, the following antimicrobial effect of *L. reuteri* ATCC PTA 6475 against pathogens, *e.g.*, *Salmonella enterica* (De Weirdt et al. 2012). Interestingly, strain ATCC 55730 may produce higher quantities of reuterin than ATCC PTA 5289 (Jones and Versalovic 2009). However, when bacterial antagonism was studied in a biofilm model, both *L. reuteri* strains ATCC PTA 5289 and ATCC 55730 decreased total anaerobe and streptococci counts, but did not decrease the amount of other oral pathogens, with or without added glycerol (Madhwani and McBain 2011).

In the mouth, *L. reuteri* has reduced MS count after probiotic consumption. In an intervention of full-mouth disinfection followed by the consumption of *L. reuteri* DSM 17938 and ATCC PTA 5289, the patients with *L. reuteri* in their saliva after the study period showed slower regrowth of *S. mutans* (Romani Vestman et al. 2013). In another study, salivary MS count was not different between test and control group after a full-mouth disinfection and *L. reuteri* intervention with the same strains as in the study by Romani Vestman et al. (Keller et al. 2012). Romani Vestman (2013) emphasized that the presence of *L. reuteri* in saliva after 3-months follow-up was associated with slower MS regrowth. The effect of *L. reuteri* DSM 17938 and ATCC PTA 5289 on early caries lesions is limited (Keller et al. 2014). Early *L. reuteri* consumption during the first year of life seems to have long-term health effects. Children who received *L. reuteri* strain ATCC 55730 had reduced caries prevalence

and less gingivitis at the age of nine. *L. reuteri* consumption does not result in a long-term colonization of probiotics or changes in the plaque microbial composition (Stensson et al. 2014). *L. reuteri* DSM 17938 and PTA 5289 may temporarily change the microbial composition as in the study by Romani Vestman *et al*. (2015), the effect was transient and was no longer present one month after the probiotic intervention.

It has been claimed that *L. reuteri* ATCC 55730 is safe for teeth since it does not degrade HA during incubation with saliva and growth medium (Nikawa et al. 2004). However, it ferments glucose, sucrose, and lactose and produces acids (Haukioja et al. 2008). *L. reuteri* PTA 5289 is poor in acid production in the presence of sugars and only weak delayed fermentation happened with, *e.g.*, glucose, lactose, and sucrose (Hedberg et al. 2008). *L. reuteri* ATCC 55730 produced EPS from sucrose and starch, but not from glucose (Arsköld et al. 2007; Bai et al. 2016).

L. reuteri intervention with strain ATCC PTA 5289 in combination with either ATCC 55730 or DSM 17938 decreases PI, GI, and PPD in patients with gingivitis, moderate periodontitis, and pregnancy gingivitis (Krasse et al. 2006; Schlagenhauf et al. 2016; Vicario et al. 2013). In periodontitis and gingivitis patients, the same strains lower the count of *P. gingivalis* and *P. intermedia* in subgingival plaque and saliva (Iniesta et al. 2012; Krasse et al. 2006; Teughels et al. 2013). Furthermore, reduced *Aggregatibacter actinomycetemcomitans* count have been observed in the plaque of periodontitis patients after an intervention with the same *L. reuteri* strains combined with SRP (Vivekananda et al. 2010). *L. reuteri* strains ATCC PTA 5289 and DSM 17938 combined with SRP in the treatment of periodontitis patients seems to result in long-lasting effects. After a 3-week probiotic intervention, clinical parameters (PI, GI, BOP, and PPD) were found to be significantly lower in probiotic subjects when compared with the subjects in the control group, even after a 1-year follow-up period (Tekce et al. 2015).

Probiotic *L. reuteri* ATCC 5289 and ATCC 55730 modulate the oral immunity by reducing pro-inflammatory cytokines IL-8, IL-17, and TNF-α in the GCF of gingivitis and periodontitis patients (Szkaradkiewicz et al. 2014; Twetman et al. 2009). A strain-dependent difference has been observed in suppression of TNF-α production. ATCC PTA 5289 supresses TNF-α production while ATCC 55730 does not (Jones and Versalovic 2009). In addition to the reduced clinical periodontal parameters in chronic periodontitis patients, Ince et al. (2015) also reported that, when *L. reuteri* intervention was combined with SRP, the MMP-8 levels in GCF decreased and those of TIMP-1 increased.

Lactobacilllus rhamnosus GG (ATCC 53103)

Lactobacillus rhamnosus GG (LGG) was isolated from the human intestine and it has well documented beneficial health effects, *e.g.*, in antibiotic diarrhoea and atopic eczema (Meurman 2005; Pace et al. 2015). With respect to the oral cavity, LGG seems to be a harmless organism, with some beneficial effects. In an adhesion experiment, LGG adhered well on s-HA, but when *F. nucleatum* was added to the test arrangement, binding was low (Haukioja et al. 2006; Stamatova et al. 2009). LGG co-aggregates poorly with *S. mutans* (Keller et al. 2011). Normally, oral colonization by LGG is temporary but long term colonization has been reported in one case in which the subject had a history of LGG consumption in childhood (Yli-Knuuttila et al. 2006).

LGG uses only glucose for acid production, while fermentation with sucrose and lactose does not result in acidic conditions (Haukioja et al. 2008). Hedberg et al. (2008) have found that LGG can ferment glucose and fructose and shows delayed reaction to sucrose and lactose.

In the studies of microbial interactions, LGG has inhibited the growth of *S. mutans* in a two-species biofilm when LGG was added 24 h before *S. mutans*, or they both were added simultaneously. However, when *S. mutans* was added 24 h before LGG to the biofilm model, no reduction in *S. mutans* count was found, but the growth of LGG was enhanced. In saliva-derived microcosms LGG decreased MS count and the amounts of *Actinomyces odontolyticus* in the presence of sucrose. It had no effect on pH or dentin demineralization. Interestingly, sucrose-exposed microcosms harboured significantly more LGG than microcosms without sucrose. LGG has also decreased the count of genus *Prevotella*, irrespective of the presence or absence of sucrose (Pham et al. 2011). LGG integrates into oral biofilms *in vitro* and inhibits the growth of *S. sanguinis* and *C. albicans*. It also lowers the ability of *F. nucleatum* to form a biofilm, with no reduction in MS count (Jiang et al. 2016). However, LGG has induced mineral loss in dentin in highly cariogenic conditions, with no reduction in *S. mutans* count. It is suggested that in favourable conditions LGG might be cariogenic (Schwendicke et al. 2014a).

The results of clinical studies of the inhibitory effect of LGG on salivary MS are in contradiction to each other. The consumption of LGG and *L. reuteri* has no effect on plaque acidogenity or salivary MS count (Marttinen et al. 2012), nor does LGG alone decrease the salivary MS count (Ahola et al. 2002). Näse et al. (2001) exposed preschool children at 1-6 years of age to LGG-containing milk for 7 months.

Significant caries reduction after probiotic consumption was observed in children of 3-4 years. Both the oral colonization of LGG and the reduction of *S. mutans* in children can be enhanced by chlorhexidine pre-treatment (Aminabadi et al. 2011).

Bifidobacterium animalis subsp. lactis BB-12

Bifidobacteria are frequently found in the gut and they inhabit especially the digestive tract of breast-fed infants. *Bifidobacterium animalis* subsp. *lactis* BB-12 (BB-12) has a proven beneficial effect on gastrointestinal health and it is a widely studied probiotic *Bifidobacterium* strain. BB-12 originates in Chr. Hansen's collection of dairy cultures (Jungersen et al. 2014).

BB-12 uses glucose for acid production, while the fermentation of sucrose and lactose is low (Haukioja et al. 2008). Its adhesion on s-HA is poor, but *F. nucleatum* enhances the adherence (Haukioja et al. 2006). MS growth is lower in *Bifidobacterium lactis* supplemented milk-formula than in non-supplemented formula (Duse et al. 2014) and a short-term consumption of BB-12 has been shown to lower the salivary MS counts both in adults and in children (Caglar et al. 2008; Singh et al. 2011). When the potential cariogenity of BB-12 was assessed in an *in vitro* study with viable and heat-inactivated BB-12, viable BB-12 could induce some mineral loss, which proved to be minor when compared to that caused by *S. mutans*. However, heat-inactivated BB-12 decreased the cariogenity of *S. mutans* by generating significantly less mineral loss, when compared to viable BB-12 combined with *S. mutans* (Schwendicke et al. 2014b). Early administration of BB-12 has resulted in poor oral colonization and has shown no beneficial effects in the action against caries, *e.g.*, lowered MS count or decrease in manifested caries (Taipale et al. 2012, Taipale et al. 2013). However, in the same study BB-12 use caused a reduced incidence of respiratory infections in the probiotic subject group during the first eight months of life (Taipale et al. 2016).

Probiotic *Bifidobacterium* (strain not indicated) has inhibited the growth of periodontal pathogens *P. gingivalis*, *F. nucleatum*, and *A. actinomycetemomitans in vitro* when inoculated as an early colonizer. In a simultaneous bacterial addition, *Bifidobacterium* no longer inhibited the pathogens (Zhu et al. 2010). However, studies of the effects of BB-12 on periodontal pathogens or periodontal disease are not available.

Oral bifidobacteria

The major habitat of bifidobacteria is the intestines, but some of them are found also in the oral cavity (Beighton et al. 2008; Turroni et al. 2014). Human milk includes several bifidobacterial growth stimulating factors and in breast-fed infant's gut, bifidobacteria are predominant. Probiotic bifidobacteria are introduced in several new-born pathologies and infant diseases, such as necrotizing entero-colitis, infantile colics and acute diarrhoea (Di Gioia et al. 2014). They reduce the risk of respiratory tract infections in early childhood (Taipale et al. 2016). In adults, bifidobacteria have positive effect in the treatment of *e.g.* ulcerative colitis (Saez Lara et al. 2015).

In the mouth, bifidobacteria are associated with dental caries and they are found in elevated quantities in the saliva of caries-active children, as well as in active occlusal and root caries lesions (Kaur et al. 2013; Mantzourani et al. 2009a; Mantzourani et al. 2009b). *B. longum* and *B. dentium* are species frequently found in caries-active subjects (Beighton et al. 2008; Mantzourani et al. 2009a). *B. dentium* is absent in an edentulous mouth (Mantzourani et al. 2010). Their counts correlate with other caries-associated bacteria, *e.g*., mutans streptococci, and lactobacilli, as well as oral hygiene and dietary habits such as the frequency of sugar consumption and the amount of dietary sugar. In a site-specific study, the lowest proportions of bifidobacteria was found on sound dental surfaces. (Kaur et al. 2013). Furthermore, bifidobacteria have been found in the denture stomatitis of edentulous patients (Mantzourani et al. 2010). However, bifidobacteria are associated with periodontal health (Hojo et al. 2007b).

An examination of the *B. dentium* Bd1 genome showed a genetic adaptation in the oral cavity, and it is a potential cariogenic pathogen (Ventura et al. 2009). Interestingly, when the resistance of bifidobacteria to acidic conditions was studied *in vitro*, *B. longum* tolerated well the wide pH range between 4.0-8.0, and *B. dentium* grew best in a narrow pH range around neutral (Nakajo et al. 2010). When the microbial diversity of dentin caries lesion was studied in relation to pH, *B. dentium* was present at sites with pH 5.5-6.0 (Kianoush et al. 2014). However, *S. mutans* has been shown to generate more acidic conditions in a dual-species biofilm with bifidobacteria than when it is present alone (de Matos et al. 2016).

3. AIMS OF THE STUDY

Currently, probiotics are considered to improve gingival health, but their effect on dental caries seems to be limited. Probiotic products, containing, *e.g.*, *L. reuteri* have been designed to improve oral health, and they are on the market. BB-12 and LGG are also widely used in probiotic products. Still, the knowledge of the cariogenic potential of probiotics is scarce. Studies of their effects on gingival health have been focused on lactobacilli, mainly on *L. reuteri*. Bifidobacteria are associated with dental caries and have a contradictory role in oral cavity. Furthermore, there are no clinical studies available on the effects of probiotic BB-12 and LGG on gingival health.

The aim of this thesis was to study the probiotic bacteria in relation to the most common oral infectious diseases, dental caries and gingivitis. The work included both *in vitro* and *in vivo* studies.

The specific aims were:

> To study the properties of probiotic *L. reuteri* stains ATCC 55730 and ATCC PTA 5289 from the cariological point of view. The work included both adhesion and biofilm formation, and studies of acidogenity and arginine degradation. The hypothesis was, that the probiotic *L. reuteri* strains adhere to and form a biofilm on dental surfaces. It is suggested that they produce acids from sugars, and alkaline pH in the presence of arginine. (Study I)
>
> To study the integration and the effects of probiotic BB-12 and oral *Bifidobacterium* isolates on the oral supragingival cariogenic and subgingival biofilms *in vitro.* Since bifidobacteria are associated with dental caries and, simultaneously, with gingival health there might be variation in their integration and growth in supragingival and subgingival biofilms. The hypothesis was that bifidobacteria can integrate into biofilms and affect, most likely inhibit, the growth of other bacteria in them. (Study II)
>
> To study the effects of probiotic LGG and BB-12 on the oral health of healthy adults after probiotic intervention. The studied effects of the probiotics were: effect on the salivary MS and LB count; effect on the composition of oral microbiota; effect on clinical outcome (plaque and gingival indices); and effect on salivary MMP-8, MMP-9, and TIMP-1 levels. The hypothesis was that probiotics have no effect on the oral microbiota or host response in healthy subjects. (Studies III and IV)

4. MATERIALS AND METHODS

The summary of materials and methods is presented in this chapter. More detailed information on study procedures can be found in the materials and methods sections of studies I-IV.

4.1. Micro-organisms and saliva preparations

4.1.1. Bacterial strains used

This study included four probiotic strains and nine oral *Bifidobacterium* isolates (listed in Table 2). Bacteria representing oral pathogens and oral commensals were used *in vitro* (I, II), and these included *Actinomyces naeslundii* ATCC 12104 (II), *Fusobacterium nucleatum* ATCC 10953 (II), *Streptococcus mutans* strains MT8148 and Ingbritt (I, II), and *Porphyromonas gingivalis* ATCC 33277 (II).

Table 2. Probiotic bacteria and oral *Bifidobacterium* isolates used in studies I-IV.

STUDY	BACTERIAL STRAIN	ROLE	ORIGIN
I	*Lactobacillus reuteri* ATCC PTA 5289	probiotic	oral cavity
I	*Lactobacillus reuteri* ATCC 55730	probiotic	breast milk
II, III, IV	*Bifidobacterium animalis* subsp. *lactis* Bb12	probiotic	Chr. Hansen's collection of dairy cultures
II	*Bifidobacterium dentium* AJ 32-1	oral isolate	oral cavity (Beighton et al. 2008; Kaur et al. 2013)
II	*Bifidobacterium dentium* AJ 47-1	– " –	– " –
II	*Bifidobacterium dentium* NH 4-1	– " –	– " –
II	*Bifidobacterium dentium* NH 6-1	– " –	– " –
II	*Bifidobacterium dentium* RC-12	– " –	– " –
II	*Bifidobacterium longum* MU 57-1	– " –	– " –
II	*Bifidobacterium longum* MU 86-7	– " –	– " –
II	*Bifidobacterium longum* MU 92-2	– " –	– " –
II	*Bifidobacterium longum* MU 93-4	– " –	– " –
III, IV	*Lactobacillus rhamnosus* GG (ATCC 53013)	probiotic	gut

4.1.2. Bacterial growth conditions

In Study I, liquid pre-cultures were used: de Man Rogosa et Sharpe (MRS; Becton Dickinson Company (BD), New Jersey, USA) medium for lactobacilli and Brain Heart Infusion (BHI; BD) broth for streptococci.

In Study II, bacterial strains were pre-cultured on agar. Mitis Salivarius Agar (MS; BD; and Le Pont de Claix, France), in biofilm assays, and on BHI (BD) medium, supplemented with 13.5 g of agar l^{-1}, in agar-overlay assays were used for MS. *A. naeslundii* was precultured on Blood agar containing 5% sheep blood (BD). *F. nucleatum* and *P. gingivalis* were precultured on Brucella agar (BD). Bifidobacteria were grown on MRS agar (BD). MRS agar for BB-12 was supplemented with 0.05% (wt vol^{-1}) cysteine hydrochloride and 5 mg ml^{-1} of mupirocin (Merck, Darmstadt, Germany) in biofilm assays.

In Study I, MS were incubated in CO_2-rich atmosphere (74% N_2 and 7% CO_2), and LB in air (HA degradation experiment) or in anaerobic conditions (80% N_2, 10% CO_2, and 10% H_2). All incubations in Study II were performed in an anaerobic atmosphere (90% N_2, 5 % CO_2, 5% H_2).

4.1.3. Saliva preparations

Saliva for adhesion (I) and biofilm studies (I, II) was collected on several occasions from healthy volunteers, who were instructed to avoid eating, drinking, and dental hygiene for 1 h before collection. Unstimulated whole saliva (I, II), paraffin stimulated whole saliva (I, II), and parotid saliva stimulated with Salivin tablets (I, Pharmacia Ltd., Vantaa, Finland) and collected with Lashley cups were collected in sterile polypropylene tubes on ice. Whole saliva was clarified with centrifugation. For adhesion study (I), whole saliva was diluted 1:1 in buffered KCl, pH 6.5, and stored on ice until used in experiments. For biofilm studies (I, II) saliva samples from several volunteers were pooled and pasteurized (Guggenheim et al. 2001). The efficacy of pasteurization was assessed by plating a sample on blood agar with 5 % sheep blood (BD). Saliva aliquots were stored at -20 °C until use.

4.2. Cariological studies of *Lactobacillus reuteri*

4.2.1. Adhesion on saliva-coated hydroxyapatite

Adhesion was studied by slightly modifying the method described by Clark (1977; Haukioja et al. 2006). Metabolically [^{35}S]-methionine-labeled lactobacilli and

streptococci were washed twice and suspended in buffered KCl. Then, suspensions of bacteria with approx. 1.0 x 10^8 CFU/ml were prepared. Hydroxyapatite beads (HA; BDH Chemicals Ltd., Poole, UK) were washed with buffered KCl, and the labeled bacteria were let to adhere on saliva-coated HA for 60 minutes in room temperature (RT). Unattached bacteria were washed out with buffered KCl, and the number of bound bacteria was counted with a liquid scintillation counter (MicroBeta Trilux; Wallac Oy, Turku, Finland). The number of attached bacteria is presented as a percentage of added bacteria.

4.2.2. Biofilm formation on saliva-coated hydroxyapatite

The method described by Guggenheim et al. (2001), to study biofilm formation, was modified for a biofilm formed by a single bacterial strain. Bacterial cultures grown overnight were washed with Fluid Universal Medium (FUM), after which bacterial suspensions with approx. 1.0 x 10^8 CFU/ml were prepared in FUM with no added sugar substitute, FUM with 3 % of sucrose, and FUM with 3 % of glucose. HA discs (Ø 5 mm, Dense Hydroxyapatite Discs, Clarkson Chromatography Products Inc., South Williamsport, PA, USA) coated with whole saliva were placed in wells, where bacterial suspensions and an equal amount of saliva preparation were added. Biofilms were incubated for 18 h in an anaerobic atmosphere at 37 °C. Samples were collected from the wells (planktonic bacteria) and on the top of thrice-washed HA discs (biofilm samples), and cultured on MRS agar for a 72- hour-incubation at 37 °C in an anaerobic atmosphere.

4.2.3. Acidity in the presence of arginine and glucose

Lactobacillus reuteri arginine metabolism was assessed with the method described by Wijeyeweera and Kleinberg (1989). Bacteria were incubated in the presence of arginine and glucose. Briefly, bacterial cultures grown overnight and washed were suspended in Fermentation Minimal Medium (FMM) to have a suspension with approx. 1.0 x 10^8 CFU/ml. Next, the pH value of the suspensions was set at 7 with 0.1 NaOH, and six different assays, as listed in Table 4, were prepared. Assays were incubated at 37 °C for 4 h and pH was measured after every 30 min.

4.2.4. Degradation of hydroxyapatite

The method described by Nikawa et al. (2004) was used with a slight modification to study the degradation of HA in different growth media. Bacteria were grown overnight and washed twice before suspending in PBS, and bacterial suspensions

with optical density ($OD_{550\ nm}$) of 0.01 were prepared on MRS, TSBY (Tryptic Soy Broth with 0.5 % Yeast Extract, BD; and Scharlau Chemie S.A., Barcelona, Spain), and BHI growth media. The suspensions were cultured for 24 h, at 37 °C in wells with 25 mg/ml of HA beads. Samples were collected after 2, 4, 6, and 24 h of incubation and the amount of released Ca^{2+} was measured spectrophotometrically by using the ortho-chresolphtalein method (Chestnutt et al. 1995).

4.3. Probiotic *Bifidobacterium animalis* subsp. *lactis* BB-12 and oral *Bifidobacterium* isolates in oral biofilms

4.3.1. Biofilm study

The Zurich biofilm model described by Guggenheim et al. (2001) was used with slight modifications to prepare two different biofilms: the supragingival cariogenic biofilm included one *S. mutans* strain (MT8148 or Ingbritt), *A. naeslundii*, and one of the *Bifidobacterium* strains listed in Table 2 simultaneously; and the subgingival biofilm included *P. gingivalis*, *F. nucleatum*, *A. naeslundii*, and one of the *Bifidobacterium* strains. Control biofilms were prepared without bifidobacteria.

Biofilm preparation is described in Study II. Briefly, bacterial cultures of each separate strain were prepared in S-FUM (FUM with 3 % of sucrose) for the supragingival cariogenic biofilm model, and in FUM for the subgingival biofilm model to have a suspension with approx. 10^8 CFU/ml. For salivary pellicle formation, HA discs were incubated with PBS and a saliva preparation for 30 min, and washed with PBS. Next, HA discs were placed in separate wells of sterile 24-well cell-culture plate, and coated with 250 µl of saliva and 250 µl of the mixture of bacterial suspension containing equal amounts (62 µl) of each bacterium. In control biofilms, *Bifidobacterium* suspensions were replaced with 62 µl of FUM or S-FUM.

Biofilms were incubated for 18 and 42 h, at 37 °C, in anaerobic conditions. After 18 h, the samples of 18 h biofilms were collected on the top of HA discs (bacteria in biofilm), and in the well (planktonic bacteria). The HA discs with 42 h biofilms were transferred into new wells with 250 µl saliva and 250 µl FUM or S-FUM for another 42-h incubation, after which the samples of 42 h biofilms were collected (Marttinen et al. 2013). Samples were cultured on agar plates and colonies were counted after incubation (2-3 d for bifidobacteria and *S. mutans*; 7 d for *A. naeslundii*, *P. gingivalis*, and *F. nucleatum*). The number of CFUs of *P. gingivalis* was counted on Tryptic Soy Agar (BD) containing yeast extract (Scharlau), hemin, and vitamin K (Sigma-Aldrich,

St Louis, MO, USA). The number of CFUs of *F. nucleatum* was counted on Brucella agar (BD) containing 4 mg l^{-1} of vancomycin (Sigma-Aldrich). MS was measured on Mitis Salivarius Agar (MS; BD; and Le Pont de Claix, France) and *A. naeslundii* on Blood agar containing 5% sheep blood (BD). MRS agar for BB-12 was supplemented with 0.05% (wt vol^{-1}) cysteine hydrochloride and 5 mg ml^{-1} of mupirocin (Merck, Darmstadt, Germany). Culture medium pH was measured after sample collection.

4.3.2. Agar overlay interference assays

The inhibition of bifidobacteria by *S. mutans* and that of *P. gingivalis* by bifidobacteria were studied based on the biofilm study results. The method described by Hasslöf et al. (2010) was used with a few modifications. Pre-grown bacteria were suspended in FUM and suspensions with approx. 10^9, 10^7, and 10^5 CFU/ml were prepared. On the first day, bacteria were suspended in molten (45 °C) sterile agar (MRS for bifidobacteria and BHI for *S. mutans*) and plates were poured. These were incubated overnight and a second layer of molten agar (MRS for bifidobacteria, blood for *P. gingivalis*) containing bifidobacteria or *S. mutans* was added on top of the first layer. After this, 50 µl of bacterial suspension (*Bifidobacterium* or *P. gingivalis*) with approx. 10^8 CFU/ml was plated on top of the agar.

Plates were incubated for 2-3 d (*S. mutans* with bifidobacteria) or 5-7 d (bifidobacteria with *P. gingivalis*) at 37 °C in anaerobic conditions.

4.4. Effects of probiotic *Lactobacillus rhamnosus* GG and *Bifidobacterium animalis* subsp. *lactis* BB-12 on oral health of healthy adults

4.4.1. Study design

The study was a randomized, controlled, double-blind trial with a 4-week run-in period and a 4-week study period (Figure 1). During the run-in period, subjects were instructed not to use commercial products containing LGG, BB-12, or xylitol. Otherwise, subjects were instructed to maintain their normal dietary and tooth brushing habits (most of them brushed twice a day). The same brand fluoride toothpaste was provided for the subjects and antimicrobial mouth rinses were prohibited during the study.

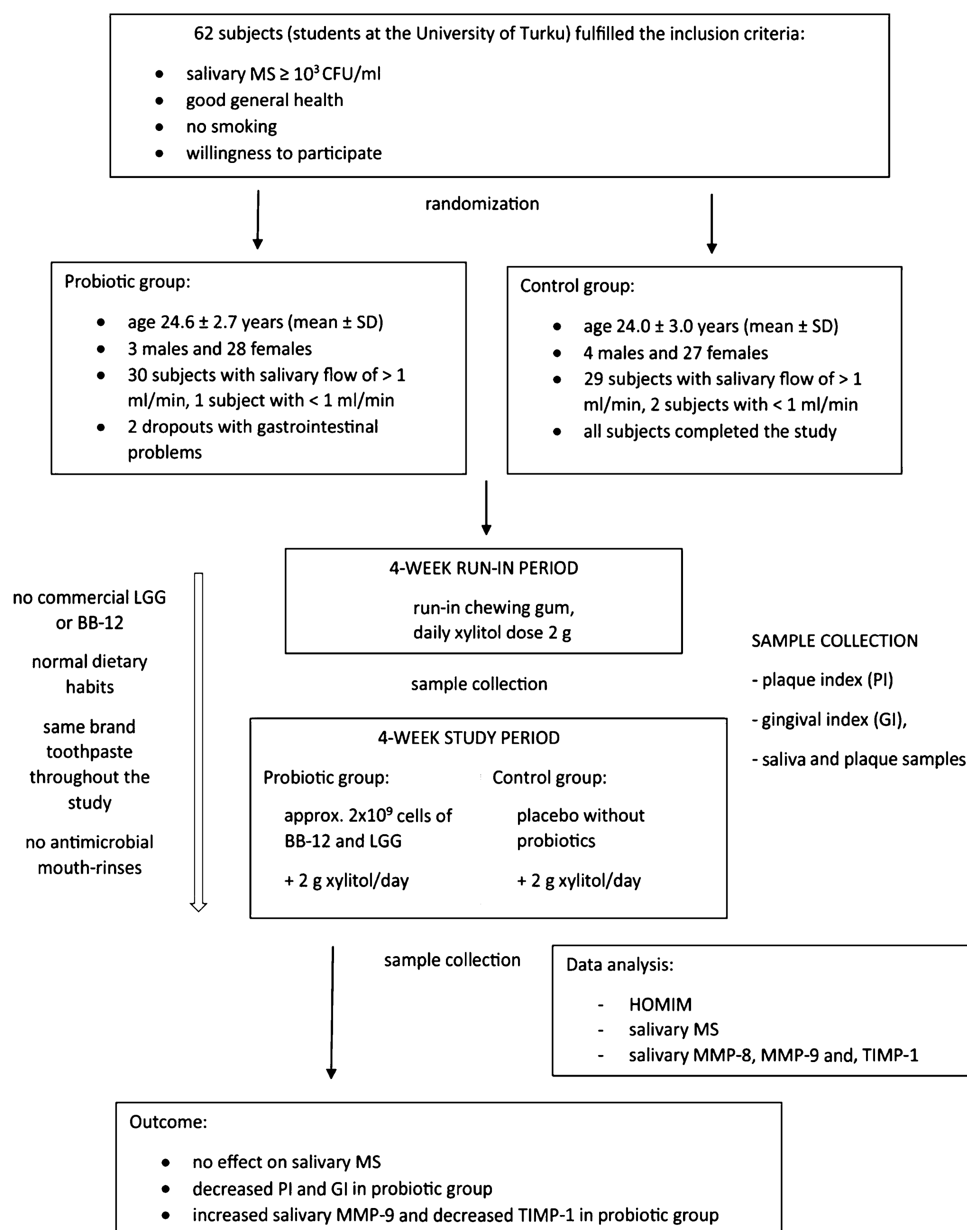

Figure 1. Overview of study design (studies III and IV).

Subjects in the two groups were recommended to use 4 pieces of the same run-in chewing gum for 4 weeks before the test period started. During the 4-week study period, the probiotic group used study-tablets with probiotics and the control group used tablets without probiotics. The recommended daily amount of 4 pieces of probiotic-containing lozenges resulted in a dose of 2 g xylitol and 2×10^9 CFU/ml for

both LGG and BB-12. The subjects were instructed to take the first of the daily tablets in the morning and the last in the evening, always after brushing their teeth.

Subjects and ethics

Study subjects were healthy volunteers who studied at the University of Turku, Finland. 62 subjects who fulfilled the inclusion criteria (good oral health, willingness to participate, and high salivary MS count ($\geq 10^3$ CFU/ml)) were chosen to participate in the study. The ethical committee of the Southwest Finland approved the study and defined the rules for experiment termination. Written informed consent was collected from all subjects.

Subjects formed two groups: group 1 consisted of 3 males and 28 females with a mean age (SD) of 24.6 (±2.7) years, and group 2 consisted of 4 males and 27 females with a mean age (SD) of 24.0 (±3.0) years. All subjects had good oral hygiene and no need for dental treatment.

During the study period, two subjects dropped out of the study: one during the wash-out period and one during the test period. In both cases the reason for dropout was gastrointestinal problems which were not associated to the use of tablets. One subject reported that she had used less than four pieces of study product daily, but otherwise compliance with the study was regarded as good.

Test products

Before the study, the run-in period was performed with noncommercial mild-tasting chewing-gum (Karl Fazer AB, Vantaa, Finland). The gum pieces weighing 1.2 g contained 42 % xylitol, 18 % sorbitol, and 5 % maltitol.

The study tablets weighed 1 g and contained 50 % xylitol and 46 % sorbitol (both probiotic and control). The probiotic tablets contained a further 4.4×10^8 CFU of *L. rhamnosus* GG (ATCC 53103; Probiotical S.p.A., Novara, Italy) and 4.8×10^8 CFU of *B. lactis* BB-12® (DSM 15954; Chr. Hansen A/S Hoersholm, Denmark). The tablets were stored in color-coded plastic bottles at room temperature.

Sample collection

At the baseline of the study (after the run-in period) and at the end of the study, clinical outcomes were measured and samples were collected. The subjects were instructed to refrain from tooth brushing for 24 h before the sample collection and not to take the test lozenge on the morning of sample collection day.

Plaque index was measured with using Silness-Loe index (PI; Silness and Loe 1964) and gingival index was measured using Loe-Silness index (GI; Loe and Silness 1963) with a periodontal probe.

An aliquot of 4 ml of paraffin-stimulated saliva was collected and the collection time was recorded. From the collected saliva samples, salivary microbial analysis; salivary MS and LB counts; and salivary MMP-8, MMP-9, and TIMP-1 levels were measured.

4.4.2. Study outcome: salivary microbial analysis and matrix metalloproteinase levels

Salivary microbial analysis was performed with human oral microbe identification microarray (HOMIM) method at Forsyth Institute (MA, USA), for which 15 salivary samples per group were randomly selected. One ml saliva samples were pipetted onto 10 µl TE buffer (Sigma-Aldrich, St. Louis, MO, USA) and shipped in dry ice. Genomic DNA was purified (MasterPure Gram Positive DNA Purification kit; Epicentre Biotechnologies, Madison, WI, USA, with modifications) and microbial profiles were generated from the image files of scanned HOMIM arrays. The concentration levels of approx. 300 oral taxa were determined by microarray hybridization using fluorescent readout reverse-capture method (Colombo et al. 2009). Positive hybridization signals were categorized into five levels from 1 (barely detectable) to 5 (maximum signal intensity).

Salivary MS and LB counts were determined by plate culturing. For *S. mutans* and *S. sobrinus* counts samples were plated on MSB agar (MS agar containing bacitracin; BD), and incubated at 37 °C in a CO_2 rich atmosphere for 2 d. For salivary LB count, samples were plated on Rogosa SL agar (BD) and incubated anaerobically at 37 °C for 2 d.

Salivary MMP-8 levels were detected by using a time-resolved immunofluorometric assay (IFMA) as described earlier by (Gursoy et al. 2010; Hanemaaijer et al. 1997). MMP-9 and TIMP-1 levels were determined with an enzyme-linked immunosorbent assay using (ELISA) commercial kits and following manufacturers' protocol; Quantikine ELISA was used for MMP-9 (R&D Systems, Minneapolis, MN, USA) and Amersham ELISA was used for TIMP-1 (Human, Biotrak, ELISA system, GE Healthcare, Amersham, Buckinghamshire, UK).

4.5. Statistics

Statistical analyses are described in detail in studies (I-IV). All *in vitro* assays were run at least twice and mean values with standard deviation were calculated. Student's t-test (for independent or paired samples), ANOVA, and Dunnett's t-test were used as parametric statistical tests, and Mann-Whitney U-test and Wilcoxon signed ranks test as non-parametric tests.

In study II, differences between biofilms containing bifidobacteria and control biofilms were studied first with one-way ANOVA, then with pairwise comparisons (Dunnett's two-sided t-test). Student's t-test for independent samples was used for the comparisons between 18 h and 42 h biofilms, and between *B. dentium* and *B. longum* groups. Pearson's test was used for correlations.

In the clinical study, the sample size was based on earlier studies. The effects of probiotic consumption on the primary outcome, salivary MS count, can be seen even with a sample size of 10 MS-positive subjects (Caglar et al. 2008). After a probiotic intervention, the values of the studied parameters (PI, GI, salivary MS count, salivary MMP-8 and -9 levels, salivary TIMP-1 levels, and saliva flowrate) were compared to the baseline levels and between groups. Thus, paired sample analyses, sensitive for even small changes in variables, could be used for baseline and after intervention comparisons. Association of PI and GI in groups was studied with multinomial logistic regression analysis.

For statistical analyses SPSS versions 19.0-23.0 (IBM Inc., New York, USA) were used. SAS version 9.3 was used for the analysis of HOMIM data. Statistical significance level was set at $p<0.05$.

5. RESULTS

5.1. Cariogenic properties of *Lactobacillus reuteri* ATCC 55730 and ATCC PTA 5289 (Study I)

5.1.1. Adhesion on saliva-coated hydroxyapatite

Adhesion of *L. reuteri* strains ATCC PTA 5289 and ATCC 55730 on saliva-coated hydroxyapatite is presented in Table 3. The two studied *L. reuteri* strains differed from each other in their adhesion (Student's paired two tailed t-test): ATCC PTA 5289 adhered well on saliva-coated hydroxyapatite while ATCC 55730 was poor in adhesion. In stimulated whole saliva, both strains' adhesion was poor. For control, the adhesion of *S. mutans* MT8148 was also studied. Of the added *S. mutans*, 0.5 % adhered to HA coated with BSA, 8.5 % adhered to HA coated with unstimulated whole saliva, 2.0 % adhered to HA coated with stimulated whole saliva, and 33.2 % adhered to HA coated with parotid saliva.

Table 3. Adhesion of *L. reuteri* on saliva-coated hydroxyapatite, data presented as % of added bacteria (Study I).

	BSA	unstimulated whole saliva	stimulated whole saliva	parotid saliva
L. reuteri ATCC PTA 5289	1.9 (± 0.3)	12.5 (± 1.9)	5.4 (± 2.0)	25.0 (±2.6)
L. reuteri ATCC 55730	2.5 (± 1.0)	2.5 (± 1.2)	3.6(± 1.6)	4.1 (± 1.3)
p-value*	0.35	**0.002**	0.28	**0.0002**

* Difference in adhesion between the strains (Student's t- test).

5.1.2. Biofilm formation

Biofilm formation on saliva-coated HA was studied in the presence of glucose and sucrose, or without added sugar. There was a difference in biofilm formation between the *L. reuteri* strains. Strain ATCC PTA 5289 formed more biofilm, ($p<0.001$, univariate ANOVA with contrast estimate) but there were no differences between the two sugars (Figure 2). In the planktonic phase, glucose enhanced the growth of ATCC 55730, when compared to sucrose ($p=0.002$) or incubation with no added sugar ($p=0.18$).

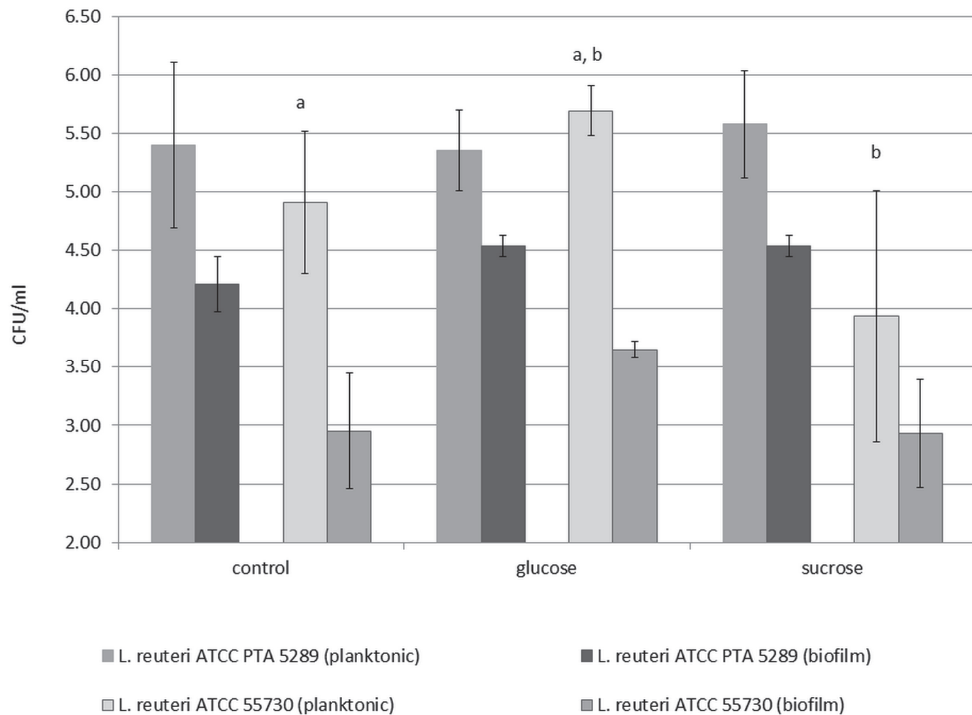

Figure 2. Bacterial number of *L. reuteri* in biofilm on s-HA and in surrounding medium (planktonic), in presence of glucose or sucrose as carbon sources (Study I). Glucose enhanced growth of strain ATCC 55730 when compared to control ([a] p=0.018) and to sucrose as a carbon source ([b] p=0.002), univariate ANOVA with contrast estimate.

5.1.3. Arginolytic properties

Both *L. reuteri* strains degraded arginine (Table 4). Both in the presence of and without any added glucose, the final pH values were higher with a greater amount of arginine in the growth media (one-way ANOVA, comparison to the control with Dunnett's t-test). Strain ATCC PTA 5289 produced more alkali in the presence of arginine and glucose than strain ATCC 55730, and this difference was observed already with 25 mM of added arginine.

Table 4. Final pH of growth medium (FMM) after 4 hours of incubation of *L. reuteri* with glucose and arginine (Study I). Comparison to control with Dunnett's t-test.

	ATCC 55730	ATCC PTA 5289
control (H_2O)	6.9 ±0.5	6.9±0.4
25 mM arginine	8.12±0.03 (p<0.001)	8.19±0.01 (p<0.001)
50 mM arginine	8.34±0.01 (p<0.001)	8.36±0.02 (p<0.001)
25 mM arginine + 50 mM glucose	5.4±0.4 (p<0.001)	7.6±0.5 (p=0.017)
50 mM arginine + 50 mM glucose	7.6±0.2 (p=0.034)	8.3±0.4 (p<0.001)
50 mM glucose	3.4±0.1 (p<0.001)	3.5±0.1 (p<0.001)

5.1.4. Degradation of hydroxyapatite

Both *L. reuteri* strains could generate acidic conditions, but the final pH of the medium was dependent on the bacterial growth rate in the medium. Growth of *L. reuteri* was slow in BHI medium, moderate in TSBY medium and fast in MRS medium. The amount of released calcium was dependent on the final pH of different growth media after 24 h incubation (growth medium pH correlated with $\log_{10}(Ca^{2+})$, $r=0.903$; Pearson correlation, $p=0.002$) and it varied between 3.7 and 1388 µg/ml. The pH varied between 4.55 and 6.10, and the highest degradation of HA was observed in MRS medium, where the final pH was lowest (4.55-4.56).

5.2. Probiotic and oral *Bifidobacterium* isolates in a biofilm model (Study II)

5.2.1. Subgingival *in vitro* biofilm

The counts of BB-12, oral bifidobacteria, *A. naeslundii*, and *F. nucleatum*, in subgingival biofilms are shown in Table 1 in Study II.

All studied bifidobacteria integrated into the biofilm with *A. naeslundii*, *F. nucleatum*, and *P. gingivalis* (Figure 3). Individual bacterial strains behaved differently (Study II). At the species level, the growth of BB-12 was most effective, when compared to the *B. dentium* and *B. longum* ($p=0.017$ and $p<0.001$, Dunnett's two-sided *t*-test). During incubation between 18 h and 42 h, the counts of the *B. longum* strains and BB-12 increased significantly ($p<0.001$ for BB-12, $p=0.002$ for *B. longum* group), while the *B. dentium* count did not increase ($p=0.228$).

When compared with the control biofilms, the number of *P. gingivalis* was significantly lower in the biofilms with BB-12 both after 18 h and 42 h of incubation ($p<0.05$, Student's t-test). At 18 h, three oral *Bifidobacterium* isolates (*B. dentium* NH 4-1, *B. longum* MU 92-2, and *B. longum* MU 93-4) had no effect on the growth of *P. gingivalis* while other oral species inhibited it significantly. The mean ± SD $\log(CFU)^{-1}$ of *P. gingivalis* was 5.99±0.95 in control biofilms, 2.8±4.2 with *B. longum* MU 57-1 ($p<0.001$), and under the detection limit (100 CFU/ml, $p<0.001$) in all other biofilms with bifidobacteria. At the 42 h, *P. gingivalis* was uncultivable in all biofilms containing oral *Bifidobacterium* isolates.

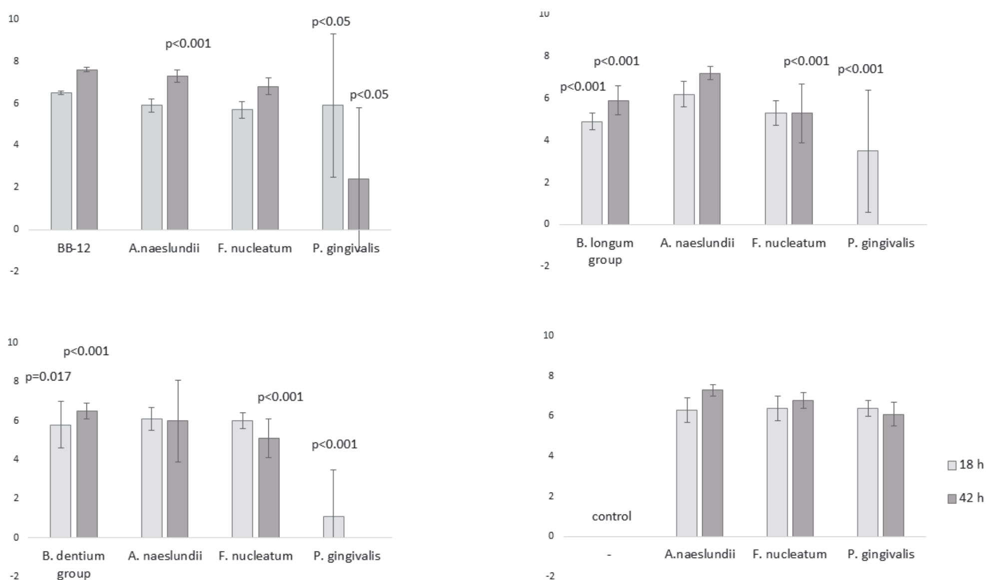

Figure 3. Bacterial count in subgingival biofilm at group level (Study II). Data are presented as log(CFU/ml). Control biofilm included no bifidobacteria. Oral bifidobacteria counts were compared to BB-12 and others to control with Dunnett's t-test.

At 18 h, the presence of BB-12, or either of two of the *B. longum* strains (MU 57-1 and MU86-7) decreased the count of *F. nucleatum*. In biofilms containing any of the *B. dentium* strains and two of the *B. longum* strains (MU 92-2 and MU 93-4), *F. nucleatum* count was similar than the control. At 42 h, the *F. nucleatum* count remained the same as at 18 h with BB-12 and two of the oral *Bifidobacterium* isolates (*B. dentium* NH 4-1 and *B. longum* MU 92-2). All other oral bifidobacteria resulted in decreased count of *F. nucleatum* (p<0.001, Dunnett's two-sided t-test). In the control biofilms, the *F. nucleatum* count increased significantly between 18 h and 42 h (p<0.001).

At 18 h, the *A. naeslundii* count was not affected by any of the bifidobacteria studied. At 42 h, the presence of *B. dentium* NH 4-1 decreased the growth of *A. naeslundii* when compared to the control (p=0.001, Dunnett's two-sided t-test). In control biofilms, the *A. naeslundii* count increased significantly between 18 h and 42 h (p<0.001).

In biofilms with BB-12, the pH remained unaffected (mean pH±SD; 6.5±0.4 at 18 h, 6.2±0.5 at 42 h). With *B. dentium* and *B. longum*, the growth medium pH decreased significantly (for *B. dentium* group 6.0±0.4 at 18 h (p=0.022) and 5.7±0.4 at 42 h (p=0.001), and for *B. longum* group 5.9±0.5 (p=0.004) at 18 h and 5.6±0.3 (p=0.001) at 42 h). All comparisons were made with the control biofilm without bifidobacteria.

5.2.2. Supragingival cariogenic biofilm

The integration of bifidobacteria into and their effects on supragingival cariogenic biofilm was studied with *A. naeslundii* and *S. mutans*. *S. mutans* strains Ingbritt and MT8148 were studied in separate biofilms. Bacterial counts in supragingival cariogenic biofilms are presented in Table 2 in study II. With *S. mutans* Ingbritt, none of the studied bifidobacteria survived in the biofilms and they had no effect on the growth of other bacteria therein. With *S. mutans* MT8148, probiotic BB-12 and all *B. dentium* strains integrated into supragingival cariogenic biofilm (Figure 4), while *B. longum* did not. Oral *Bifidobacterium* isolates had no effect on *S. mutans* MT8148 count. In preliminary studies, also *F. nucleatum* was inoculated into supragingival cariogenic biofilm with BB-12. It was uncultivable in supragingival cariogenic biofilms.

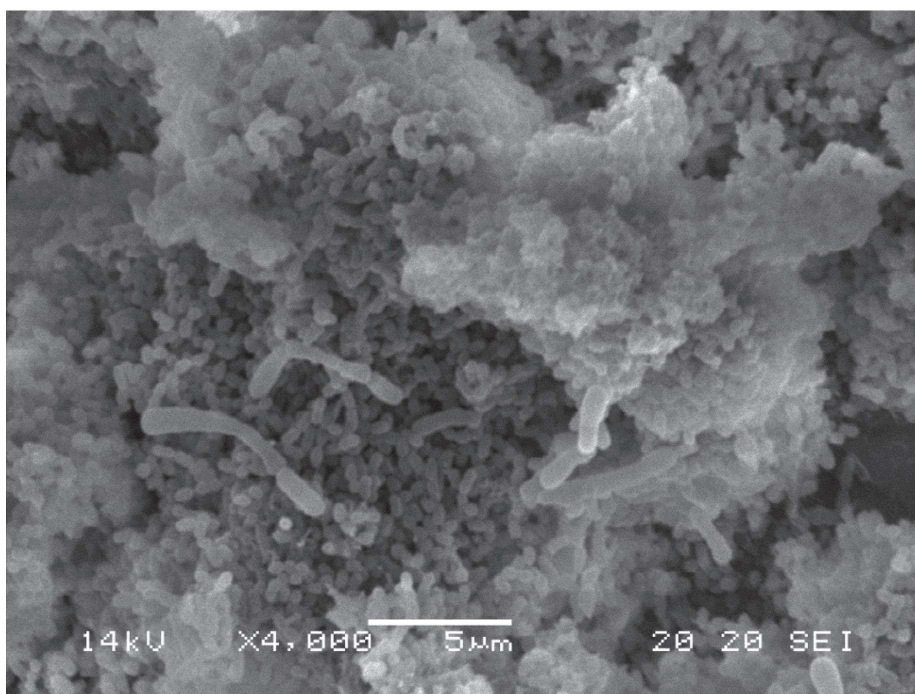

Figure 4. Scanning electron microscope (SEM) image of *S. mutans* and *A. naeslundii* in biofilm with BB-12 (Study II). Data from unpublished preliminary studies.

The counts of most *B. dentium* strains increased during incubation between 18 h and 42 h, with the exception of two strains: *B. dentium* AJ 47-1 remained under the detection limit (100 CFU/ml) at 18 h, and a lower count was detected also in the 42-h biofilm when compared with BB-12; furthermore, a lower *B. dentium* NH 4-1 count

was also detected both after 18 h and 42 h of incubation (Dunnett's two-sided t-test).

The *S. mutans* MT8148 count remained unaffected in biofilms with bifidobacteria. *A. naeslundii* grew poorly in control biofilms and BB-12 did not have any effect on it either. Until 18 hours of incubation, the presence of any of the *B. dentium* strains enhanced the growth of *A. naeslundii* (p=0.005, Dunnett's two-sided t-test), but after 42 h of incubation, it grew well only in the presence of *B. dentium* AJ 47-1. At 42 h, the growth of *A. naeslundii* was slow both in control biofilms and with *B. dentium* AJ 32-1, and it remained under the detection limit in all biofilms with BB-12 and other *B. dentium* strains.

The pH values varied between 4.1 and 4.6 in control biofilms, between 4.2 and 4.5 in biofilms with BB-12, and between 4.3 and 4.7 in biofilms with *B. dentium*. Differences between groups were not statistically significant. At 42 h, the pH of the biofilm with *B. dentium* NH 6-1 was higher than that of the control (p=0.001, Dunnett's two-sided t-test).

5.2.3. Agar overlay interference assay

In agar overlay assays with all the studied bacterial concentrations, *S. mutans* Ingbritt and MT8148 completely inhibited the growth of all bifidobacteria, and all the bifidobacteria used inhibited the growth of *P. gingivalis*.

5.3. Effect of probiotic *Lactobacillus rhamnosus* GG and *Bifidobacterium animalis* subsp. *lactis* Bb12 on oral health of healthy adults (Studies III and IV)

5.3.1. Clinical parameters: gingival and plaque indices, salivary secretion rate

PI and GI values are presented in Table 5. In the probiotic group, mean PI and GI decreased significantly (PI; p=0.016 and GI; 0.013, paired samples t-test) during the study period, although no differences between groups were observed (Table 5). Decreased PI and GI values in the probiotic group correlated (r=0.389, p=0.037) and were positively associated (OR 5.8, 95 % CI 1.0-30). Saliva secretion rate remained unaffected during the study period in both groups.

Table 5. Median [minimum, maximum] values of PI and GI in probiotic and control groups (Study III). a) Between baseline and end of study (paired samples t-test). b) Between groups (independent samples t-test).

	PI*			**GI***		
	control	LGG + BB-12	p-value [b]	control	LGG + BB-12	p-value [b]
baseline	0.88 [0.11, 2.00]	1.00 [0.46, 1.79]	0.112	0.67 [0.04, 2.00]	0.71 [0.21, 1.17]	0.929
4 wk	0.79 [0.04, 2.10]	0.94 [0.30, 1.71]	0.176	0.63 [0.08, 1.67]	0.56 [0.08, 1.00]	0.571
p-value [a]	0.064	**0.016**		0.094	**0.013**	

5.3.2. Salivary microbial analysis: mutans streptococci and lactobacilli counts and total microbiota

According to the inclusion criteria of the study, all subjects harbored *S. mutans* in their saliva. Of these, 14 subjects in the probiotic group and 19 subjects in the control group had high salivary MS counts (>10^5 CFU/ml). One subject had the combination of *S. mutans* and *S. sobrinus* in their saliva. Salivary MS counts did not change in either of the groups during the study period. At the baseline, 15 subjects in the probiotic group and 14 subjects in the control group had a cultivable number of lactobacilli in their saliva. No changes in salivary lactobacilli count were observed during the study in either of the groups.

In HOMIM analysis, the microbiota at the baseline was similar in both groups. *P. gingivalis*, *P. intermedia*, or *A. actinomycetemcomitans* were not detected in any of the subjects and only three subjects had detectable amounts of lactobacilli in their saliva. No changes in salivary microbiota were observed between the baseline and end of the study period.

5.3.3. Salivary matrix metalloproteinases and their inhibitor levels

Salivary levels of MMP-8, MMP-9, and TIMP-1 in the probiotic and control groups at the baseline and at the end of the study are presented in Figure 5.

No intergroup differences in salivary MMP-8, MMP-9, or TIMP-1 levels were observed either at the baseline or at the end of the study. In the probiotic group, salivary MMP-9 level increased (p=0.007, Wilcoxon signed ranks test) and TIMP-1 level decreased (p=0.003) significantly during the study. Also, the molar ratio of MMP-9/TIMP-1 changed significantly from the baseline value in the probiotic group (p=0.002).

Both at the baseline and at the end of the study, GI and MMP-9 correlated weakly when all samples were studied (r=0.260, p=0.045 at baseline and r=0.354, p=0.005 at 4 wk, Pearson correlation). When different groups were studied, correlation between GI and salivary MMP-9 was observed only in the control group. The change in the molar ratio

of MMP-8/TIMP-1 during the 4-week intervention correlated significantly with the change in GI (p=0.03) in the control group, but not in the probiotic group.

No correlation with other clinical parameters (PI, salivary MS and LB counts, or saliva secretion rate) and salivary MMP-8, MMP-9, and TIMP-1 levels were observed.

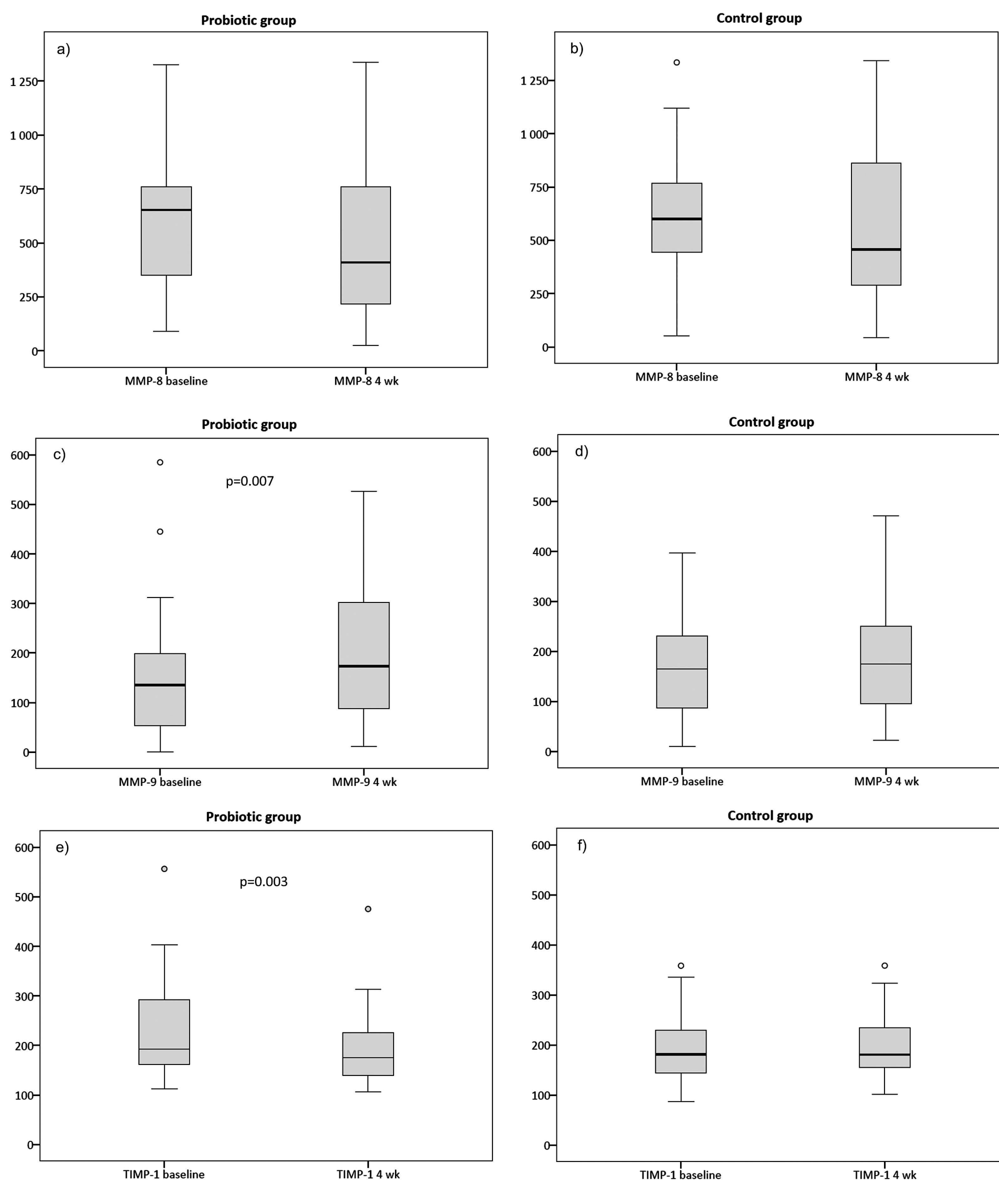

Figure 5. Salivary MMP-8, MMP-9, and TIMP-1 levels in probiotic and control groups before and after probiotic intervention (Study IV). p-values between baseline and 4 wk, Wilcoxon signed ranks test.

6. DISCUSSION

Probiotics are widely studied for their effects on general and oral health. They seem to improve gingival health but the effects on dental caries might be limited (Gruner et al. 2016). This thesis aimed to study probiotic bacteria in relation to dental caries and gingivitis with *in vitro* and clinical studies. *L. reuteri* strains ATCC 55730 and ATCC PTA 5289 were studied from the cariological point of view. They were shown to express cariogenic properties in favourable conditions. The strains differed in their actions, which highlights the importance of the studies with individual strains. Studies of probiotic BB-12 and oral bifidobacteria in oral biofilms showed that they could integrate in biofilms, but there were differences between studied strains and biofilms. Furthermore, the effect of the combination of probiotic LGG and BB-12 on oral health was studied with healthy adults. No effect on salivary MS/LB counts or oral microbiota were observed after probiotic consumption but probiotic intervention showed promising results on gingival health and oral immunity. This study provides new information about the adhesion and biofilm formation properties, as well as cariogenity of *L. reuteri*, integration and actions of BB-12 and oral bifidobacteria in oral biofilms, and clinical effects of LGG and BB-12 on gingival health in healthy adults.

6.1. *L. reuteri* and dental caries

The cariogenic bacteria adhere on dental surfaces; they produce acids from fermentable carbohydrates and survive in acidic conditions (Gibbons 1989; Loesche 1986). However, for direct cell-cell interactions and probiotic effects in the oral cavity, probiotic bacteria should adhere to and at least temporarily colonize dental surfaces (Meurman 2005). In this study, probiotic *L. reuteri* strains ATCC PTA 5289 and ATCC 55730 differed in their adhesion and biofilm formation on s-HA. While the adhesion of strain ATCC PTA 5289 has not been studied before, the low adhesion of strain ATCC 55730 was in line with earlier results (Haukioja et al. 2006). Strain ATCC PTA 5289 was more efficient in biofilm formation, and these results are in line with earlier findings. Jones and Versalovic (Jones and Versalovic 2009) observed a 10-fold bacterial density in biofilms with *L. reuteri* ATCC PTA 5289, when compared with ATCC 55730. Adhesion and biofilm formation *in vitro* reflect the potential to colonize the oral cavity *in vivo*. *L. reuteri* ATCC 55730 colonizes oral cavity only temporarily (Caglar et al. 2009), and there are strain-dependent differences in the colonization of different *L. reuteri* strains (Krasse et al. 2006). Furthermore, the isolation site of

L. reuteri strains may reflect their colonization potential in the oral cavity. Strain ATCC 55730 has been isolated from breast milk, while the origin of strain ATCC PTA 5289 is the mouth (Spinler et al. 2008).

The *L. reuteri* strains studied here could generate acidic conditions when incubated with glucose. However, the addition of arginine to the test arrangement caused the pH to remain above neutral. This indicated the ability of *L. reuteri* to protect itself from an acidic environment, thus suggesting it as a non-cariogenic species. Arginine metabolism modifies plaque pH and promotes dental health. Slightly acidic conditions start alkali production in dental plaque as a reaction to the acid pulse generated by acidogenic bacteria (Wijeyeweera and Kleinberg 1989). *L. reuteri's* ability to maintain an alkalic pH in the presence of arginine and glucose reflects its potential of caries-prevention in the mouth (Huang et al. 2015). *L. reuteri* is regarded as non-cariogenic. It has been claimed, that it does not decrease the pH of oral biofilms (Madhwani and McBain 2011) or degrade HA *in vitro* (Nikawa et al. 2004). Furthermore, clinical evidence, although limited, suggests that a combination of *L. reuteri* ATCC 55730 and PTA 5289 does not influence the acidogenity of dental plaque in orally healthy adults (Marttinen et al. 2012). However, when arginine sources are limited and carbohydrates available, *L. reuteri* can generate acidic conditions (Hedberg et al. 2008). This is in accordance with the results of this study, where the potential of *L. reuteri* to dissolve Ca^{2+} from HA was dependent on the environmental factors, *e.g.*, carbohydrate availability. Furthermore, the exopolysaccharide production of *L. reuteri* varies with different sugars as the source (Arsköld et al. 2007). Therefore, environmental factors determine the cariogenic potential of *L. reuteri*.

A limitation of this study was that; the pH values of biofilm were not measured. The biofilm pH values with the EPS production, and microbial interactions of *L. reuteri* in multispecies biofilms would give valuable information on the cariogenic potential of *L. reuteri.* Arginine metabolism was indicated by measuring pH without the detection of released ammonia. These additional studies would have resulted in more specific information on the bacterium's cariogenic properties, *e.g.*, its biofilm formation, ability to inhibit oral pathogens, and arginine metabolism.

In the light of this and other studies, *L. reuteri* can be considered as a harmless organism from the cariological point of view (Keller and Twetman 2012; Marttinen et al. 2012; Nikawa et al. 2004). However, *L. reuteri* has only a limited effect on salivary MS (Keller et al. 2012). Lactobacilli are usually found in cavitated caries lesions and their role in the initiation of caries process is limited (Kianoush et al.

2014). Thus, the increased risk of dental caries induction by probiotic consumption, without other predisposing factors, is mainly theoretic. The two strains used in this study differed from each other; therefore, probiotic characteristics should be considered as strain-specific, and any generalization should be avoided.

6.2. Probiotic and oral bifidobacteria in dental biofilms

Bifidobacteria are members of health-associated gut microbiota, but their role in oral health is contradictory (Turroni et al. 2014). On one hand, bifidobacteria are found in caries lesions and their count correlates with caries occurrence (Mantzourani et al. 2009b). On the other hand, bifidobacteria have been found in periodontally healthy subjects more frequently than in subjects suffering from periodontal disease (Hojo et al. 2007b). BB-12 has no significant effect on MS colonization or caries incidence in small children (Taipale et al. 2012; Taipale et al. 2013). In children aged 6-12 years and also in adults, BB-12 alone or combined with *L. acidophilus* La-5 reduces salivary MS counts temporarily (Ashwin et al. 2015; Caglar et al. 2008; Singh et al. 2011). There are no studies available of the effects of BB-12 or other probiotic bifidobacteria on the periodontal disease.

In this study, the studied bifidobacteria differed in their actions in supragingival cariogenic and subgingival biofilms. None of the studied bifidobacteria survived in biofilms with *S. mutans* Ingbritt, but BB-12 and all oral *B. dentium* isolates integrated into the biofilm with *S. mutans* MT8148. This may be related to Ingbritts efficient ability to generate acidic conditions (Hamilton and Ellwood 1978).

Bifidobacteria grow best in neutral or slightly acidic conditions (Nakajo et al. 2010), but they are found in caries lesions (Mantzourani et al. 2009b). The advancing front of the lesion is not as acidic as its other parts (Kianoush et al. 2014). It is possible that bifidobacteria are located in the deepest parts of caries lesions and they may be associated with the progression of caries. BB-12 or *B. dentium* strains did not influence the acidity of biofilms, or on the growth of *S. mutans*.

All studied bifidobacteria survived in the subgingival biofilm model. In subgingival biofilm, bifidobacteria decreased the number of *P. gingivalis* and most of them inhibited also *F. nucleatum*. Bifidobacteria use vitamin K, which is also needed for the growth of *P. gingivalis*. The growth inhibition of *P. gingivalis* might be achieved by using vitamin K or by some other direct antagonistic measures (Hojo et al. 2007a). BB-12 had no effect on biofilm pH, but oral bifidobacteria decreased the pH

significantly. *P. gingivalis* is sensitive to acidic conditions, and poor growth with bifidobacteria may also be caused by a low pH (Takahashi et al. 1997).

Bifidobacterium longum did not integrate into the supragingival cariogenic biofilm, but all strains studied integrated in the subgingival biofilms with *F. nucleatum*. *F. nucleatum* increases the adhesion of bifidobacteria to s-HA (Haukioja et al. 2006), and bifidobacteria are absent in early dental biofilms (Huang et al. 2011). Therefore, they can be suggested as secondary colonizers in dental biofilms. In our study, *B. longum* and *F. nucleatum* did not integrate in the supragingival cariogenic biofilm. In other biofilm studies, oral commensal *Veillonella* has enhanced the growth of *F. nucleatum* (Periasamy and Kolenbrander 2010). The absence of *Veillonella*, or too acidic conditions generated by *S. mutans*, may be the reason for absence of *F. nucleatum* in cariogenic biofilm. Inclusion of *F. nucleatum* in the model could also have enhanced the growth of bifidobacteria in the supragingival cariogenic biofilm.

The studied bifidobacteria should be discussed in the light of their probiotic potential in the oral cavity. *B. dentium* is absent in edentulous mouth, and is thus considered to belong to dental microbiota (Mantzourani et al. 2010). In this study, *B. dentium* and BB-12 integrated into both supragingival and subgingival biofilms and showed inhibition of *P. gingivalis*, *F. nucleatum,* and slightly of *A. naeslundii*. The probiotic effects of *B. dentium* and BB-12 in periodontal disease should be studied further. Since there was no effect on MS count or the acidity of supragingival cariogenic biofilm, the potential cariogenic effects should be taken into consideration. *B. longum* may be introduced in the mouth in probiotic food products (Beighton et al. 2008). In this study, it did not integrate in the supragingival cariogenic biofilm and the inhibition of *P. gingivalis* and *F. nucleatum* in subgingival biofilm was strain-dependent. The probiotic effects of *B. longum* by direct bacterial interactions in the oral cavity may be limited.

6.3. Lactobacillus rhamnosus GG and Bifidobacterium animalis subsp. lactis BB-12 in oral health

In this study, the use of the combination of LGG and BB-12 did not influence salivary MS; salivary LB count also stayed the same. In general, the salivary MS count is relatively stable. With, *e.g.*, a full-mouth disinfection and/or probiotic intervention, only a temporary reduction in the MS count has been reported (Aminabadi et al. 2011; Caglar et al. 2008). However, contradictory to the results of this study, both LGG and BB-12 have affected salivary MS counts in other studies: LGG in

combination with *L. rhamnosus* LC 705 reduced the risk of the highest salivary MS level (Ahola et al. 2002) and BB-12 alone, or in combination with *L. acidophilus* La-5 reduced salivary MS counts (Caglar et al. 2008b, Singh et al. 2011). LGG has also reduced the *S. mutans* count in dual-species biofilms and saliva-derived microcosms *in vitro* (Pham et al. 2011). The results of this study support the assumption that probiotic lactobacilli and bifidobacteria have only a limited effect on salivary MS count (Cagetti et al. 2013).

Probiotic *L. reuteri* DSM 17938 and PTA 5289 consumption causes a temporary shift in the oral microbiota. The count of oral commensals, *e.g.*, *S. mitis* group, is increased and that of pathogens, *e.g., S. mutans* and *F. nucleatum*, is decreased. The effect disappears in a month after the termination of intervention (Romani Vestman et al. 2015). Hypothetically, probiotics cause a transient shift in the composition of oral microbiota, resembling their effect that in the gut (Voreades et al. 2014). In this study, the total oral microbiota determined by the HOMIM method remained the same during the study period. At the time of the study, the method to study oral microbiota detected approx. 300 microbial species. Today, a more enhanced method to detect over 600 microbes is available (http://homings.forsyth.org/index2.html). It would be interesting to see whether a more detailed study method could find differences between the groups. However, oral microbiota seems to be relatively stable over time (Utter et al. 2016).

Even though an oral colonization by LGG and BB-12 was not an outcome of this study, LGG was detectable in LB cultures and the HOMIM method could detect BB-12. Both microbes were under the detection limit in the post-trial samples of the probiotic group. The subjects had taken a probiotic tablet less than 24 h before the sample collection, and they restrained from oral hygiene before the sample collection. LGG has earlier been detected in the salivary samples of some of the subjects directly after a probiotic intervention (Marttinen et al. 2012, Yli-Knuuttila et al. 2006). BB-12 was not found in most of the subjects' samples even during the intervention (Taipale et al. 2012). After a follow-up period, both LGG and BB-12 were undetectable in oral samples. Thus, they can be regarded as poor colonizers of the oral cavity (Taipale et al. 2012, Yli-Knuuttila et al. 2006).

Probiotic consumption has increased the salivary flow in elderly suffering from hyposalivation (Hatakka et al. 2007). In adults, chewing gum with xylitol and probiotics does not affect the saliva flow rate or salivary composition (Gueimonde et al. 2016). The participants in our study were healthy and had normal salivary flow rates, and no changes in saliva flow were determined after probiotic consumption.

Enhanced gingival health by reduced PI and GI, was a very interesting finding in this study. There are numerous studies available of the effects of BB-12 and LGG, used either in combination or alone, on caries risk factor, salivary MS count, but none of them report their effects on gingival health (Ahola et al. 2002, Aminabadi et al. 2011, Caglar et al. 2008b, Marttinen et al. 2012, Näse et al. 2001, Singh et al. 2011, Taipale et al. 2013). This is the first study to report the enhanced gingival health with this probiotic combination. Subjects were generally healthy and had no risk factors (*e.g.*, diabetes or smoking habits), of periodontal disease. No periodontal pathogens were detected in salivary samples. When a significant change is seen in subjects with healthy gingiva even greater effects may, hypothetically, be gained in subjects with periodontal disease.

As the subjects of this study were generally healthy, had no periodontal risk factors and had low mean GI, which indicates healthy gingiva, immunomodulation by probiotics was hypothetically the mechanism causing reduced gingival and plaque indexes. Probiotic *L. reuteri* can modulate the inflammatory response in GCF by reducing TNF-α and IL-8 (Twetman et al. 2009). There are no studies available of oral immunomodulation by BB-12 and LGG, either alone or in combination. Earlier studies report changes, generally decreased, in MMP and TIMP levels after probiotic interventions. MMP-3 levels in GCF decrease in gingivitis patients (Staab et al. 2009). MMP-8 levels decrease and TIMP-1 levels increase in chronic periodontitis, when probiotic consumption is added as a supporting therapy to scaling and root planing (Ince et al. 2015). In this study, salivary MMP-8 remained unaffected, but probiotic consumption increased salivary MMP-9 and decreased TIMP-1 levels. MMP-8 has a strong association with periodontal disease (Sorsa et al. 2016). There is one study available of the effects of probiotic LGG on MMP-9; MMP-9 levels in the lung tissue were decreased after probiotic consumption in test animals with asthma (Wu et al. 2014). There is a contradiction between the results of this study and those reported by Wu et al. MMP-9 seems to have a more complicated role in periodontal tissues than MMP-8. When MMP-8 correlates strongly with periodontal disease, MMP-9 levels in mild periodontitis might be similar to those in healthy subjects (Nedzi Góra et al. 2014). Even though increased levels of both MMP-8 and MMP-9 are generally associated with tissue destruction, they are shown also to have beneficial defensive effects (Hernández et al. 2011; Kuula et al. 2009; Saarinen et al. 2016).

There are no studies available of probiotic BB-12 and LGG in periodontal disease. In the light of this study, they seem to have a potential for immunomodulation.

Immunomodulatory effects might be promising as an additional therapy in periodontal disease and further studies are needed in this field.

6.4. General discussion

Probiotics have physiological benefits for humans; the classification of a microbe as a probiotic requires scientifically demonstrated beneficial effects, human origin, safety for human use, stability to acid and bile, and adherence to mucosal and dental surfaces. (Isolauri 2001; Meurman 2005). Despite numerous studies of probiotics in oral diseases, only limited effects are reported. The study periods are usually short and studies report surrogate or intermediate risk effects, *e.g.*, salivary MS count. For example, in dental caries, MS have only limited effect on the multifactorial disease process. No statements of the effect on caries can be given with only the effects on salivary MS counts (Cagetti et al. 2013). Future studies should report also accepted markers or indicators of caries and periodontal diseases in addition to surrogate markers. Probiotics may have a role in the prevention of oral diseases and their effects on periodontal diseases are more promising than in caries. The results of this study are in line with this assumption. However, with current knowledge, no clinical guidelines can be stated (Gruner et al. 2016).

In this study, no effect on MS counts either in the clinical study or in the biofilm model were observed. The biofilm formation starts rapidly on clear tooth surfaces. The delivery of probiotics in tablets or in dairy products is probably ineffective for enhancing oral health. Thus, an addition of probiotic bacteria, for example, in tooth paste, could transport bacteria directly to the action sites and include them in the initial biofilm formation (Cagetti et al. 2013). Furthermore, a distraction of oral microbial community prior to probiotic therapy may increase probiotic effects. Full mouth disinfection with chlorhexidine reduces salivary MS count and, after a probiotic intervention, results in prolonged oral colonization (Aminabadi et al. 2011). However, despite the limited local actions, the immunomodulatory effects of probiotics are relevant also for oral health (Staab et al. 2009). Probiotic effects are found even with heat-inactivated probiotics or cell-free supernatants of probiotics (Holz et al. 2013; Nissen et al. 2014; Salehi et al. 2014; Schwendicke et al. 2014b).

In general, the knowledge of human microbiome and its association with oral health and disease has lately hugely increased. Earlier studies have focused on specific bacterial strains, and pathogenic bacteria associated with certain diseases have been mentioned (Loesche 1986). Oral microbiota seems to be relatively stable and

biofilm is a natural habitat for organisms living in the oral cavity. The colonization resistance inhibits the oral colonization by probiotics and beneficial effects are not gained when bacteria do not integrate into oral biofilms (Marsh et al. 2015). Traditionally, probiotics are isolated from the gut and primarily used at body sites other than the oral cavity. Oral commensals may give more promising results for oral health and potential oral probiotics are searched among oral microbiota. In addition to high antimicrobial activity, oral lactobacilli express a tolerance to environmental stress in the oral cavity (Kõll et al. 2008). Probiotic streptococci, *e.g.*, *S. salivarius* M18, *S. uberis* KJ2, *S. oralis* KJ3, and *S. rattus* JH145, have proved promising in caries prevention in children (Di Pierro et al. 2015; Hedayati Hajikand et al. 2015). However, oral commensal streptococci may cause bacteraemia and infectious endocarditis (Carley 1992; Ge et al. 2016; Longman et al. 1991). Thus, the use of potential pathogens as probiotics is questionable.

Today, the role of total microbiota in oral diseases is recognised. The introduction of single probiotic bacterial strains seems to have only a limited ability to achieve health effects. The research of bacteriotherapy has recently moved towards whole microbiome transplants. For example, faecal microbiome transplants are vigorously studied for the treatment of gut-associated diseases. The idea is to introduce a healthy gut microbiome into a diseased bowel. Promising results are reported in the treatment of diseases, *e.g.*, antibiotic-associated diarrhoea, inflammatory bowel syndrome, and metabolic syndrome. (Kelly et al. 2015) In the oral cavity, a microbiome transplant is suggested for the treatment of periodontitis (Pozhitkov et al. 2015). More studies are desperately needed in this field. The bacteriophage communities affecting oral microbiome in health and disease should also be studied (Edlund et al. 2015). Bacteriophage therapy might have a potential to alter the dysbiotic oral microbiota in disease. Even more promising results may be gained, when bacteriophage therapy is combined with probiotics or microbiome transplants.

7. CONCLUSIONS

In the present study, probiotic *L. reuteri* strains ATCC 55730 and ATCC PTA 5289 were acidogenic, and the amount of produced acid was dependent on the availability of fermentable carbohydrates. The strains also differed from each other in adhesion and biofilm formation. The findings reflect the difference in their oral colonization potential and, furthermore, in their cariogenic potential.

BB-12 and oral bifidobacteria differed in their integration into and actions in oral biofilms. The integration of bifidobacteria into oral biofilms seems to be affected by oral bacteria. The possible probiotic effects of bifidobacteria in subgingival biofilms may be gained by the inhibition of periodontal pathogens. Inhibition may be effected by direct bacterial interactions or by the generation of an acidic environment, suboptimal for periodontal pathogens.

Probiotic consumption seems to have beneficial effects on gingival health even in periodontally healthy subjects. Since the probiotic intervention had no effects on microbiota, interaction with the host was suggested. Increased salivary MMP-9 and decreased salivary TIMP-1 levels indicate possible immunomodulation. The combination of BB-12 and LGG, and their health benefits in periodontal disease should be studied further.

In the light of current knowledge, probiotics are not recommended to manage caries, but they might be used in managing periodontal diseases (Gruner et al. 2016). This view is supported by the findings of this thesis. Probiotic actions are strain-specific. Both *in vitro* and clinical studies show only a limited effect on the MS. Possible health-improving effects after probiotic consumption may be gained in periodontal status. In addition to direct bacterial interactions, immunomodulatory effects should be considered as important probiotic action mechanisms in the oral cavity.

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