Effect of Probiotics on Caries-related Variables

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Cover illustration: Scanning electron microscopy image of *Limosilactobacillus reuteri* DSM 17938 (magnification 4.00 K X) produced by the Centre for Cellular Imaging, Core Facilities, the Sahlgrenska Academy, University of Gothenburg, and Ludwig Lundberg at the Swedish University of Agricultural Sciences and BioGaia. Copyright BioGaia AB

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To my family with love…

ABSTRACT

In relation to the caries disease, the aims of this thesis were to investigate the effect of probiotic drops on different caries-related variables *in vivo* (Papers I & III), on interference capability and genetic response to different metabolites *in vitro* (II) and systematically to review the best mode and dose of administration based on the examined oral outcomes (IV). The effects on colonisation and cariogenic bacteria were studied in plaque and saliva via plating and qPCR-analyses in teenagers and orthodontic patients after short-term exposure. Moreover, the changes in plaque acidogenicity were evaluated in orthodontic subjects using the "pH strip method". The interference capability of the endogenous lactobacilli on a panel of 13 *Streptococci* strains after probiotic intervention was examined *in vitro*. Further, *S. mutans* strains from both active and inactive caries subjects were used for genetic evaluation to probiotic exposure using qRT-PCR expression tests. The optimal mode and dose for using probiotics were studied in a systematic review following the PRISMA checklist. The probiotics had the ability to colonise saliva and dental biofilm after short-term use. Temporary colonsation was seen in young adults up to five weeks following use. They had the opportunity to reduce the number of salivary *S. mutans* in young adults, while no such effect was found during orthodontic treatment. Using probiotics during orthodontic treatment also increased the pH of the dental biofilm in comparison to the placebo group. The behaviour of the endogenous lactobacilli changed after *L. reuteri* administration and was shown to produce an antibacterial effect against oral *streptococci*. A variation in susceptibility to probiotic bacteria and endogenous lactobacilli was found among the tested panel. The various metabolites induced different genetic responses on *S. mutans* in relation to caries activity. No clear optimal vehicle or dose was identified via the systematic review. Probiotics in the form of drops have the ability to colonise the oral cavity after short-time exposure and to be an additional tool in caries prevention in order to reduce plaque pH and change the oral ecosystem. Further studies are needed to identify the optimal mode and dose required.

Keywords: Bacterial interference, Caries, Caries prevention, Dental biofilm, Dental plaque, Lactobacilli, *Lactobacillus reuteri*, *Streptococcus mutans*, Plaque acidogenicity, Probiotics, qPCR, Saliva, Virulence.

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SAMMANFATTNING PÅ SVENSKA

Syftet med denna avhandling var studera effekten av en probiotisk dropp-produkt på olika kariesrelaterade variabler *in vivo* (Paper I & III), interferensförmåga och genetiskt svar av metaboliter *in vitro* (II) och via en systematisk litteraturgenomgång utvärdera administrationssätt och dosering för största möjliga effekt (IV). Koloniseringsmönster och effekt på kariogena bakterier studerades i plack och saliv efter en kortvarig exponeringsperiod, via konventionell odlingsteknik och genom qPCR-analyser, hos tonåringar respektive unga vuxna som genomgick behandling med fast ortodontisk apparatur. Även förändringarna i det dentala plackets syrabildande förmåga utvärderades hos ortodontipatienter med hjälp av den pH strip-metoden. Interferensförmågan hos endogena laktobaciller studerades *in vitro* mot en panel med 13 streptockockstammar efter påverkan av probiotiska bakterier. Effekten på mutansstreptokocker från aktiva och inaktiva kariesindivider utvärderades genom genetiska expressionstester. Den mest optimala administrationsformen samt dos vid användning av probiotika utvärderades i en systematisk litteraturgenomgång som genomfördes enligt PRISMAs instruktioner. Probiotiska laktobaciller hade förmågan att kolonisera saliv och biofilm efter kortvarig exponering. Kvarvarande kolonisering sågs hos unga vuxna upp till fem veckor efter användning. En reduktion i antalet *S. mutans* i saliv hos unga vuxna observerades medan ingen sådan effekt kunde ses under ortodontisk behandling. Användning av probiotika under ortodontisk behandling reducerade pH-fallet i den dentala biofilmen jämfört med en placebogrupp. Beteendet hos endogena laktobaciller förändrades efter administrering av *L. reuteri* och visade sig ge en antibakteriell effekt mot den testade panelen av streptokocker. En variation i känsligheten för probiotiska bakterier och endogena laktobaciller sågs bland de testade bakterierna. De olika metaboliterna inducerade olika genetiskt svar på *S. mutans* i förhållande till kariesaktivitet. Ingen tydlig optimal produkt för administration av probiotika eller dos identifierades via den systematiska granskningen. Probiotika i form av munsköljning har förmågan att kolonisera munhålan efter kort tids exponering och att vara ett ytterligare verktyg för att förebygga karies då det reducerar det dentala plackets syrabildande förmåga och förändrar det orala ekosystemet. Ytterligare studier behövs för att identifiera optimal administrationsform och vilken dos som krävs vid behandling av probiotika.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. Alforaidi S, Bresin A, Almosa N, Lehrkinder A, Lingström P. Oral colonisation after the administration of drops containing *Lactobacillus reuteri*. Oral Health Prev Dent 2020; 18 :1017-1023.

II. Lehrkinder A, Alforaidi S, Simark-Mattsson C, Almosa N, Lingström P. *Lactobacillus reuteri* treatment induces a genetic response in *Streptococcus mutans* and interference in endogenous *lactobacilli sp*. *2021 Submitted for publication.*

III. Alforaidi S, Bresin A, Almosa N, Lehrkinder A, Lingström P. Effect of drops containing Lactobacillus reuteri (DSM 17938 and ATCC PTA 5289) on plaque acidogenicity and other caries-related variables in orthodontic patients. BMC Microbiology 2021: accepted.

IV. Alforaidi S, Almosa N, Zafar H, Ashi H, Lingström P. Administrative mode and dose-related effect of probiotics on caries-related variables: A systematic review. *2021 Submitted for publication.*

CONTENTS

ABBREVIATIONS

ANOVA	Analysis of Variance
atpD	ATP synthase subunit beta gene
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
$AUC_{7.0}$	Area under the curve at pH value of 7.0
BHI	Brain Heart Infusion agar
cDNA	Complementary DNA strain
CFU	Colony Forming Unit
DMFS	Number of decayed, missed and filled tooth surfaces
DNA	Deoxyribo Nucleic Acid
EFSA	European Food Safety Authority
FAO	Food and Agriculture Organization
GRAB	Generally Recognized as Safe
LAB	Lactic Acid Bacteria
L.B	Lactobacilli
ldh	Lactate dehydrogenase gene
LGG	*Lactobacillus rhamnosus* GG
GIT	Gastrointestinal tract
gtf	Glucosyltransferase genes
M17	Bacterial growth medium with disodium-β-glycerophosphate
Max pH fall	Difference between baseline pH and minimum pH
Min pH	Minimum pH value after a sugar challenge
MRS	de Man, Rogosa & Sharpe broth / agar
MS	Mitis Salivarius agar
MSB	Mitis Salivarius Bacitracin agar
PBS	Phosphate buffer saline
PICO	Patient/Population, Intervention, Comparison and Outcomes
PRISMA	Preferred Reporting Items for Systematic Reviews and Meta-Analyses
qPCR	Quantitative Polymerase Chain Reaction
qRT-PCR	Reverse Transcription Quantitative Polymerase Chain Reaction
REST	Relative expression software method
RNA	Ribonucleic Acid
rRNA	Ribosomal RNA
S.M.	*Streptococcus mutans*
TE buffer	Tris EDTA buffer
TH broth	Todd-Hewitt broth

vicR	Putative response regulator gene
VMG II	Viable Medium Gothenburg II
WHO	World Health Organization
WSL	White Spot Lesion

1 INTRODUCTION

1.1 Dental caries

Dental caries or tooth decay is local hard-tissue destruction caused by acidic by-products from the bacterial fermentation of dietary carbohydrates (Fejerskov et al., 2003). The teeth will develop carious lesions if a demineralisation or mineral loss process takes place, but the disease process itself starts in the dental biofilm which covers the tooth surface (Selwitz et al., 2007, Le et al., 2014). Dental caries can lead to complications such as pain, discomfort when chewing, sleep disturbances and issues with one's appearance (Figueiredo et al., 2011).

The promotion of evidence-based approaches, along with clinical guidelines to help individuals to receive the most optimal prevention and treatment for their particular needs, can now be achieved in dentistry. In dental caries management, the emphasis has been on the prevention of caries in children, but caries is a disease that needs to be controlled over the lifespan of a person (Ismail et al., 2004). Study findings are pointing to an international change in clinical practice, turning away from invasive procedures and pushing towards different preventive strategies (Pitts et al., 2004). As awareness of the disease process has increased, an increased number of management techniques have emerged, including multiple approaches aimed at preventing or reversing the demineralisation process (Winston et al., 1998).

1.1.1 Aetiology

Dental caries is not a single-factor disease. It has a multifactorial origin and is primarily the result of the interaction between fermentable carbohydrates, host factors and cariogenic bacteria (Fig. 1). Aside from these, a large number of contributory factors are responsible for raising or reducing the speed of the disease, including the frequency and type of food intake, salivary quality and quantity, as well as oral hygiene practices (Selwitz et al., 2007). Furthermore, there are a variety of factors that are considered significant, such as socioeconomic status, biological factors and genetics, which have also been shown to be relevant to dental caries (Opal et al., 2015; Chapple et al., 2017).

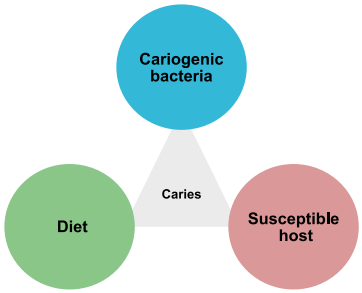

Figure 1. The three key factors behind the caries disease.

Dental caries is believed to be the result of an alteration in the resident microflora caused by a disturbance in the oral balance leading to the enrichment of potentially more cariogenic bacteria within the microbial population. This is a result of repeated conditions of low pH in dental biofilms, as a result of a shift in diet or a decrease in saliva production, for example (Marsh et al., 2010). When fermentable carbohydrate consumption increases, the dental biofilm is found for a longer period below the essential pH for enamel and dentine demineralisation (approximately pH 5.5 and pH 6.2 respectively). As a result, a higher number of cariogenic micro-organisms, such as mutans streptococci and lactobacilli, are contained in the biofilm, which leads to more acid being generated at a faster rate, thereby making hard-tissue destruction more predictable and leading to the destabilisation of biofilm ecology. Repeated low pH and the inhibition of competing species increase the likelihood of successful colonisation by bacteria, such as mutans streptococci or lactobacilli. This series of events explains the disturbances in the ecology which may in turn lead to disease initiation (Fig. 2) (Marsh et al., 2010). One of the best known cariogenic pathogens are mutans streptococci, which are highly acidogenic and have the ability to produce short-chain acids that dissolve the hard tissues of the teeth. In particular, they utilise sucrose, which also creates extracellular polysaccharides, which help them bind to the tooth surface and promote biofilm formation (Islam et al., 2007). On the other hand, lactobacilli are usually isolated from deep carious lesions and often present at a later stage of the process (van Houte et al., 1994). However, it is now known that a large number of bacteria contribute to this process and that the action cannot be claimed to be the result of one specific species (Mira et al., 2017).

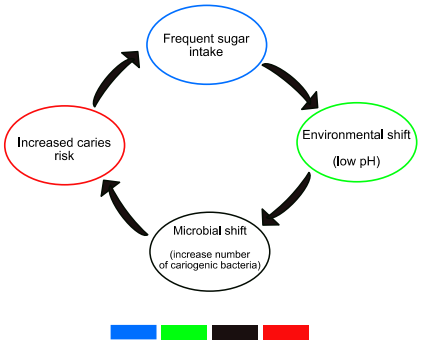

Figure 2. The ecological plaque hypothesis.

1.1.2 Prevalence

Over the past few decades, several studies have reported that there has been a substantial decrease in caries prevalence and that the disease continues to decline in many individuals (König et al., 2004, Marthaler et al., 2004). The use of preventive measures such as fluoridated toothpaste, sealants, dietary changes and oral health education has led to a decline in dental caries (Lee et al., 2013). On the other hand, recent studies have shown that caries is rising at an alarming rate in specific age groups. These increases occur in both children and adults, in both primary and permanent teeth and on coronal and root surfaces. A lower socioeconomic status, being immigrants and younger age appear to be significant factors in relation to this increase (Bagramian et al., 2009). A very recent systematic review on a global basis has concluded that, internationally, dental caries has been found to be present in 46% of the primary dentition and 54% of permanent teeth (Kazeminia et al., 2020). Conversely, in certain areas in the world such as Saudi Arabia, the caries disease may still be regarded as a pandemic and the incidence has been reported to be around 83% (Alhabdan et al., 2018).

1.1.3 Dental caries in orthodontic patients

Specific patient groups and different treatments may enhance the risk of caries occurring. This applies to the placement of fixed orthodontic appliances, which often hinders natural cleaning and also makes it difficult for the orthodontic patient to maintain optimal oral hygiene. When the components of the appliance are fitted (brackets, bands, wires and ligatures), these areas can house both new and increased sets of micro-organisms and help to block

normal access to the tooth surfaces to improve the cleaning of saliva, as well as the tongue and cheeks. It has been found that the positioning of bands and arch wires in orthodontic patients significantly increases the number of lactobacilli on dental surfaces (Topaloglu et al., 2011). Studies have also shown that the numbers of Streptococcus mutans in the saliva of these patients increase during treatment in comparison to before or after treatment (Jing et al., 2019). As a result, white spot lesions (WSLs) are one of the most common side-effects of orthodontic treatment and the most common areas in which WSLs have been recorded are the labial surfaces surrounding braces and near the gingival margins (Tanner et al., 2012). WSL incidence has been stated to be between 32-73% and the activity of cavitated lesions has been shown to increase over the course of orthodontic treatment (Richter et al., 2011; Akin et al., 2015). As a result, the early detection of WSLs is critical, as it allows clinicians to introduce preventive measures at an early stage in order to help slow down or even reverse the demineralisation process in order to avoid the progression of a lesion.

1.2 Preventive strategies

To obtain a better picture of the risks of dental caries, various causal factors must be assessed and analysed. The determination of the caries risk is used in daily practice in order to evaluate a particular patient's risk, to classify major causal factors and to implement appropriate preventive measures on an individual level.

During treatment, caries-risk assessments can be used as a screening tool to ensure treatment success. Risk assessment may also be useful for detecting caries-prone subjects who need more comprehensive preventive treatment in community-based preventive programmes. Dental caries is regarded as a preventable non-communicable disease, affecting the majority of the population over their lifetime. Preventing tooth decay has traditionally included the use of fluoride application, appropriate diet and good oral hygiene primarily, but other strategies such as antibacterial substances and fissure sealants are also used. Although standard routines remain intact, new technological applications based on ecological concepts have the ability to target and change the oral biofilm (Twetman et al., 2018). There are currently various methods that can be used to preserve a stable oral microbiome. Some different strategies that could influence the stability of the biofilm now follow.

1.2.1 Fluoride

The main causes of dental caries in developing countries are excess sugar intake and insufficient fluoride exposure (World Health Organisation, 2015; Prüss-Üstün et al., 2019) . The use of fluoride is a large public health advance. The addition of fluoride to drinking water, so-called artificial water fluoridation, in communities where fluoride concentrations are below optimum levels, has been shown to have a cariostatic impact. Since the middle of the last century, there has been an effective reduction in dental caries in many parts of the world and it can be attributed to the increased use of fluoride. Fluoride toothpaste has now been available for decades and it is thought to be the main contributor to the reduction in dental caries seen by people in developed countries (Petersen et al., 2016). Unfortunately, toothpaste is not always widely used, owing in particular to the cost factor that prevents vulnerable population groups from using these preventive measures. It has been demonstrated that fluoride inhibits the demineralisation of the crystal structures, while also increasing the opportunity for remineralisation (Featherstone et al., 1999). Water fluoridation, mouthrinses and applied fluoride products like gels and varnishes are examples of other different fluoride application methods.

1.2.2 Sugar reduction

A reduction in sugar consumption is an essential preventive approach for caries prevention. According to a new World Health Organisation (WHO) guideline, free sugar consumption should be kept to less than 10% of total energy intake or even less than 5% (World Health Organisation, 2015). Both the amounts and frequency of sugar intake should be considered when assessing caries risk. The key finding of the Vipeholm Study (1954) was that the severity of caries was strongly linked to sugar frequency (Gustafsson et al., 1954). Recently, Loveren et al. (2019) have shown that reducing the amount but not the frequency does not appear to be an effective caries-prevention strategy. The findings also imply that, when fluoride is utilised correctly with appropriate toothbrushing, the link between sugar consumption and caries is minimal or non-existent. To date, the latest evidence related to a reduction in sugar use comes from motivational interviewing and face-to-face counselling (Gao et al., 2014).

1.2.3 Oral hygiene

Regular, gentle mechanical disruption of the dental biofilm is considered essential for maintaining dental health and for preventing the creation of a

mature community which could lead to disease, especially at sites where biofilm is most commonly retained (Mira et al., 2017). With appropriate and routine twice-daily toothbrushing, the oral microbiota is preserved in a favourable state (Stone et al., 2017). However, systematic studies show that mechanical plaque measures alone, without fluoride, are ineffective in the prevention and treatment of dental caries (Figuero et al., 2017).

1.2.4 Antibacterial approach

Antibacterial agents have also been developed for use in controlling plaque accumulation and selectively reducing pathogens. However, the long-term effects of chlorhexidine, the gold standard, on oral biofilms are regarded as uncertain (Twetman et al., 2018). As a result, it has been suggested that the bacteriocidal use of broad-spectrum antibacterial agents should be used as a supplement to mechanical plaque removal in the short term. Unfavorable side effects such as taste disturbances and staining are frequently reported (Grover et al., 2021).

1.2.5 Bacteriotherapy

Due to the above-mentioned limitations, efforts are being made to identify new preventive strategies to reduce the number of cariogenic micro-organisms. Modulation of the oral microbiome in order to change the ecological balance has been proposed (Mira et al., 2018). Probiotics have long been related to gut health, with the majority of clinical research focusing on the prevention and treatment of gastrointestinal infections and illnesses (Gupta et al., 2009). However, a growing variety of health benefits from probiotic bacteria have been documented in recent decades, including the improved adaptive immune response treatment or the prevention of urogenital and respiratory tract infections, as well as the prevention or relief of allergies and atopic illnesses in new born (Ettinger et al., 2014).

Moreover, probiotics are also gaining in popularity as a preventive measure in dental care. In recent years, the use of probiotic bacteria to fight biofilm dysbiosis in particular has increased (Twetman et al., 2017). Different vehicles and modes for delivering probiotics are currently being specifically marketed for oral health. To date, probiotic therapy in the preservation of oral health has been considered to be one of the most advanced clinical preventive strategies in relation to dental caries (Haukioja et al., 2010).

1.3 Probiotics

Many definitions of probiotics have been suggested, but the one given by United Nations and World Health Organisation is the most widely used "Live micro-organisms, which, when administrated in adequate amounts, confer a health benefit on the host" (FAO/WHO 2002; Wildemann et al., 2002). The word probiotics during the middle of last century suggested as an antithesis to the term antibiotic (Lilley and Stillwell, 1965). The idea of probiotics arose at the turn of the twentieth century from a theory suggested by Nobel Laureate and Ukrainian bacteriologist Elie Metchnikoff in 1908, who laid the theoretical groundwork for probiotics (Stamatova et al., 2009). Probiotics have since been used as one possible way to treat different medical and oral disorders. Their use has been linked to a variety of gastrointestinal health benefits, including the relief of impaired enzymatic digestion and the reduction of symptoms including diarrhoea and stomach pain (Su et al., 2020). The extensive use of probiotics administrated orally as preventive and therapeutic products for gastrointestinal health has attracted also the attention of oral healthcare workers and led to strategies in order to improve oral health.

The term "prebiotics" can also be found in the literature when addressing this area. It refers to a non-digestible food ingredient that affects and stimulates the growth of beneficial micro-organisms (Davani-Davari et al., 2019).

1.3.1 Probiotics and oral health

Due to various particular actions and inhibitory effects on pathogen development, the potential of probiotics in the oral cavity is attracting increasing attention (Cagetti et al., 2013). Probiotics can, in fact, have a preventative impact on the development and progression of common oral diseases which, apart from dental caries, also include periodontitis, fungal infection and halitosis.

Since dental caries occurs when the bacterial equilibrium is disrupted and when an unbalanced population of bacteria exists, clear-cut efforts are being made to return to a healthy, balanced oral microflora (Sanz et al., 2017). So, to improve the conventional disease control strategies of dental caries, the concept of probiotics, during which interference with cariogenic pathogens' colonisation takes place, has been developed. Probiotics are able efficiently to normalise the pathological microbiota by reversing the imbalance in the oral ecological equilibrium and modulating the pathogenic potential of the biofilm (Parvez et al., 2006). The identification of potent probiotic strains, formulation, dosage,

intervention period, and host-microbe interaction status all play important roles in the final effect of the probiotic supplementation (Alok et al., 2017).

1.3.2 Mechanism of action

Probiotics may exert their effects through a number of different mechanisms, including the alteration of pH, antagonising pathogens through the synthesis of antimicrobial substances, competing for pathogen binding and receptor sites, and stimulating immunological modulatory cells to produce lactase (Haukioja et al., 2010). The positive effect of probiotics on the caries and dental biofilms are specific to each strain and the result of a complex interaction between competing actions (Sivamaruthi et al., 2020). The probiotic *Streptococcus A12* strain competes with cariogenic *Streptococcus mutans* by raising plaque pH, colonising the tooth surface and producing the extracellular protease challisin, which blocks *S. mutans* from bacteriocin production (Huang et al., 2016). *Streptococcus dentisani*, on the other hand, produce bacteriocin, which kills cariogenic bacteria while also balancing the pH of dental plaque (López-López et al., 2017).

Moreover, *L. reuteri* is a heterofermentative resident in the human gastrointestinal tract and it has been reported to produce antagonistic metabolites, such as reuterin (Mu et al., 2018) and reutericyclin (Gänzle et al., 2000), which are water-soluble, wide-spectrum antimicrobials that are effective across a broad pH range and are also resistant to proteolytic and lipolytic enzymes (El-Ziney et al., 1998). Although there is growing scientific evidence supporting the use of probiotics against different oral diseases, the specific mechanisms by which they work against oral micro-organisms is still the subject of debate.

1.3.3 Probiotic micro-organisms

Probiotics can be found in a variety of forms, including bacteria, yeast and moulds (Alok et al., 2017; Fernandes et al., 2019;), but the most frequent are bacterial species. The two genera of probiotics that are most often utilised are *Lactobacillus* and *Bifidobacterium*. Streptococci strains are rarely used. Studies have shown that breast milk contains both lactobacilli and bifidobacteria, implying early exposure to both bacteria in the oral cavity (Gueimonde et al., 2007; Abrahamsson et al., 2009). They are also known to be manufactured in the dairy industry (Parihar et al., 2015).

Lactobacilli represent 1% of the total cultivable oral flora in the oral cavity (van Houte et al., 1981). Davis et al., (1955) attempted to classify lactobacilli isolated from human mouths in the 1950s. A total of 473 strains were divided into five groups: *L. acidophilus, L. casei, L. plantarum, L. fermenti,* and *L. bravis*. Some of these strains are known for their cariogenic effect, while others could be act as a beneficial or friendly bacterium. *Lactobacillus fermentum, L. gasseri,* and *L. salivarious*, three species of lactobacilli, were recently found in the most caries-active subjects and were missing in caries-free subjects (Shimada et al., 2015; Xu et al., 2015). *Lactobacillus rhamnosus* GG (ATCC 53103), *lactobacillus reuteri,* recently classified as *Limosilactobacillus reuteri* (Zheng et al., 2020), and *lactobacillus plantarum,* are other examples of beneficial strains used for probiotic strategies (Bernardeau et al., 2006). One commonly used combination of two *L. reuteri* strains is DSM 17938, which is a daughter strain that was initially isolated from a Peruvian mother's breast milk and ATCC PTA 5289 which was discovered in the mouth of a Japanese woman who had excellent oral hygiene are commonly used today (Reuter et al., 2001).

Bifidobacteria, which are found naturally in the colonic microbiota, account for up to 25% of cultivable faecal bacteria in adults and up to 80% in newborns. They have been mostly examined as probiotic agents for the prevention and treatment of a wide range of animal and human gastrointestinal illnesses, including colonic transit disorders, intestinal infections and colonic adenomas (Picard et al., 2005). Bifidobacteria is fairly acidogenic in the oral cavity and may play a role in deep dentine caries development rather than early enamel demineralisation (Becker et al., 2002). *Bifidobacterium lactis* Bb-12 is an example of a strain that has some application in the oral cavity (Çaglar et al., 2008), although there is limited information is known about this genus in comparison to lactobacilli.

The use of streptococci as probiotic microorganism has a limited application in relation to oral health. The only streptococci suggested to be used for this purpose is *S. dentisani* (Ferrer et al., 2020) which has been found able to significantly increase salivary flow and reduce plaque amount.

1.3.4 Effect in the oral cavity

Since the end of the last century, the efficacy of Probiotics on dental caries has been studied worldwide, using different strains aiming to suppress the level of some known cariogenic bacteria such as *S. mutans* and lactobacilli. The findings are promising and point to significant advancements in this area.

Studies using strains such as *L. rhamnosus* GG and *L. reuteri* have established their ability to reduce the number of *S. mutans* (Näse et al., 2001; Ahola et al., 2002; Nikawa et al., 2004; Meurman et al., 2008), implying a role for probiotics in caries prevention. In addition, the consumption of products containing probiotic bifidobacteria has been used in several trials, resulting in a decrease in the number of mutans streptococci in saliva (Ahola et al., 2002; Çaglar et al., 2008; Çaglar et al., 2009a). However, not all studies have reported similar results. It is noteworthy that only a few studies have shown a significant rise in the amount of lactobacilli in salivary samples (Ahola et al., 2002; Montalto et al., 2004). The discrepancy between the results may be due to small sample groups, short duration, study design, the vehicle for probiotic administration and the prescribed dose. However, more studies are needed before conclusions about the most optimal strategy can be drawn.

Few studies have investigated the effect of probiotics with caries development as the final outcome. A study by Taipale et al. (2013) found no significant effect of using probiotic tablets supplemented with a pacifier between the age of one to two months up to two years on caries occurrence at the age of four. A randomised clinical trial in preschool children found a significant caries reduction in the test group using probiotic milk with fluoride in comparison to the placebo group after 21 months based on the dmfs score (Stecksén-Blicks et al., 2009). The effect of probiotic milk on primary root caries lesions has also been investigated in older adults. The authors found a significantly higher number of reversed lesions when probiotic milk was used with or without fluoride was used for 15 months (Petersson et al., 2011).

Moreover, the periodontal disease, which is an inflammatory disease affecting the structure around the tooth, has been found to benefit from the use of probiotics. In addition to improved oral hygiene, it is primarily treated using mechanical subgingival debridement. However, researchers have started to focus on adjunctive therapies, including the use of probiotics. Studies of probiotics and periodontal disease are especially scarce and currently only a few clinical studies have tested the effectiveness of probiotic organisms in this field (Gruner et al., 2016). *Lactobacillus reuteri* and *Lactobacillus brevis* are two species that have been shown to have a beneficial impact on gingivitis and plaque composition. The results showed that, after a two-week probiotic administration, gingival bleeding and gingivitis were found to be significantly reduced (Riccia et al., 2007). However, further research is needed in this field in order to draw more definite conclusions.

Probiotics has also been suggested to have a preventive and therapeutic effect against oral candidiasis with a particular focus on elderly (Ai et al., 2017;

Mundula et al., 2017). A fifty percent reduction of candida albicans counts has been found after administration of tablets containing *L. reuteri* (Kraft Bodi et al., 2015).

Different aspects of probiotic application in the oral cavity include treatment to oral malodour. Halitosis is not a disease, but it is described as an irritation or discomfort where the cause may be located in the oral cavity in nearly 90% of all cases (Delanghe et al., 2005). Only a few clinical trials have shown that various probiotic strains such as *S. salivarius* K12, and *E. coli* Nisle 1917 could be helpful in the treatment of oral malodour (Henker et al., 2001; Burton et al., 2006), but probiotics are currently being marketed for the treatment of both oral and gut related halitosis.

1.3.5 Probiotic administration

Probiotics are found naturally in a number of foods, including a supplement-free way to reap the benefits of these beneficial bacteria. Yogurt, milk, kefir and cheese are examples of the most common foods with a natural content of probiotics. There are other non-dairy products into which probiotics have been artificially incorporated; they include lozenges, chewing gums, drops, mouthwashes, toothpaste etc. To achieve optimal effects of probiotics, a number of conditions must be met. Sufficiently long interaction time between probiotics and different oral components, such as saliva and dental biofilm, and a specific concentration of the daily consumption, 10^6–10^{10} viable cells, has been suggested to achieve the best results (Lee et al., 1995). Also, product characteristics may play a role in influencing particularly contact time. It is therefore essential to increase our knowledge of the best vehicles for use in probiotic administration.

1.3.6 Safety

A safety evaluation is a crucial criterion for any food-grade organism, particularly for new strains with no prior history of use. In healthy humans, lactobacilli and bifidobacteria are both major groups of the typical GIT microflora (Ruiz-Moyano et al., 2012). Most lactic acid bacteria (LAB) strains, particularly lactobacilli, have been categorised as ‘GRAS' (Generally Recognised As Safe), due to long-term usage and safe human exposure (Floch et al., 2013). Furthermore, the European Food Safety Authority (EFSA) has granted a number of Lactobacillus species for Qualified Presumption of Safety (QPS) status, based on their safety review standards in terms of possible pathogenicity and end use (Ricci et al., 2017). Among the rare cases of

infections, some strains have been identified as the causal agents of bacterial infective endocarditis (Suarez-Garcia et al., 2012).

As the prescribed dose of probiotics is relatively high, any virulence features may have negative implications (Bunesova et al., 2015). As a result, probiotics should be safe and pose no threat to the host. In addition, probiotics should be extensively evaluated for the existence of any pathogenic characteristics or virulence properties before being used or tested in humans. A number of clinical studies have evaluated the effect of probiotics on the oral health, with no side-effects being reported (Petersson et al., 2011; Pahumunto et al., 2019; Zare Javid et al., 2020).

2 AIMS

This thesis was designed to investigate the following aspects of probiotics in relation to caries related variables. The work was performed in a clinical study of healthy subjects (Paper I), *in vitro* (Paper II), in a clinical study of orthodontic subjects (Paper III) and in a systematic review (Paper IV). The following aims and hypotheses were formulated for the different papers:

Paper I

- To investigate the extent to which two *Lactobacillus reuteri* strains were detectable in the oral cavity after four weeks' of administration of probiotic-drops
- To study the short-term effect of probiotic drops on the level of salivary *S. mutans* and lactobacilli
- The null hypothesis was that the selected strains would only be detectable for two weeks after probiotic cessation and that probiotic drops would not alter the bacterial levels

Paper II

- To evaluate changes in endogenous caries microflora after a four-weeks' treatment with probiotics by investigating the modification of host *lactobacilli* interference capability (Part I)
- To examine the genetic response of *Streptococcus mutans* to probiotic metabolites (Part II)
- The hypothesis was that no effect of probiotics would be found

Paper III

- To study the effect of probiotics on biofilm acidogenicity and on the number of salivary *Streptococcus mutans* and lactobacilli in orthodontic patients
- The null hypothesis was that the effect of probiotics would not differ from that in a placebo-treated control group

Paper IV

- Systematically to review the best vehicle for probiotic administration and to examine the dose-response effect on caries-related variables

3 MATERIALS AND METHODS

The current thesis work, presented in four papers (I-IV), is based on three studies (I-III). Study 1 includes two parts where part I is a clinical investigation and part II an *in-vitro* examination. The characteristics of the study subjects and the design are presented (Table 1).

Table 1. Characteristics of the included studies.

Studies	Number of subjects and mean age	Power analysis	Design	Title
Study I, Part I	13 (6 males/7 females) 25.7 ± 3.6 yrs	40% reduction in *S. mutans* counts	Clinical study	Oral colonisation after the administration of drops containing *lactobacillus reuteri*
Study I, Part II, A	10 subsamples	40% reduction in *S. mutans* counts	*In vitro*	*Lactobacillus reuteri* treatment induces a genetic response in *Streptococcus mutans* and interference in endogenous *lactobacilli sp*
Part II, B	10 caries active (6 males/4 females) 52.7 ± 17.3 yrs 10 caries free (3 males/7 females) 27.5 ± 7.9 yrs	-	*In vitro*	
Study II	28 (14 males/14 females) 17.3 ± 1.1 yrs	pH drop of 0.4 ± 0.35	Clinical study	Effect of drops containing *Lactobacillus reuteri* on plaque acidogenicity and other caries-related variables in orthodontic patients
Study III	Systematic review of best administrative mode and optimal dose of probiotics			

3.1 Study subjects

3.1.1 Study I (Part I & Part II, A)

Healthy young adults, recruited via an advertising poster, were included in Study I. A saliva screening test was used to identify participants with the required number of *Streptococcus mutans*. During a period of two months, 40 subjects were screened and, of those, thirteen subjects were included. A subsample of 10 subjects was included in Paper II. The study was performed at the Department of Cariology, Institute of Odontology, University of Gothenburg, Sweden.

The inclusion criteria were:

- Healthy
- $>10^4$ CFU of *S. mutans* per ml saliva
- Non-smokers

3.1.2 Part II, B

Streptococcus mutans clinical isolates were obtained from the University of Gothenburg's Department of Cariology research laboratory's existing collection of bacteria for gene expression investigation. Ten strains from caries-active and ten strains from caries-inactive patients were selected.

3.1.3 Study II

A saliva screening examination was conducted on 60 subjects undergoing treatment with bimaxillary fixed orthodontic appliances at the Specialist Clinic of Orthodontics, Public Dental Service, Gothenburg, Region Västra Götaland, Sweden. This was done in order to identify participants with a high number of *Streptococcus mutans* ($>10^4$ CFU/ml of saliva). Out of the screened subjects, 28 were included, twenty subjects were not willing to participate, and twelve did not meet the inclusion criteria (Fig. 3).

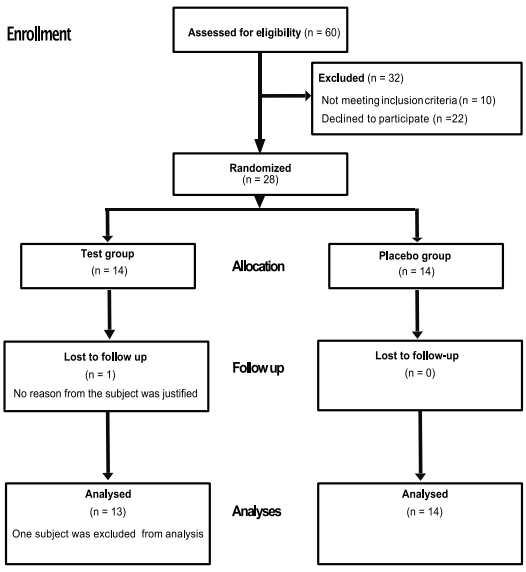

Figure 3. Flow chart for subject allocation (Study II).

They had to fulfil the following inclusion criteria:

- Healthy
- $>10^4$ CFU of *S. mutans* per ml saliva
- Bimaxillary fixed orthodontic appliances
- Eight months since the onset of orthodontic treatment

All the subjects had good oral hygiene ahead of starting the clinical trial and brushed their teeth with fluoridated toothpaste twice/day. The subjects were told to refrain from oral hygiene before sampling (Fig. 4).

For Studies I and II, subjects with a history of probiotics/anti-inflammatory drugs/antimicrobial substances taken during the last four weeks were excluded.

Figure 4. Prior to each sampling session, the participants were asked to refrain from proximal cleaning for 48 hours and toothbrushing for 24 hours prior to the test.

3.2 Study design

3.2.1 Study I, Part I

The study was designed as a short term prospective clinical trial lasting nine weeks (Fig. 5).

During the study period, the participants were told to use the probiotics drops as a mouthwash twice a day, after brushing morning and evening. They used the product for rinsing for 60 sec and then spat it out.

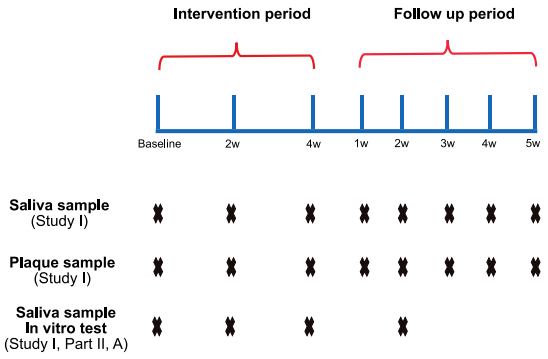

Figure 5. Time points for collection of saliva and plaque samples (Study I, Part I) and saliva sample (Study I, Part II, A).

3.2.2 Study I, Part II, A & B

Samples collected at baseline, two and four-week intervention and after two-week follow-up were further handled *in vitro* (Fig. 5). As an additional part of the *in vitro* examination, the laboratory bacterial strains were used.

3.2.3 Study II

The study was designed as a randomised, double-blinded, controlled clinical trial (Reg Clinicaltrial.gov: NCT04593017) with two parallel arms, lasting for three weeks (Fig. 6).

Figure 6. Plaque pH was measured and saliva & plaque samples were collected at each time point.

Randomisation and blinding

The subjects were first stratified based on the gender and the number of *S. mutans*, after which the randomisation was performed using an Excel program (Version 15.33) where the subjects were assigned to either the test or the placebo group. In order to avoid bias, a third independent person was assigned

for the coding, so that none of the participants nor the investigator was aware of the group they were enrolled in. The codes were revealed after data analysis.

3.2.4 Study III

The systematic review was conducted based on guidelines of the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) (Moher et al., 2010).

Definition of the search question and search strategy

The literature search was performed according to the PICO criteria to answer the research question: in healthy adolescents, what will be the effect of probiotics compared with placebo/control on caries-related variables?

Two electronic databases (PubMed and Scopus) were searched for published articles without filtering. Participants were described as an adolescent or adult, intervention was described as probiotics or replacement therapy or bacterial interference or bacteriotherapy, the comparison group was either a control or a placebo group, and the outcome was dental caries, tooth decay, cariogenic bacteria, *Streptococcus mutans* or lactobacilli.

Selection criteria

For the title, abstracts, and articles, the following inclusion criteria were set:

- Randomised and controlled clinical trials
- Human studies of healthy subjects
- Studies in English
- Studies of subjects wearing fixed orthodontic appliances
- Comparison made between probiotics and placebo/control group

Additional articles were identified from the reference lists of the retrieved papers.

Data selection and data extraction

Two independent researchers reviewed all the abstracts with regard to the inclusion criteria. When important information was missing, the complete text of the possibly included research was examined. Inter-examiner disagreement was overcome by a third-party consultation and a detailed discussion of each article until consensus was reached. Irrelevant papers were removed after reading the whole text, and the explanation was noted.

All included studies had their data extracted using an extraction sheet, which included the following information:

All included studies had their data extracted using an extraction sheet, which included the following information: (1) Authors, (2) Results, (3) Number of participants and their ages, (4) Probiotic micro-organism and its concentration, (5) Delivery vehicle, (6) Regular probiotic dose, (7) Comparison group, (8) Design, intervention duration and follow-up time, and (9) Findings.

Risk of bias assessment

Cochrane's collaborative tool for assessing the risk of bias in randomised clinical trials was used to assess the quality of all the included articles (Higgins et al., 2011). Each study was evaluated separately by two reviewers. Six categories of bias were checked which include seven domains were checked and each study was assessed as having a low, moderate, or high risk of bias according to the analysis of the domains (Table 2). Any disagreements were settled through discussion before a resolution was found or, if necessary, involving a third party.

Table 2. Assessing risk of bias using the Cochrane's collaborative tool.

Domain	
Low risk of bias	When all seven domains were judged as having low risk
Moderate risk of bias	When one or two out of the seven criteria were assessed as having a high risk of bias or unclear
High risk of bias	When three or more criteria were assessed as having a high risk of bias or unclear

3.3 Study drops

The probiotic drops, BioGaia Prodentis drops (BioGaia AB, Stockholm, Sweden), contained two different freeze-dried bacterial strains, namely *L. reuteri* DSM and *L. reuteri* ATCC PTA 5289 (>2x10^8 CFU/10 drops) suspended in an oil. Prior to each rinsing session, the test participant made a fresh probiotic solution by mixing five drops of probiotic oil with 5 ml of distilled water. When not in use, the water and drops were kept in the refrigerator.

Rinsing took place twice a day, in the morning after breakfast and toothbrushing and in the evening before going to bed.

3.4 Clinical variables

Table 3. Data collection in the studies.

Papers	Saliva	Plaque	Plaque pH
I	✓	✓	
II	✓		
III	✓	✓	✓

All the samples were analysed at the Department of Cariology, Institute of Odontology, University of Gothenburg, Sweden.

3.4.1 Saliva samples

By chewing on 1 g of paraffin wax and spitting into a graded test tube, whole stimulated saliva (5 ml) was obtained, and the secretion rate was expressed in ml/min. One ml was used for buffer capacity, 1 ml was transferred to VMGII medium for microbiological analysis (Study I, *Part I* and Study II), and 1 ml was used for further qPCR analysis (Study II). Ericsson's technique was used to evaluate buffer capacity, which was expressed as final pH (Ericsson et al., 1959).

3.4.2 Plaque samples

Plaque samples were collected using a sterile tooth pick. For Study I, *Part I*, pooled plaque samples were collected from both upper and lower jaws to identify probiotic strains using the qPCR technique. For Study II, deep interproximal plaque samples were obtained between the upper lateral incisors and canines on both sides and then immediately transferred into microtubes with TE buffer for qPCR identification and quantification of the probiotic strains.

3.4.3 Plaque acidogenicity

For Study II, the pH of plaque was determined using the strip method (Carlén et al., 2010). Plaque pH was measured using a pH indicator strip

(Spezialindikator, Merck, Darmstadt, Germany) ranging between (4.0-7.0). Each strip was cut into three parts and placed in the interproximal area of the lateral incisors/canines in the left and right upper regions before (0 min) and up to 30 min after a one-minute mouthwash with 10 mL of a 10% sucrose solution (Fig. 7). Comparisons were made between the colour on the inserted strip and the index chart provided by the manufacturer in order to assess the pH values.

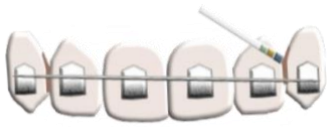

Figure 7. An illustration indicating the sites from which the plaque samples were collected.

3.4.4 Endogenous lactobacilli collection

In Study I *Part II, A & B*, all clinical lactobacilli isolates were gathered from subjects participating in Study I, Part I. Due to a lack of baseline data, three patients were excluded from the study. Lactobacilli isolates with distinctly different colony morphology from *L. reuteri* were selected for an interference test. Isolates were kept frozen (-80^{o} C) until testing.

3.6 Microbiological analyses

3.6.1 Cultural analysis

The saliva samples were serially diluted in phosphate buffer (PBS) into (10^1 to 10^6). *Streptococcus mutans* strains were cultured on Mitis Salivarius (MS) agar (DIFCO Laboratories, Michigan, USA), supplemented with bacitracin (Gutiérrez de Annan et al., 1997), and Rogosa agar for the total number of lactobacilli (Rogosa et al., 1951). The CFU was determined by identifying characteristic colony morphology. In the interference test, universal M17 (Sigma-Aldrich, Gmbh, Steimheim, Germany) agar was employed to acquire the growth of all streptococci. All cultures were incubated at 37^{O}C in anaerobic conditions. All cultures were incubated at 37^{O} C in anaerobic conditions.

3.6.2 Quantitative polymerase chain reaction

Prior to qPCR analysis, tubes containing plaque or saliva samples were shaken for 10 minutes at 95°C and 1,000 rpm in a thermoshaker (TS-100C, Biosan SIA, Latvia) to release genomic DNA. An MIC analyser was used to perform the qPCR relative quantification analysis (Bio Molecular Systems, Upper Coomera, Australia). In the relative quantification analysis, a conserved region (16S rRNA) in the ribosomal genome, which is present in all bacteria, was used as the reference gene, and both strain-specific and species-specific primers for *L. reuteri* (Study I, Part I and Study II) (Vestman et al., 2013 & 2015), *S. mutans*, lactobacilli, and streptococci (in Study II) were used as the gene of interest (Table 4).

The analysis was carried out using the relative expression software method (REST) on the MIC analyser (Bio Molecular Systems, Australia).

3.6.3 Agar-overlay interference test

A *lactobacillus* colony was selected from each participant in Study I at each time point based on morphological characteristics.

For the agar-overlay test against a panel of 13 different streptococcal strains:

1- *S. mutans* group: Ingbritt, OMZ65, two wild types (20T3, So22)
2- *S. sobrinus* group: B13, OMZ175, two wild types (43a, S20)
3- Streptococci group: *S. salivarius*, *S. mitis*, *S. gordonii*, *S. orealis*, *S. sanguinis*

Both drops and separate *L. reuteri* strains (DSM 17938 and ATCC PTA 5289) were used for comparison and no lactobacilli were used as a control.

The agar-overlay test was performed as described by Rönnqvist et al. (2009). Briefly, two agar layers were prepared; the first bottom layer contained collected *lactobacillus* strain suspension mixed with MRS agar, then plates were incubated anaerobically at 37°C overnight. The second top layer contained M17 agar was poured and allowed to congeal. The tested streptococci panel (5 ul of each bacteria suspension) was inoculated and plates were incubated anaerobically, overnight at 37°C. Tests were run in triplicates and growth inhibition was scored from 0 (total inhibition) to 5 (no inhibition). Finally, pH was measured on top of the M17 agar layer with a pH electrode (Orion 8220BNWP, Thermo Scientific, USA).

Table 4. The details of each assay and primer sequence.

	Primer sequence 5'-3'	**qPCR program**
Streptococcus mutans	Forward: CTACACTTTCGGGTGGCTTG	95℃ 2 min
	Reverse: GAAGCTTTTCACCATTAGAAGCTG	40 x 95℃ 10 s, 61℃ 20 s, Plate read
Total bacteria (16S rRNA)	Forward: TGGAGCATGTGGTTTAATTCGA	94℃ 4 min
	Reverse: TGCGGGACTTAACCCAACA	40 x 94℃ 20 s, 62℃ 20 s Plate read
Total lactobacilli	Forward: TGGAAACAGRTGCTAATACCG	98℃ 2 min
	Reverse: GTCCATTGTGGAAGATTCCC	40 x 98℃ 10 s, 62℃ 15 s Plate read
***Lactobacillus reuteri* DSM 17938**	Forward: TTAAGGATGCAAACCCGAAC	98℃ 2 min
	reverse: CCTTGTCACCTGGAACCACT	40 x 98℃ 5 s and 60℃ 15 s Plate read
***Lactobacillus reuteri* ATCC PTA 5289**	Forward: GACAGTGGCTAAACGCCTTC	98℃ 2 min
	Reverse: AATTCCACTTGCCATCTTCG	40 x 98℃ 5 s and 60℃ 15 s Plate read
Total Streptococci	Forward: YGTGCAATTTTTGGATAAT	95℃ 3min,
	Reverse: TTCTATAAGCCATGTTTTGT	40 x 94℃ 20 s, 52℃ 30 s Plate read

3.6.4 Phosphate-buffered saline filtrate test

One colony of each: *Lactobacillus reuteri* DSM 17938, *Lactobacillus reuteri* ATCC PTA 5289, endogenous *lactobacilli*, with observed highest interference ability, and 10 ul of the probiotic product were grown separately for 18 hours in MRS broth under recommended environmental conditions. After centrifugation, the pellet was washed three times in PBS buffer and finally

resuspended in 10 ml of prewarmed PBS buffer. The suspensions were incubated for five hours at 37˚C and centrifuged (15 min, 8˚C, 4000 rpm). The obtained supernatant with metabolites was adjusted to pH 5.5 and 7.0, and sterile filtered into Eppendorf tubes (1 ml). Samples were kept frozen (-20˚C) until use in the gene expression test and in the PBS filtrate test. In PBS filtrate test streptococci suspension (10 ul) was incubated with obtained metabolites, samples to determine bacteria growth (CFU) were taken at baseline and up to 24 hours after incubation (37˚C). As a control, a clean PBS buffer was used.

3.6.5 Gene expression test

Twenty *S. mutans* strains were cultured anaerobically on MSB agar for 48 hours at 37˚C, while one colony was utilised to inoculate BHI broth (0.1 percent sucrose; 0.5 percent yeast extract). Obtained overnight cultures (100 µl) with OD550 0.4, indicating midlogarithmic growth phase, were added to freshly thawed PBS filtrates (1 ml) supplemented with BHI broth (100 µl). The samples were incubated at 37˚C for four hours, with gentle mixing in between. The supernatant was removed after centrifugation (4ºC, 5000 rpm, 4 min). To protect RNA, pellets were treated with NucleoProtect® RNA solution (Macherey-Nagel, Düren, Germany) according to the manufacturer's protocol. Until RNA extraction and analysis, tubes were kept at -40˚C.

3.6.6 RNA extraction and cDNA synthesis

The NucleoSpin® RNA kit (Macherey-Nagel, Düren, Germany) was used to isolate RNA, according to the manufacturer's instructions. To remove any DNA contamination, the samples were processed with RNase-free DNase. NanoDrop (NanoDrop 2000, Thermo Scientific, USA) was used to assess RNA concentration and purity (A260/A280 >1.9). Finally, 1 µl of the total RNA sample was reverse transcribed into complementary DNA using the qPCRBIO cDNA Synthesis Kit (PCRBio, London, UK) (cDNA) (Study I, *Part II, B*).

3.6.7 Quantitative real-time polymerase chain reaction (qRT-PCR)

The effect of metabolites derived from probiotic and endogenous lactobacilli strains on the expression levels of six target genes *(gtfB, gtfC, gtfD, atpD, ldh, vicR)* in two groups (active and non-active) of *S. mutans* was investigated using quantitative real-time PCR (qRT-PCR) (Study I, Part II, B). The primers

utilised for amplification have previously been described and validated (Wu et al., 2020)

The internal reference gene was *Streptococcus mutans* 16S rRNA, while the control was *S. mutans* strains not treated with PBS filtrate. The qRT-PCR was carried out using a MIC analyser (Bio Molecular Systems, Upper Coomera, Australia). 1x qPCRBIO SyGreen mix (PCR BioSystems, London, UK), 400 nM of each forward and reverse primers (Sigma-Aldrich Co., LLC), and 2.0 µl cDNA template were included in the reaction mixture of 20 µl. In MIC Tubes and Caps, all amplifications were made in duplicate (BioMolecular Systems, Upper Coomera, Australia). MIC software was used to analyse all of the data (BioMolecular Systems, Upper Coomera, Australia).

3.8 Ethical considerations

The clinical trials included in this thesis were registered and approved by the Research Ethics Committee at Sahlgrenska Academy at the University of Gothenburg with a registration number 260-18 for Study I, and 788-18 for Study II. The research protocol followed the Helsinki Declaration of Human Rights (World Medical Association, 2013). The subjects received both verbal and written information and they gave their informed consent. For Study II, for subjects aged <18 years, informed consents was obtained from both the participants and their guardians prior to entering the trial.

3.9 Statistical analysis

Table 5 shows the summary of the statistical analyses used in different studies. Both descriptive and statistical analyses were performed using GraphPad Prism software (version 8.2.0 (272)). Statistical significance was set at a p-value of $p < 0.05$.

In Study I, Part I, the level of the probiotic strains at different time points in saliva was compared through identification and quantification using qPCR gene expression analyses.

In Part II, B, gene expression was analysed by MIC software (BioMolecular Systems, Upper Coomera, Australia) using the Pfaffl method (Pfaffl et al., 2001).

$$ratio = \frac{(E_{target})^{\Delta CP_{target}(control-sample)}}{(E_{ref})^{\Delta CP_{ref}(control-sample)}}$$

The ratio of a target gene is expressed in a sample versus control compared with a reference gene.

In Study II, the mean pH for the two sites at each of the different time points, the minimum pH, maximum pH fall and the area under the curve ($AUC_{7.0}$), was calculated. The mean ± SD and 95% confidence interval for the *S. mutans* and lactobacilli concentrations in saliva were calculated for each group.

Table 5. Summary of statistics used for the separate studies.

Statistical analysis	**Study 1, *Part I***	**Study 1, Part II, A**	**Study 1, Part II, B**	**Study 2**
One-way ANOVA followed by Tukey's comparison	Salivary level of *S. mutans* and lactobacilli The two probiotic strains in saliva			Quantification of investigated probiotic and other bacterial species in plaque
Two-way ANOVA followed by Tukey's multiple comparisons		Differences in interference between groups		Changes in pH value within the test and placebo groups Salivary cariogenic bacteria between groups
Sidak's multiple comparisons				Changes in pH value between the test and placebo groups
Paired t-test				Detailed information regarding the pH Changes in the buffer capacity between the two groups
MIC software			Gene expression	

4 RESULTS

4.1 Microbiological data from saliva and plaque samples

Colonisation of probiotic strains based on saliva samples in healthy adults (Study I, Part I)

At baseline, two subjects carried the *L. reuteri* DSM 17938 strain, while one subject carried the *L. reuteri* ATCC PTA 5289 strain in saliva based on the qPCR analysis. The four-weeks' sample in comparison to the baseline showed that 11 (84.6%) and 13 (100%) of all individuals were positive for *L. reuteri* DSM 17938 and ATCC PTA 5289 in saliva respectively. Five weeks after administration, four individuals were colonised with the DSM 17938 strain and two individuals with the ATCC PTA 5289 strain, suggesting that the probiotic bacteria were steadily disappearing after completing administration. The saliva samples demonstrated that the DSM 17938 strain is slightly better at colonisation compared with the ATCC PTA 5289 strain for the dental biofilm at nine weeks, with 4/13 and 2/13 subjects respectively (Figs. 8 and 9). Table 6 shows the individual pattern during and after intervention.

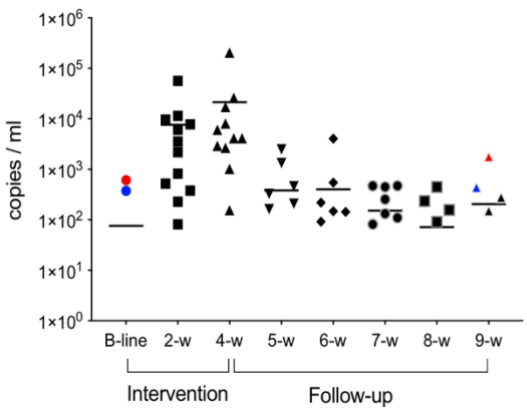

Figure 8. Detection of L. reuteri DSM 17938 in saliva assessed via qPCR. The line indicates the mean and the symbols represent individual subjects who are positive for the DSM 17938 strain. The coloured dots indicate the two subjects already harbouring the bacteria already at baseline.

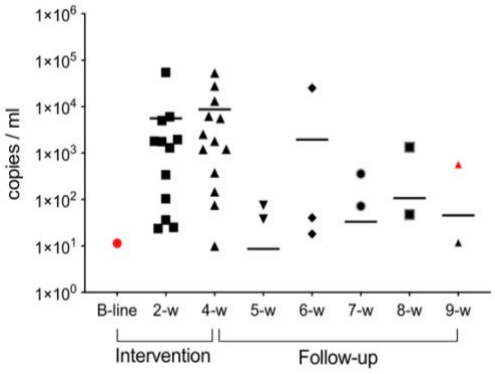

Figure 9. Detection of L. reuteri ATCC PTA 5289 in saliva assessed via qPCR. The line indicates the mean, while the symbols represent individual subjects who are positive for the ATCC PTA 5289 strain. The coloured dot indicate the one subject already harbouring the bacteria already at baseline.

Table 6. Individual pattern for individuals included after probiotics administration (DSM 17938 and ATCC PTA 5289) indicating the colonisation process.

DSM / **PTA** **Subjects**	**Baseline**	**2 weeks**	**4 weeks**	**1 w follow up**	**2w follow up**	**3w follow up**	**4w follow up**	**5w follow up**
1	0/0	1/1	0/1	0/0	0/0	1/0	1/0	0/0
2	0/0	1/1	1/1	1/1	1/1	1/1	1/1	1/1
3	0/0	1/1	1/1	1/0	1/1	1/1	1/1	1/0
4	0/0	1/1	1/1	0/0	0/0	0/0	0/0	0/0
5	0/0	1/1	1/1	0/0	0/0	0/0	0/0	0/0
6	0/0	1/1	1/1	1/1	1/1	0/0	0/0	0/0
7	0/0	1/0	1/1	1/0	1/0	1/0	0/0	0/0
8	1/0	1/1	1/1	1/0	1/0	1/0	0/0	1/0
9	0/0	1/1	1/1	0/0	0/0	0/0	0/0	0/0
10	0/0	1/1	1/1	0/0	0/0	0/0	0/0	0/0
11	0/0	0/1	0/1	0/0	0/0	0/0	0/0	0/0
12	0/0	1/1	1/1	0/0	0/0	1/0	0/0	0/0
13	1/1	1/1	1/1	1/0	1/0	1/0	1/0	1/1

Effect of probiotics on salivary *S. mutans* and lactobacilli in healthy adults (Study I, Part I)

At the end of the intervention period, the culture analysis of saliva showed a statistically significant decrease in the number of *Streptococcus mutans* ($p<0.05$) and a statistically significant increase in the lactobacilli level ($p<0.05$). Apart from week four, all time points showed a similar standard deviation. During the follow-up period, no major changes in bacterial levels were observed. (Table 7). The salivary secretion rate and buffer capacity showed no significant changes during the whole study period.

Table 7. Differences in salivary levels of Streptococcus mutans and lactobacilli between baseline and the different time points. Mean difference, 95% CI and p-values are shown.

	Mean difference	95% CI	P value
S. mutans			
Baseline vs 2 weeks	0,1020	-1,288 to 1,492	>0.05
Baseline vs 4 weeks	1,537	0,1464 to 2,927	**<0.05**
Baseline vs 1-week follow-up	0,3784	-1,012 to 1,769	>0.05
Baseline vs 2-week follow-up	0,4405	-0,9498 to 1,831	>0.05
Baseline vs 3-week follow-up	0,1614	-1,229 to 1,552	>0.05
Baseline vs 4-week follow-up	0,2324	-1,158 to 1,623	>0.05
Baseline vs 5-week follow-up	0,2080	-1,182 to 1,598	>0.05
Lactobacilli			
Baseline vs 2 weeks	-0,4664	-1,670 to 0,737	>0.05
Baseline vs 4 weeks	-0,6112	-1,190 to -0,032	**<0.05**
Baseline vs 1-week follow-up	0,4551	-1,111 to 2,021	>0.05
Baseline vs 2-week follow-up	-0,2546	-2,021 to 1,512	>0.05
Baseline vs 3-week follow-up	0,01455	-1,770 to 1,799	>0.05
Baseline vs 4-week follow-up	-0,5207	-1,597 to 0,556	>0.05
Baseline vs 5-week follow-up	-0,2354	-1,452 to 0,982	>0.05

Colonisation of probiotics strains based on plaque samples in healthy adults (Study I, Part I)

The plaque qPCR analyses showed a similar trend to the saliva analysis, but neither of the two strains was identified at baseline (Figs. 10 and 11).

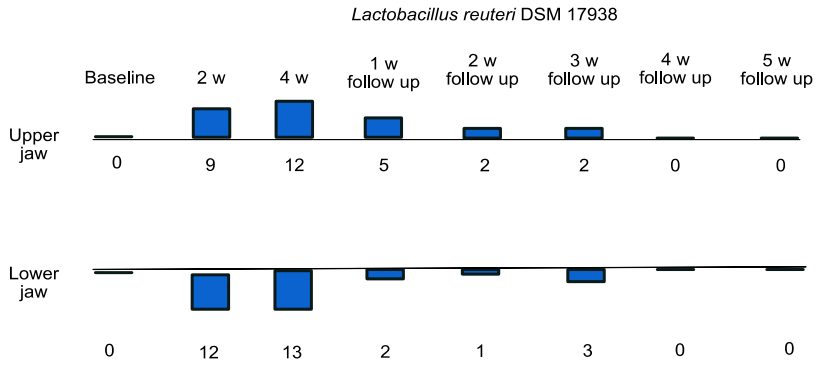

Figure 10. Positive detection of probiotics DSM strain 17938 in plaque samples using the strain-specific qPCR technique. Data represents the number of subjects.

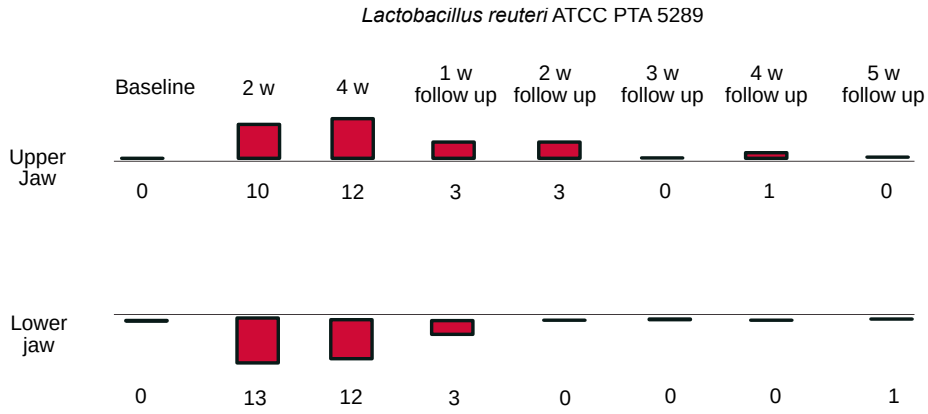

Figure 11. Positive detection of probiotic ATCC PTA 5289 strain in plaque samples using the strain-specific qPCR technique. Data represents the number of subjects.

Effect of probiotics on salivary *S. mutans* and lactobacilli in orthodontic subjects (Study II)

Changes in the level of *S. mutans* and lactobacilli between one and three weeks' use of probiotics are shown (Tables 8 and 9). Based on the cultural analysis, there was no statistical difference in the salivary level of *S. mutans* nor the level of lactobacilli between the test and placebo groups during the trial period. ($p>0.05$).

Table 8. Salivary prevalence of S. mutans in the test and placebo groups at baseline, one week and three weeks post intervention (p<0.05).

S. mutans	**Test group**		
	Mean difference	95% CI	p value
Baseline vs. 1 week	0,1816	-0,7953 to 1,158	>0.05
Baseline vs. 3 weeks	0,5305	-0,4464 to 1,507	>0.05
1 week vs. 3 weeks	0,3489	-0,6280 to 1,326	>0.05
Placebo group			
Baseline vs. 1 week	-0,3462	-1,288 to 0,5952	>0.05
Baseline vs. 3 weeks	-0,001659	-0,9430 to 0,9397	>0.05
1 week vs. 3 weeks	0,3445	-0,5968 to 1,286	>0.05

Table 9. Salivary prevalence of total lactobacilli in the test and placebo groups at baseline, one week and three weeks post intervention (p<0.05).

Lactobacilli	**Test group**		
	Mean difference	95% CI	p value
Baseline vs. 1 week	-0,7336	-1,997 to 0,5299	>0.05
Baseline vs. 3 weeks	-0,6346	-1,544 to 0,2751	>0.05
1 week vs. 3 weeks	0,09901	-1,110 to 1,308	>0.05
Placebo group			
Baseline vs. 1 week	0,3870	-0,5654 to 1,339	>0.05
Baseline vs. 3 weeks	-0,1300	-0,5733 to 0,3132	>0.05
1 week vs. 3 weeks	-0,5170	-1,305 to 0,2711	>0.05

The salivary *S. mutans* and lactobacilli levels were more stable in the test groups compared with the placebo group, which showed an increase during the trial period. No statistically significant differences were seen within or between the groups. A numerically but not statistically significant increase in buffer

capacity was found for the test group, but no such difference was found for the control group.

The number of salivary *Streptococcus mutans* and lactobacilli did not differ statistically between the test and placebo groups at baseline, one week and at the end of the intervention period (p=0.1) (Tables 10 and 11).

Table 10. Changes in the salivary levels of S. mutans at baseline between the test and placebo groups. Data presented as the mean difference, 95% confidence interval and level of significance (p<0.05).

S. mutans				
		Mean difference	**95% CI**	**p value**
Baseline	*S. mutans* test vs *S. mutans* placebo	0,066	-0,906 to 1,038	>0.05
One week	*S. mutans* test vs *S. mutans* placebo	-0,098	-1,089 to 0,893	>0.05
Three weeks	*S. mutans* test vs *S. mutans* placebo	-0,081	-1,116 to 0,954	>0.05

Table 11. Changes in the salivary levels of lactobacilli at baseline between the test and placebo groups. Data presented as the mean difference, 95% confidence interval and level of significance (p<0.05).

Lactobacilli				
		Mean difference	**95% CI**	**p value**
Baseline	Lactobacilli test vs lactobacilli placebo	-0,043	-1,064 to 0.978	>0.05
One week	Lactobacilli test vs lactobacilli placebo	0,221	-0,814 to 1,256	>0.05
Three weeks	Lactobacilli test vs lactobacilli placebo	0,204	-0,790 to 1,199	>0.05

Colonisation and effect of probiotics on cariogenic bacteria based on the dental biofilm in orthodontic subjects (Study II)

The two *Lactobacillus reuteri* strains, DSM 17938 and ATCC PTA 5289, were both identified in the dental biofilm in the test group at one week and at the three-week follow-up, with an increased value relative to baseline ($p<0.05$), according to the qPCR analysis (Table 12).

Table 12. Relative quantification of investigated bacteria species in the dental biofilm using qPCR gene expression analysis. ($p<0.05$).

p-value in relation to baseline		**Week 1**	**Week 3**
		Whole mouth	**Whole mouth**
***Lactobacillus reuteri* DSM/total lactobacilli**	**Test**	p<0.01 (up)	p<0.01 (up)
	Placebo	Too few samples	Too few samples
***Lactobacillus reuteri* ATCC PTA/total lactobacilli**	**Test**	p<0.05 (up)	p<0.01 (up)
	Placebo	Too few samples	Too few samples

However, in relation to the overall bacterial counts in the test and placebo groups, neither strains had any effect on the total lactobacilli or total streptococci in the biofilm during the intervention period.

4.2 Interference capability of lactobacilli and genetic response in *S. mutans*

Interference capability of endogenous lactobacilli on number of streptococcal groups (Study I, Part II, A)

When cultured in agar enriched with higher glucose (0.5%), host lactobacilli showed better interference capabilities than when cultured in agar loaded with lower glucose (0.25%). As a result, the 0.5% concentration was used for further analyses. The obtained findings were recalculated to demonstrate the change

in interference in relation to baseline (for endogenous lactobacilli) and control (for probiotics) (Figs. 12 and 13).

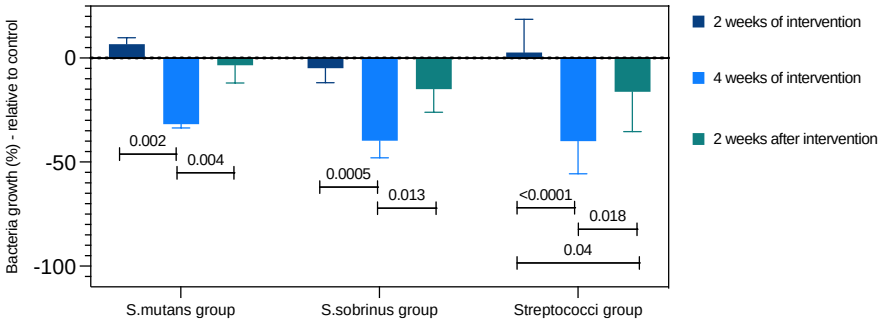

Figure 12. Endogenous lactobacilli interference in groups of oral streptococci (MV, SD) during and after probiotic intervention in relation to baseline. Significant differences between groups are marked with the p-value. (p<0.05).

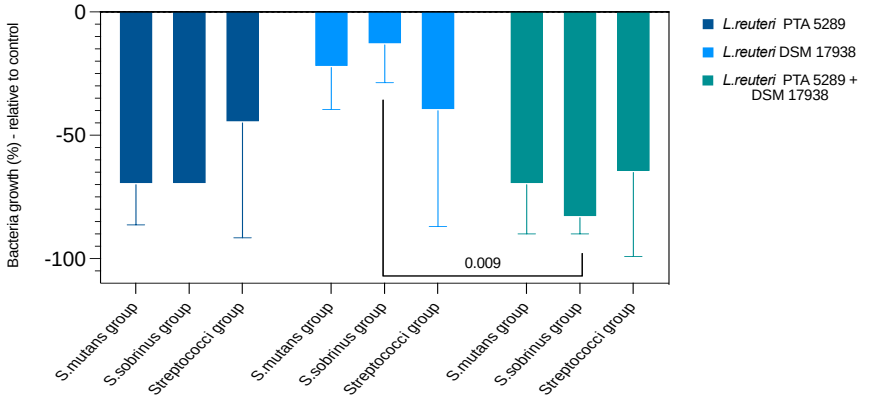

Figure 13. Probiotics interference in groups of oral streptococci (MV, SD) in relation to control. The significant difference between groups is marked with the p-value. (p<0.05).

After two weeks of probiotic intervention, endogenous lactobacilli strains promoted the development growth of *S. mutans* (4.5-11.1%) and streptococci (1.7-12.7%), while inhibiting the growth of all tested strains from 30% to 70% after four weeks. A significant difference in interference between each visit was discovered using a two-way ANOVA test ($p < 0.05$). During the follow up period, the inhibitory effect was decreased, ranging from 45% inhibition to 6% promotion.

Clinical and laboratory strains were found to behave in a similar way. The *S. mutans* group was more resistant to lactobacilli metabolites than the *S. sobrinus* group. Out of all the oral streptococci examined, the *S. salivarius* strain proved to be the most susceptible.

The probiotic drops inhibited streptococcal growth by 20% (*S. gordonii*) to 100% (*S. mutans, S. salivarius, S. sanguis*). The interference capability of *L. reuteri* ATCC PTA 5289 was higher than that of *L. reuteri* DSM 17938.

Phosphate-buffered saline filtrate test (Study 1, Part II, A)

PBS filtrate with active metabolites was obtained from the probiotic drops and lactobacilli strains from a patient with the most distinct inhibition-change pattern. The growth of oral streptococci was monitored by establishing bacterium concentrations (CFU/ml) after two, four, eigh, and 24 hours of incubation (Fig. 14).

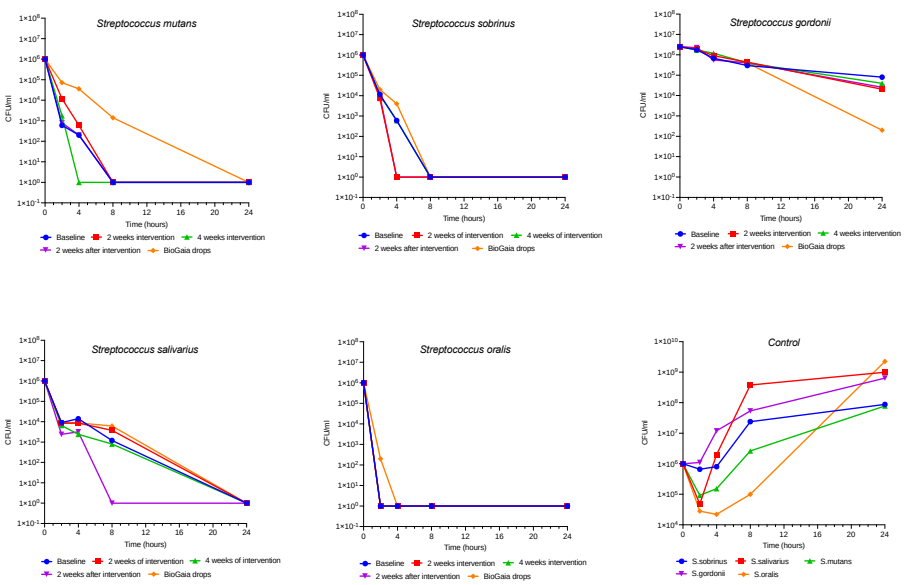

Figure 14. Growth inhibition of tested bacterial strains with PBS filtrates from endogenous lactobacilli collected at different intervention times and probiotics drops.

The test demonstrated that *S. oralis* was the most susceptible to probiotic metabolites, where no growth was seen after two hours, and *S. gordonii* was the most resistant.

Gene expression analysis (Study 1, Part II, B)

After each treatment, the expression of all the examined genes differed between caries-active and caries-free *S. mutans* isolates. Metabolites from both *L. reuteri* strains showed a significant reduction (p=0.02-0.07) in *gtfB* and *gtfC* expression, indicating less stable biofilm formation. Endogenous lactobacillus and metabolites from test probiotic strains had a smaller effect on glucotransferase expression (p=0.15-0.92). In the caries-active group, the probiotic-metabolite treatment resulted in a higher stress response (*vicR*) (p=1.29-2.08), implying greater bacterial adaptability than in the caries-free group (p=0.10-0.68). Acidogenicity (*ldh*) was downregulated in all cases, although acidurity (*atpD*) was upregulated (p=1.93) in an active-caries group by probiotic PBS filtrate (Fig. 15)

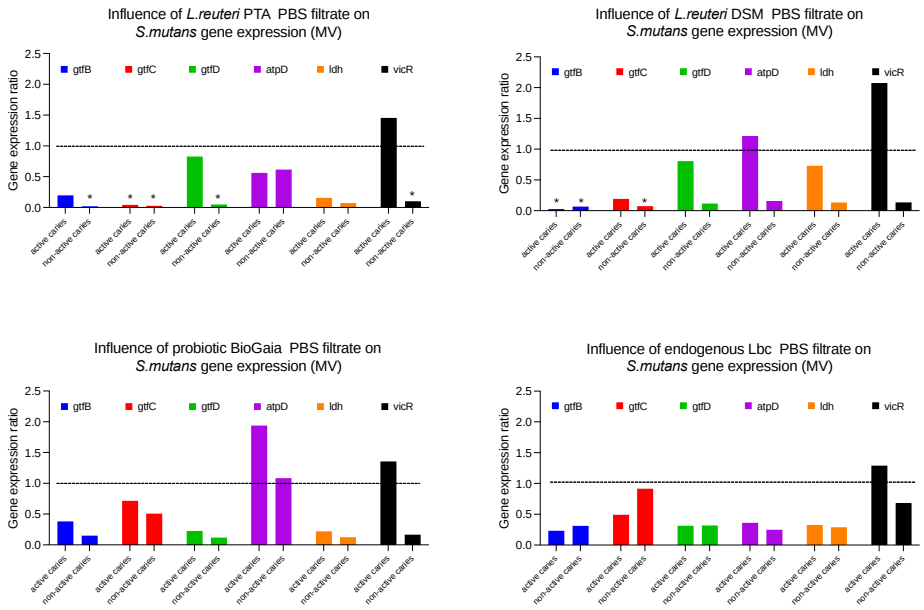

*Figure 15. Effect of PBS filtrate on the gene expression of S. mutans isolates. Gene expression was quantified by qRT-PCR with 16S ribosomal RNA as the internal control. The results represent the mean of two independent experiments performed in duplicate. * p < 0.05, ** p< 0.01, significantly different from the control group, indicated by a broken line.*

4.3 Plaque acidogenicity

Changes in plaque acidogenicity after probiotics intervention in orthodontic subjects (Study II)

The plaque-pH values of the test and placebo group at baseline, one week and three weeks post intervention are illustrated (Fig. 16). There was no statistically significant difference in plaque pH between the test and placebo groups at baseline ($p=0.1$) A statistically significant increase in plaque pH was observed in the test group between baseline and the one-week follow-up, as well as between baseline and the end of the intervention ($p<0.05$). The placebo group, on the other hand, showed no statistically significant difference at any of the time points.

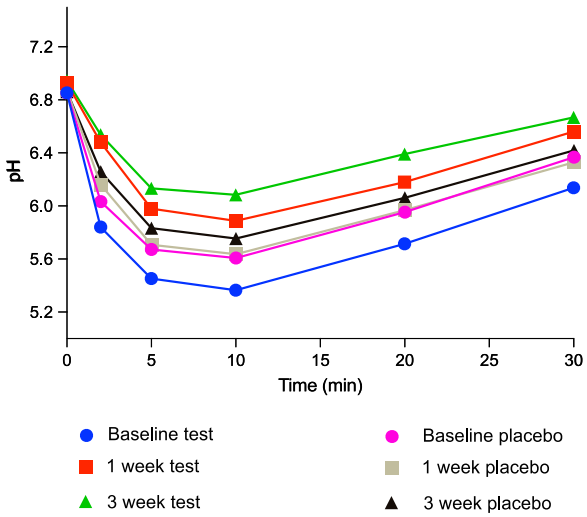

Figure 16. The figure presents plaque-pH curves obtained at baseline, one week and three weeks' use of probiotics for the test and placebo groups.

Results for pH changes in the dental biofilm are presented (Table 13). Based on the baseline pH value at all three time points, the findings showed a statistically insignificant difference between the test and placebo groups ($p>0.05$). However, only the maximum pH fall was found to be significant at baseline ($p<0.05$).

At baseline and one week post intervention, minor insignificant changes in the mean minimum pH value were observed, but, three weeks post intervention, the value began to differ significantly between the two groups ($p<0.05$).

Moreover, the differences in $AUC_{7.0}$ values between the test and placebo groups were significant at both baseline and one week post intervention. It is worth noting that the greatest difference between the two groups was observed at the end of the intervention period ($p<0.01$).

Table 13. Detailed information regarding the pH measurements at baseline, one week and three weeks. The star symbol indicates statistically significance differences.

	Test	**Placebo**	**t-test**
	Mean (±SD)	Mean (±SD)	p value
Baseline			
Baseline	6.85 (± 0.37)	6.85 (± 0.28)	>0.999
Max pH drop	1.60 (± 0.3)	1.35 (± 0.32)	0.047*
Final pH	6.14 (± 0.44)	6.37 (± 0.5)	0.217
Min- max	4.70 - 7.00	4.55 - 7.00	
Mean min pH	5.2 (± 0.33)	5.4 (± 0.39)	0.105
$AUC_{7.0}$	38.6 (± 4.6)	32.0 (± 4.79)	0.001*
1 week			
Baseline	6.93 (±0.15)	6.87 (±0.26)	0.474
Max pH drop	1.14 (±0.5)	1.37 (±0.29)	0.152
Final pH	6.56 (±0.43)	6.33 (±0.37)	0.147
Min- max	5.15-7.00	4.70-7.00	
Mean min pH	5.7 (±0.55)	5.5 (±0.34)	0.153
$AUC_{7.0}$	24.2 (±5.4)	31.3 (±4.2)	0.0007 *
3 weeks			
Baseline	6.95 (±0.13)	6.86 (±0.23)	0.227
Max pH drop	1.02 (±0.31)	1.26 (±0.42)	0.106
Final pH	6.67 (±0.34)	6.42 (±0.35)	0.072
Min- max	5.45-7.00	4.80-7.00	
Mean min pH	5.9 (±0.35)	5.5 (±0.41)	0.038 *
$AUC_{7.0}$	19.3 (±4.4)	28.3 (±4.6)	0.02 *

4.4 Compliance and drop-out rate

For Study I, all 13 subjects participated on all sampling occasions and compliance was classified by the app as good for 98.1 % of all subjects. For study II, 27 participants successfully completed the trial period and, with only one drop-out subject in the test group registered after baseline, the compliance

was recorded as high (99 %). No negative side-effects were identified in any of the studies during the trial period.

4.5 Systematic review – best administrative mode and dose-response effect (Study III)

Study selection

Figure 17 shows the study selection. After screening databases, 488 records were found, with 366 remaining after duplicates were removed. Following title and abstract screening, 260 papers were excluded due to exclusion criteria. A total of 106 articles, plus three that were found through a manual search, were screened for full text and eligibility. Out of those 109, 72 records were excluded due to either a comparison group, or an age limit, or a different outcome. As a result, finally 38 papers were finally considered for this review.

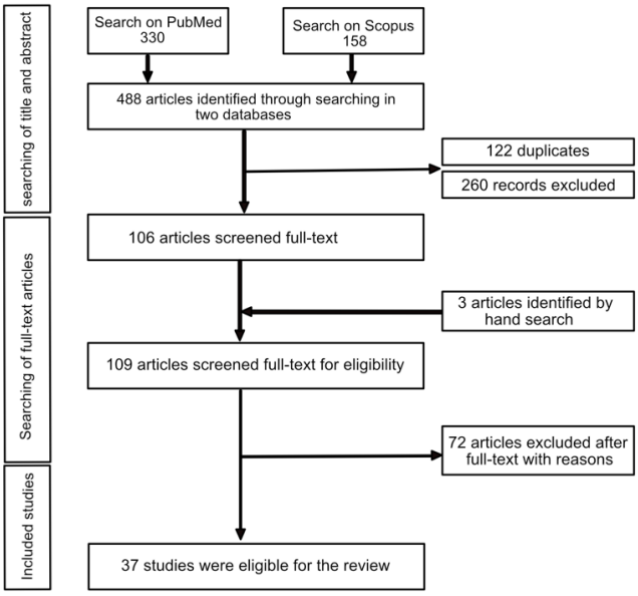

Figure 17. Flow chart of study selection for the systematic review conducted in Study III.

Characteristics of included studies

All of the analysed studies were randomised controlled trials examining the role of probiotics using a parallel design in which the test group was compared with either a placebo or a control group. It included both subjects undergoing orthodontic treatment and non-orthodontic subjects. The age range varied, with the majority focusing on younger individuals (18 to 37 yrs), one study looking at root caries in elderly patients (Petersson et al., 2011) and three studies with a wider age range (Suzuki et al., 2012; Vestman et al., 2015; Ferrer et al., 2020).

The clinical parameters used were microbial changes in saliva (87%) or dental plaque (18%), or biofilm acidogenicity (8%). Both a chair-side kit and a cultural analysis were commonly used and PCR was only used in eight studies (Sinkiewicz et al., 2010; Suzuki et al., 2012; Jose et al., 2013; Vestman et al., 2013; Vestman et al., 2015 Pahumunto et al., 2019 & 2020; Ferrer et al., 2020). Various lactobacilli strains were used in 15 studies, Bifidobacterium strains in seven and *Streptococcus dentisani* in one study (Ferrer et al., 2020). In 39% of the studies, there was a combination of strains from the same genus or two different genera.

Two studies did not mention the active micro-organism (Jose et al., 2013; Karuppaiah et al., 2013), and eight did not define the probiotic micro-organism concentration (Jose et al., 2013; Karuppaiah et al., 2013; Pinto et al., 2014; Bhalla et al., 2015; Youssuf et al., 2015; Srivastava et al., 2016; Alp et al., 2018; Pahumunto et al., 2019).

Risk of bias assessment

The risk of bias was assessed using Cochrane's collaboration tool. Of the thirty-eight studies analysed, seven articles were assessed as having a low risk of bias (Çaglar et al., 2009a; Chuang et al., 2011; Teanpaisan et al., 2014; Vestman, 2015; Yousuf et al., 2015; Srivastava et al., 2016; Zare Javid et al., 2020), the vast majority were judged as moderate, and a high risk of bias was found in nine papers (Petersson et al., 2011; Juneja et al., 2012; Mortazavi et al., 2012; Chinnappa et al., 2013; Bhalla et al., 2015; Jothika et al., 2015; Toiviainen et al., 2015; Gizani et al., 2016; Ferrer et al., 2020a).

Mode/vehicle for probiotic administration in non-orthodontic subjects

Thirty-three studies were identified using non-orthodontic subjects. Each one looked at the effect of probiotics on oral health in terms of changes in salivary and plaque microbial counts, plaque acidogenicity and plaque amount.

The most commonly used probiotic delivery methods were milk and lozenges. Other vehicles included dairy products such as yogurt, cheese, milk, ice cream and curd, as well as media such as tablets, chewing gum, powder and lozenges

Of the seven studies using milk in order to deliver probiotics (Lexner et al., 2010; Petersson et al., 2011; Juneja et al., 2012; Teanpaisan et al., 2014 & 2015; Pahumunto et al., 2019 & 2020), five presented a significant reduction in salivary *S. mutans* counts (Juneja et al., 2012; Teanpaisan et al., 2014 & 2015; Pahumunto et al., 2019 & 2020), with four showing a significant increase in lactobacilli. No effect on reducing the microbial count in saliva was found in two studies (Lexner et al., 2010; Petersson et al., 2011). Lozenges, being the second most common vehicle, reported a significant reduction in *S. mutans* counts in saliva for only two studies (Çaglar et al., 2008) on agar plating and (Vestman et al., 2013) based on PCR analysis. One study in which probiotic lozenges had been used by 60 adults for one month reported a significant reduction in the plaque index (Toiviainen et al., 2015).

Ice cream and/or curd were used in seven studies, with positive findings in terms of their ability to reduce *S. mutans* counts in saliva after short term of administration (Çaglar et al., 2008; Singh et al., 2011; Chinnappa et al., 2013; Karuppaiah et al., 2013; Bhalla et al., 2015; Nagarajappa et al., 2015; Srivastava et al., 2016). A significant increase in salivary pH was found after using *Lactobacillus acidophilus* provided by curd for seven days (Srivastava et al. (2016). While cheese revealed no significant changes in salivary microbial counts when used in two studies (Ahola et al., 2002; Mortazavi et al., 2012), positive outcomes were reported for yoghurt as a vehicle for administration (Çaglar et al., 2009; Zare Javid et al., 2020). Conflicting results were found for probiotic mouthwash, powder sachet, straw, gel tray, liquid and capsule, chewing gum, and oil drops, all of which were used infrequently.

Appropriate dose of probiotics in non-orthodontic subjects

No standard recommendations for the optimal dose could be identified. Various dosages had been used and the majority of the included studies (54%) instructed participants to take the probiotic once daily. Fifteen studies found a significant reduction in *S. mutans* counts in either saliva or plaque, while two studies found no changes in *S. mutans* or lactobacilli levels when the probiotic was administered once daily (Lexner et al., 2010; Petersson et al., 2011). Moreover, the use of a probiotics curd once a day showed a significant reduction in the amount of plaque (Karuppaiah et al., 2013).

Two daily dosages were also identified in 21% of the studies, with three articles reporting a significant reduction in *S. mutans* counts (Juneja et al., 2012; Vestman et al., 2013; Jothika et al., 2015), three articles showing no changes (Sinkiewicz et al., 2010; Keller et al., 2012a; Mortazavi et al., 2012), and one study finding a significant increase in lactobacilli counts (Vestman et al., 2015). Three daily dosages were reported, in which only one study showed a significant reduction in mutans streptococci counts after three weeks of use (Çaglar et al., 2007). No significant effect on the level of bacterial counts was found for the studies recommending a daily dosage of four or five times (Ahola et al., 2002; Toiviainen et al., 2015). Only one study did not specify how many times a day the participants were supposed to take the probiotic (Montalto et al., 2004). A probiotic gel applied every second day to the buccal surface with a dental splint was found significantly to reduce the amount of plaque and improve salivary flow (Ferrer et al., 2020a).

Appropriate dose, mode/vehicle for probiotic administration in orthodontic subjects

Only five studies, using four different vehicles and evaluating the effects of probiotics on microbial counts in subjects wearing orthodontic appliances, were found to be eligible for this review. Four of these studies identified significant microbiological changes. The use of kefir and toothpaste twice a day for six weeks resulted in a significant reduction in salivary *S. mutans* and lactobacilli levels (Alp et al., 2018). It was also found that using either toothpaste or curd once a day over a short period of time significantly reduced the level of *S. mutans* in the plaque (Jose et al., 2013). The level of mutans streptococci in saliva was significantly reduced following the daily application of yoghurt for one study (Cildir et al., 2009), while the other study found no significant changes based on cultural analysis (Pinto et al., 2014). No

significant effect was found on the level of *S. mutans* or on the level of lactobacilli after 17 months of using a lozenge every day (Gizani et al., 2015).

5 DISCUSSION

The current thesis looked at a novel strategy for delivery of probiotics in the form of drops and its survival in the oral cavity based on saliva and plaque samples after a short period of administration, as well as the ability of endogenous lactobacilli to interact with a community of streptococci after probiotic intervention. Furthermore, the impact of probiotics on caries-related variables, including salivary microbial counts, cariogenic bacteria in plaque, and pH levels in subjects with and without orthodontic appliances, was also evaluated. In addition, a systematic review was conducted to determine the superiority of the vehicle and the optimal dosage in terms of the oral effect of probiotics.

5.1 Colonisation of probiotics strains in the saliva and in the dental biofilm after short-term use

To date, there are a limited number of studies of the oral colonisation of probiotics and the issue requires further investigation. Therefore, it was considered important to determine whether the two Lactobacillus reuteri strains will colonise the saliva and/or the dental biofilm after a few weeks of administration in a group of young adult volunteers based on qPCR analysis.

The key finding from the qPCR analyses was that both the *L. reuteri* DSM 17938 and ATCC PTA 5289 strains had the potential to be introduced into the saliva and oral biofilm starting from the second week of intervention when using probiotic drops as a mouth rinse. It is worth mentioning that a few participants harboured the two strains at the start of the analysis. They all claimed, however, that they had not used any probiotic products in the four weeks prior to the baseline samples. The presence of the two strains at baseline can be attributed to the fact that those strains could be found genetically or they may exist naturally in certain food products (Giraffa et al., 2010; Oh et al., 2010; Duar et al., 2017).

A downturn was noted after ending the administration period, in which only four and two individuals were positive for the DSM 17938 and ATCC PTA 5289 strains, respectively, as identified via saliva samples taken after five weeks of follow-up. Previous studies have found that *Lactobacillus rhamnosus* GG (LGG) is able to colonise in the oral cavity between one to five days after taking a product containing this bacterium for a short time (Yli-Knuuttila et al., 2006; Meurman et al., 2008). Additionally, it has previously been demonstrated

that no lactobacilli were present in saliva samples taken from volunteers one week after they consumed a bio-yoghurt containing two *lactobacilli* strains and a *Bifidobacterium bifidum* strain (Busscher et al., 1999). The present results were also in consistent with previous research work by Caglar et al. (2009b) in which the number of *L. reuteri* carriers steadily decreased and, after one week, only 8% of the subjects carried the bacterium. The pattern and the persistence of the two strains administered in the present study were found to vary among individuals based on the samples that were taken which could be referred to inter-individual variations such as biofilm composition, pH and sensitivity to oxygen exposure.

In Study II, the results showed that the two *Lactobacillus reuteri* strains, DSM 17938 and ATCC PTA 5289, were both significantly detected for the test group at one week and at three weeks based on the qPCR analysis of the dental biofilm in comparison to the placebo group. This aspect in orthodontic subjects has not been explored and further long-term studies are needed. The qPCR technique was uniquely used in the two studies. As previously demonstrated, it has been shown superiority of the PCR technology concerning its sensitivity and specificity in comparison to the traditional cultural plating and the chair-side kit (Bustin et al., 2009).

For oral probiotics, colonisation, or transient colonisation, is a critical issue in the sense of the long-term effect. Taking probiotics at an early age could improve its colonisation and survival in the oral cavity. Yli-Knuuttila et al. (2006) has reported that, despite discontinuing the use of LGG-containing products, one of the subjects who had received LGG milk as a supportive treatment for atopic dermatitis for a year at the age of 10 was still LGG positive in saliva after nine years. This indicates that permanent colonisation could have occurred during childhood. A recent systematic review has found a small but statistically significant effect by adding probiotics early in life for the prevention of early childhood caries (Twetman and Jørgensen, 2021). For this reason, further trials studying colonisation are still necessary with larger materials and in various patient groups and ages. The current situation also suggests that consistent *L. reuteri* uptake is needed to achieve a beneficial effect.

5.2 Effect of probiotics on cariogenic bacteria from saliva and plaque samples

In Study 1, saliva samples were taken to investigate the effect of probiotics on the cariogenic bacteria, namely *S. mutans* and lactobacilli, using selective agar media on a group of 22-29 years old subjects. The results showed that, during the intervention phase, salivary levels of *S. mutans* were found to be significantly reduced after a four-week intervention, accompanied by an increase during the follow-up period. On the other hand, the level of lactobacilli in saliva increased at the end of the intervention, followed by a reduction in log CFU/ml during the post-rinsing period. Using probiotics drops appears promising when it comes to reducing the number of pathogenic bacteria. However, the effect was temporary, indicating the necessity for the long-term use in order to achieve the desired oral effect.

The present findings were in line with those in previous studies. Zare Javid et al. (2020) showed that, in 66 subjects in the age group between 18-30, a probiotic yogurt was found to reduce the number of salivary *S. mutans* significantly after two weeks of intervention. Chinnappa et al. (2013) have demonstrated a similar statistically significant reduction in mutans streptococci after seven days of probiotics curd and ice cream administration. On the other hand, Petersson et al. (2011) and Toiviainen et al (2015) found no effect of probiotic milk and lozenges respectively on the microbial counts of the tested subjects.

In agreement with the current findings, in a randomised, double-blinded clinical trial, Montalto et al. (2004) found an increase in the salivary lactobacilli in a number of healthy subjects after forty-five days of probiotic capsule and liquid administration (Montalto et al., 2004). These findings indicate the need to monitor the subjects, especially those with active carious lesions.

A systematic review by Cagetti and coworkers (2013) found that, in two-thirds of the involved studies, probiotics have the ability to minimise MS counts in saliva and/or plaque in a short-term perspective, while the impact of probiotics on the development of carious lesions appears promising. However, the authors concluded that there are insufficient RCTs on this subject to provide scientific clinical evidence.

The present study includes only one group of subjects. Inclusion of a control group would have been of value in order to answer the research question related to the effect of probiotics on the cariogenic bacteria. However, the main

outcome in this study was the colonisation of the two strains after short administration. Moreover, the level of the bacteria was examined as a pilot for future trials. It is important to bear in mind that administration was carried out via drops. To our knowledge, this mode of delivery for probiotics has previously only been explored in two studies on children (Cildir et al., 2012; Tehrani et al., 2016). It is impossible to tell whether any other vehicle would have given a different result. Individuals were told to rinse with the present product, which is believed favourable in comparison to many other modes of administration, as a solution is easily distributed in the oral cavity.

The literature review showed the potential of probiotics to reduce the colony-forming units (CFU) counts of oral pathogens, so using probiotics could be helpful for maintaining oral health. To validate their effectiveness in caries prevention, further randomised clinical trials with long-term follow-up are required.

Three outcomes of probiotic drops were investigated in orthodontic patients. They were the effect on plaque pH, salivary microbial counts, and colonisation of the probiotics strains in the dental biofilm. Micro-organism accumulation in the oral cavity is thought to be facilitated by fixed orthodontic appliances (Sukontapatipark et al., 2001). Carious lesions are more likely to form around brackets bonded to the labial surface of teeth and, in addition, bands, wires, and other auxiliary apparatuses that impede the administration of standard hygiene measures, allowing dental biofilm to accumulate around the base of the brackets and associated appliances (Tukkahraman et al., 2005). Enamel demineralisation around brackets is fairly rapid process in the presence of fermentable carbohydrates, and the incidence of new enamel lesions in orthodontic patients is fairly high (Derks et al., 2004). Different strategies should therefore be applied during the course of orthodontic treatment in order to reduce the risk of caries to developing.

The present study found no significant changes in both groups at the level of *S. mutans* and lactobacilli in the saliva, but the level of cariogenic bacteria was found to be more stable in the test group during the intervention in comparison to the placebo group, which is contrary to the typical oral environment in orthodontic subjects, where the level of cariogenic bacteria was found to increase (Jing et al., 2019). Pinto et al., (2014) concluded that there were no changes in the microbial counts after two weeks of probiotics yogurt administration in 26 young adults undergoing orthodontic treatment. On the other hand, probiotics curd and toothpaste were found to reduce the level of *S. mutans* after 30 days in 60 subjects (Jose et al., 2013). The opposite results

could be justified by the duration of the intervention which indicates the need for a longer duration to obtain the desired oral effect.

The participants in the present study were enrolled after eight months after bonding, as bonding may increase the number of cariogenic bacteria to a peak level after this time period (Jing et al., 2019). Another possibility could be to start the intervention at baseline, before bonding and to follow the subjects until after debonding.

In addition, the current work showed no significant impact of using probiotics on the level of cariogenic bacteria in the plaque sample in orthodontic subjects. Only a few studies were identified in the literature. In one study, the author concluded that there was no effect on the level of bacteria in the dental plaque after two weeks after the delivery of probiotics through a dairy product in a cross-over design in 26 subjects (Pinto et al., 2014). On the other hand, a study by Jose et al. (2013) found a significant reduction after 30 days based on the PCR analysis, which could be as a result of different study design, treatment duration, vehicle for administration, and the active strain.

Saliva and plaque samples were both used to assess colonisation and the level of cariogenic bacteria, and, contrary to previous work where one sample mostly taken, they showed differences in the bacterial composition. It has been demonstrated that saliva and supragingival plaque have variations in term of microbial diversity and composition. (Segata et al., 2012; Xu et al., 2015). Shi et al. (2018) showed that saliva harboured a less even and less diverse community than plaque. However, since the supragingival biofilm is the primary habitat of cariogenic bacteria as a result of being closer to the tooth surface where demineralisation occurs, some researchers have argued that utilising salivary microbiota as a surrogate for supragingival microbiota may not produce meaningful conclusions in dental caries research. However, because the supragingival environment is constantly bathed in saliva, which contains bacteria shed from tooth surfaces, salivary microbiota could be used to monitor supragingival microbiota (Yang et al., 2012; Belstrøm et al., 2017). Saliva collection has advantages of being non-invasive, easy to perform and store, safe to handle and inexpensive, as well as containing high-quality DNA (Gura et al., 2008; Zhang et al., 2016).

5.3 Probiotics effect on plaque acidogenicity in orthodontic subjects

Plaque acidogenicity, when evaluated in terms of the area under the curve at a critical pH of 7 and the mean minimum pH, was found to be significantly reduced in the test group in comparison to the placebo group after three weeks' intervention. Although both groups were matched at the baseline, however, there was a significant difference in the maximum pH drop and the area under the curve, which could be due to individual differences. It is worth noting that the same pattern for both parameters can be seen throughout the intervention period, with the probiotic group showing more effect in comparison to the placebo group.

Possibilities to modify the dental biofilm are important as the dental biofilm plays a central role in the caries process. As a result, according to the ecological plaque-hypothesis (Marsh et al., 1999), a fall in plaque pH will cause the acidogenic bacteria to activate, leading potentially to tooth demineralisation. Furthermore, Marsh et al. (2010) have shown that raising the pH level of the biofilm is important when it comes to interrupting the caries process.

It is worth noting that none of the previous research works on probiotics has examined the changes in the pH level in a group of subjects undergoing orthodontic treatment. Only three studies on non-orthodontic subjects examining the changes in pH level after intervention with probiotics were found (Keller et al., 2012b; Suzuki et al., 2012; Srivastava et al., 2016). Contrasting results, which could be attributed to the length of the intervention period and the mode of administration, were found. Srivastava and coworkers (2016) found a statistically significant increase in the salivary pH using a probiotic curd once a day in young adult subjects after seven days of administration. On the other hand, a study by Suzuki et al. (2012) found no significant changes in the pH level when using oil drops as a vehicle for probiotic administration.

The explanation for the observed positive changes in biofilm acidogenicity could be attributed to various mechanisms, such as normalisation of the oral microbiota, metabolic activities and the modulation of the immune response (Haukioja et al., 2010). The two first strategies are anticipated to play a key role in pH adjustment.

Subjects undergoing treatment with fixed orthodontic appliances should be regarded as high-risk subjects. These positive findings encourage long term trials in order to investigate the effect of probiotics in this particular patient

group and further evaluate whether they can be used as a preventive strategy in this and other high-risk groups.

5.4 Endogenous lactobacilli, interference capability and a genetic response in *S. mutans* after probiotic administration

The strength of the used method - the semi-quantitative agar overlay technique – made it possible to investigate the potential inhibition of the endogenous lactobacilli against more than 20 streptococci strains at the same time. Lactobacilli produces and releases metabolites that permeate through the agar and inhibit pathogen growth, without any surface contact between streptococci and the probiotics. The most interesting observation was that endogenous lactobacilli strains showed a statistically significant interference capacity after four weeks' treatment with probiotics, reaching almost 50% of the interference capability of a commercial probiotic product. However, after the intervention was stopped, the impact gradually faded. The present study evaluated the behaviour of endogenous lactobacilli from caries-free individuals. The current data corresponds well with previous work by Simark-Mattsson et al. (2007) in which host Lactobacilli of caries-free subjects have been found to inhibit the growth of mutans streptococci better compared to caries-active individuals. On the other hand, the agar-overlay test showed that probiotics administration reduced the growth of commensal streptococci by up to 65%. So, while using probiotics, the method of encouraging the growth of beneficial bacteria while suppressing possibly pathogenic bacteria appears to be the most ideal.

The RT-qPCR technique was used to assess the genetic response of twenty clinical *S. mutans* isolates to probiotic metabolites, with the emphasis on genes involved in glucan synthesis, acidurity, and acidogenicity regulation. When studying the genetic response of *S. mutans* to various products, one strain type is commonly investigated (He et al., 2019; Lai et al., 2021). It is worth noting that around 52 distinct genotypes of *S. mutans* have been reported and found in saliva and dental biofilms (Bedoya-Correa et al., 2019). This line of research could lead to an improved knowledge of different probiotic mechanisms of action.

The majority of studies have up to today used either lactobacilli or bifidobacteria-derived probiotics. It is worth noting that, Haukioja et al. (2006) tested the colonisation ability of various commercially available probiotics including strains of *Lactobacillus* and *Bifidobacterium* collected from the dairy

industry in an *in vitro* study based on the survival of bacteria in saliva and their adhesion to oral surfaces. The study concluded that lactobacilli had better colonisation features than bifidobacteria. However, whether one strain or two bacterial strains in a product is more preferable in terms of oral impact has to be addressed. So far, the combination of two strains in comparison to a single strain has only been found to enhance the probiotic effect in a synergistic way in relation to GIT application (Juntunen et al., 2001). It is believed that also in relation to oral conditions, due to the great variations in the ecological ecosystem among individuals, a combination of strains would be of advantage. The present data on probiotic interference on oral streptococci indicate a stronger effect when *L. reuteri* DSM 17938 and *L. reuteri* ATCC PTA 5289 were combined compared to individual usage.

5.5. Appropriate vehicle and optimal dose for probiotics administration on the oral health.

The literature search aimed to identify the best medium for probiotic administration, as well as the optimal dose in terms of oral effect considering caries-related variables, it revealed a growing number of studies in the field of probiotics and their effect on oral health, the majority of which were published in the last decade. The findings revealed that dairy products, such as milk, cheese, and yogurt, are the most commonly used vehicles for probiotics administration. The bacteria are found naturally in these products, while they are added or incorporated in other modes of administration such as lozenges, oil drops, chewing gum, and tablets.

The method by which the probiotic is administrated is thought to play an important role in its the cariogenic potential (Teughels et al., 2008). It is important to consider the overall composition of the product. Correspondingly, the buffer capacity of milk has been found to reduce the production of acid when lactic-producing bacteria are ingested in milk products (Gadalia et al., 1991). Furthermore, it is important to consider the total composition of a product in relation to the oral health, for example no sugar should be included.

According to the findings in this systematic review, 77% of probiotics consumed via dairy products can significantly reduce salivary or plaque mutans streptococci levels. However, in some studies, an increasing level of lactobacilli was observed, indicating the need closely to track those subjects consuming probiotics over time. However, if this is the result of an increase in number of probiotic strains, it should not be of concern.

On the other hand, the short-term use of probiotics delivered through nondairy products such as lozenges, chewing gum, gel, or mouthrinse, resulted in a 60% reduction in microbial counts. Sucking on a *L. reuteri* ATCC 55730 tablet once daily for three weeks significantly reduces the growth of cariogenic bacteria in the mouth, and this effect appears to be linked to direct interaction between the tablet and the oral biofilm (Çaglar et al., 2006).

A probiotic micro-organism must bind to the oral surfaces and become part of the oral biofilm in order to have positive effects in the mouth. Additional factors, such as the level of cooperation and the optimal dose for probiotics administration, should also be given a great consideration.

The optimal dose is another important aspect when considering probiotics. The definition given by the FAO/WHO is fairly ambiguous, since the required dose is not justified (WHO, 2002). There appears to be no consensus on the optimal dosage of probiotics to use for oral health, and there is a considerable variation in the included studies. Different doses have been applied, with the majority of the studies using 10^8 dose colony-forming units. However, at the end of the trial period, half of those studies showed no noticeable effects. A higher dose was found in two studies (Juneja at al., 2012; Ferrer et al., 2020a), but one of those trials revealed no significant effect at the end of the intervention. Today, a variety in the number of bacterial strains and the total number of bacteria is found in different products. For this reason, a pilot study is recommended to be conducted prior to any clinical trial to ensure the number of bacteria living in the probiotic and to ensure their stability during manufacture and storage (Lee et al., 1995; Salminen et al., 1998; Tuomola et al., 2001).

Controversy exists regarding whether multiple doses are to be preferred in comparison to a single dose in order to obtain the strongest oral effect. There is limited information on this aspect. Only one study has been performed in which the benefit of a multi-dose over a single dose resulting in increasing salivary pH was found (Ferrer et al. 2020b). However, clear recommendations cannot be given and further studies are needed to answer this research question.

To date, the use of drops as a vehicle for the administration of probiotics is regarded as a novel strategy. As previously mentioned, only a limited number of studies have evaluated this way of administration (Cildir et al., 2012; Tehrani et al., 2016). The theory behind this is that the drops could be easily accessible around the complicated design of the fixed appliances and, as a result, the adhesion could improve. Drops may be beneficial also for other target groups such as dry mouth subjects.

Probiotics represent an interesting strategy for use in caries prevention, where the positive results dominate. This will hopefully encourage further researchers to investigate this field from many angles and answer questions comparing it to other oral health-prevention strategies.

5.6 Limitations in previous research work.

With respect to dental caries, most of the previous work has been done in a relatively small study groups, with short clinical trials and disregarding power analysis. This makes it somewhat difficult to draw conclusions and even more difficult to recommend the probiotics as a future alternative preventive strategy.

In addition, most studies have evaluated one or two variables known to be related to the caries disease. Bearing in mind that the risk of dental caries is influenced by a large number of factors, the long-term impact of a single factor can be hard to base conclusion on. Moreover, risk assessment using the Cochrane Collaboration tool's revealed drawbacks in many studies, as randomisation, allocation concealment, blinding, incomplete outcome data, selective reporting and other bias had not been considered. Future well-designed randomised clinical trials are needed in order to make suggestion which then can be based on a solid scientific foundation.

5.7 METHODOLOGICAL CONSIDERATION

Design of Studies I and II

For Study I, the design of the trial consisted of a four-week intervention and a five-week follow-up period to examine both the change in bacterial level and the colonisation based on the saliva and plaque samples. The results showed that a few participants were still positive for the probiotics strains at the end of the follow-up. It would be more appropriate to extend taking samples until negative colonisation was recorded for all subjects in order to obtain a clearer picture. Nevertheless, the present study was unique in the light of the trial duration, as very few studies have addressed the same research question and the majority have been short-term studies.

For Study II, one of the drawbacks was that it was a short-term clinical study with only three weeks of intervention. It would have been more clinically relevant if the study had been extended.

Exclusion criteria

The criteria for exclusion in our clinical trial included cleft lip and palate syndrome, handicapped patients, people with systemic disorders or illnesses that might interfere with the study, smokers, and a history of probiotics, anti-inflammatory medications, or antimicrobial substances administered in the four weeks prior to the baseline test were all excluded from the study. During the study, participants were also advised to refrain from other probiotic-containing products, xylitol chewing gums, and antibiotics. The main reason was to avoid any confounders that might affect the experiment results. It has been demonstrated that xylitol chewing gum may significantly reduce the level of *S. mutans* and *S. sobrinus* in the saliva (Bahador et al., 2012) . In addition, smoking was regarded as a confounder, according to a recent systematic review, where a positive association between tobacco use and an increased risk of dental caries was reported (Jiang et al., 2019). On the other hand, one and the same toothpaste was distributed to all the subjects in order to assure that each subject within the same group or between the two groups was following the same oral hygiene regimen.

Chair-side kit vs culture analysis vs PCR technique

The results of the systematic review revealed that different laboratory tests, such as chair-side kits, cultural plating and PCR, have been used to investigate the effect of probiotics on caries variables. When comparing the chair-side test with a conventional cultural test, it was discovered that there was a substantial difference in MS regrowth (Hildebrandt et al., 2006). Furthermore, PCR tests has been shown to be a more reliable, precise, and sensitive method for bacterial analysis in comparison to cultural investigation (Bustin et al., 2009). It was therefore difficult to perform a meta-analysis.

Strip method

For Study II, the plaque-pH measurement was evaluated using the strip method. The microelectrode could also be possible for usage (Carlén et al., 2010). Each of the two methods has its advantages and disadvantages. The strip method is being inexpensive and easy to handle. For the present study, the power analysis was based on a pH fall of 0.4 and an SD of 0.35, which can compensate for such a limitation. Carlen et. al. (2010) have concluded that the strip method is an equally effective tool as the microelectrode technique in plaque-pH measurements.

Cooperation

Clinical research outcomes may be influenced by patient compliance with the intervention. To date, no method for measuring compliance is fully adequate, and some of the most commonly used methods are insufficient. Compliance should be regarded as an integral part of clinical research, especially in the long term. Diaries, telephone communication and left-over doses are examples of previously reported cooperation checks. In two clinical studies, compliance was followed using a special smartphone app called MyMedschedule^{R} Plus. It monitors the percentage of probiotic mouth rinses used during the study period using a timed reminder. This application could both act as a reminder and ensure that the drops are taken at the same regular time. Even if compliance was thought to be a matter of concern, especially for the younger age group, the app technique which was used to follow this showed good compliance.

5.8 ETHICAL CONSIDERATIONS

Both of the clinical trials (I & II) were conducted in accordance with the Helsiniki Declaration's ethical guidelines. Prior to the start of each study, participants were given detailed verbal and written information about the study, including potential threats and side-effects. Each subject willingly agreed to take part in the study and signed the consent form (I & II). Prior to the study, parents or legal guardians had signed consent forms for teenage participants (II). All information remains anonymous and no personally identifying information has been released. The pH measurements, plaque samples, and saliva samples were all taken in a clinical setting governed by health and safety regulations.

6 CONCLUSIONS

The main conclusions from this thesis are that:

- Probiotics have the potential to colonise the oral cavity during usage, but this gradually fades after the consumption period (Study 1, Parts I & II, Study II)

- Probiotics reduces the amount of *S. mutans* in the saliva and could help with caries prevention (Study I, Part I)

- Probiotics can modify the behaviour of the endogenous lactobacilli and induce a genetic response in the *S.* mutans (Study I, Part II)

- The administration of probiotics in subjects undergoing orthodontic treatment reduces the plaque acidogenicity after a short-term use (Study II)

- Further studies are needed to examine the dose-response effect of probiotics and compare different vehicles for probiotics administration in terms of oral health (Study III).

7 FUTURE PERSPECTIVES

The use of probiotic strains to prevent caries has been encouraging, even though very few studies are comparable in terms of study design and thus in clinical results. As a result, the scientific evidence still remains insufficient. Recently, existing protocols for caries prevention have been linked to some side-effects and it is therefore worth trying to establish a new strategic or an adjunctive approach, such as probiotics, that has not been linked to adverse effects. To investigate probiotics for preventive usage, appropriately designed prospective clinical trials are required. Future studies should focus on the exact mechanisms of probiotics action in long-term clinical trials.

In general, suitable oral products, the participation of a well-defined population, large sampling of the host microbiota, the use of validated research instruments in the determination of specific, clinically significant endpoints, and adequate statistical power are all desirable characteristics in clinical studies.

The consumption of probiotic-enriched dairy products appears to be an auxiliary method for influencing the microbial ecology of the oral biofilm and for use in caries prevention. Future research should concentrate on determining the best vehicle, the most efficient probiotic bacteria, the optimum probiotic concentration, and the frequency of consumption. Studies comparing different vehicles for probiotics administration are rarely existed, and it is therefore of great importance to explore whether one mode is superior to another in terms of adhesion and long term oral effects.

Moreover, further clinical researches should concentrate on evaluating the dose-response impact on various clinical variables related to oral health in order to formulate a consistent dose-prescribing guideline. It is for future studies also important to evaluate the combination of bacterial strains in combination to each individual strain. In addition, the number of intakes needs to be addressed. It is likely that a consistent, almost daily intake is required; this may be a cooperation issue to consider. Taking probiotics in an everyday preventive product, such as toothpaste, may be one method to consider for administration.

For orthodontic patients, probiotics can be a useful preventive strategy, because fixed appliances have been related to substantial biofilm formation, placing patients at a higher risk of caries (Davis et al., 2014; Chadwick, 2016). However, research focusing exclusively on the clinical impact on orthodontic patients is limited and the subject has not been highly explored, despite

encouraging and promising results. Moreover, other high risk groups for dental caries should also be considered.

This work has focused on the effect of probiotics on the caries disease. However, other areas in which probiotics could be beneficial, such as gingivitis, periodontitis, oral malodour, candida and peri-implantitis, need to be investigated in more detail.

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APPENDIX

Paper I-IV