Biofilm management with oral probiotics
in patients with fixed orthodontic appliances

Doctor of Clinical Dentistry (Orthodontics)

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Abstract
ABSTRACT

Introduction: The risk of biofilm formation in orthodontic patients is even higher than the general dental population due to the presence of microniches and increased surface area provided by orthodontic brackets and appliances. If left untreated, oral biofilm can cause white spot lesions, gingivitis and halitosis. Traditional mechanical and chemical methods of managing biofilm formation all have limitations, which warrants the search for novel ways of biofilm management. Probiotics have shown to be beneficial in the oral health of general dental patients. Therefore the aim of this study was to investigate the efficacy of the oral probiotic Streptococcus salivarius M18 in managing biofilm formation in patients wearing fixed orthodontic appliances and to assess its effects on the oral microbiome of these patients.

Methods: The study was designed as a prospective, randomised, triple-blind, two-arm parallel-group, placebo-controlled trial. Sixty-four patients undergoing fixed treatment consumed 2 lozenges daily of probiotic (n = 32) or placebo (n = 32). The outcome measures were plaque index (PI), gingival index (GI) and halitosis-causing volatile sulphur compound (VSC) levels. Oral microflora was analysed utilising next-generation sequencing of the bacterial 16S rRNA gene.

Results: No significant differences in PI and GI scores were found between probiotic group and placebo-control group (p > 0.05). The level of VSCs significantly decreased in both probiotic group (VSC reduction = -8.5%, p = 0.015) and placebo group (-6.5%, p = 0.039) after 1-month. However, after the 3-month follow-up, VSC levels of the placebo-control group returned to baseline levels whereas those of the probiotic group decreased further compared to baseline readings (-10.8%, p = 0.005). The next-generation sequencing showed that the oral ecology of both groups was similar and that there was a significant increase in the abundance of streptococci in both the probiotic and placebo group over time.

Conclusion: Oral probiotic S. salivarius M18 reduced the VSC levels in patients with fixed appliances but did not decrease their plaque or gingival indices. The influence of probiotic S. salivarius M18 on oral microflora seems to be minimal. A longer intervention and follow-up period are needed.
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Chapter 1

Probiotics and oral health: a review
ABSTRACT

Oral biofilms can cause a number of oral diseases such as dental caries, periodontitis, gingivitis, halitosis and candidosis. Mechanical and chemical methods have traditionally been used to remove oral biofilms but they have limitations. Oral probiotics have shown potential in affecting pathogens found in biofilms without impacting negatively on the normal oral microbiota and thus their application in dental patients has garnered much attention. Probiotics come in a number of strains, all of which have similar mechanisms of action exerting their effects on pathogens either directly or indirectly. Their use in dental patients has shown a good proportion of positive effects on caries, periodontitis, gingivitis, candidosis and halitosis.

The risk of biofilm formation in orthodontic patients is even higher than the general dental population due to the presence of microniches and increased surface area provided by orthodontic brackets and appliances. For this reason, the effect of probiotics on orthodontic biofilms is gaining a lot of interest. There have only been a handful of studies investigating the effect of probiotics in orthodontic patients, although the effects have been somewhat positive. However, studies to date are difficult to compare and often conducted quite poorly. There are a number of obvious gaps in the current literature that make comparing studies and drawing accurate conclusions on this subject somewhat difficult. Hence this review on probiotics and oral health concludes with suggestions of possible future directions probiotic studies should take when tested in dental populations. By doing this, it is intended that their true relevance in dentistry and orthodontics can be realised.
**Introduction**

Biofilm-related oral diseases, including dental caries and periodontal disease, have historically been considered the most important global oral health burdens (Petersen, 2008). These diseases cause pain, infections and tooth loss, severely impacting on the ability to eat, systemic health, self-esteem and quality of life in both children and adults (Rosenoer and Sheiham, 1995; Griffin et al., 2012; Jansson et al., 2014; Li et al., 2014). In developed countries, oral diseases are the fourth most expensive to treat with an estimated 5-10% of public health budgets going towards managing these diseases (Quock, 2015).

The significant role of biofilms in oral health and disease is shown in an extensive number of epidemiological and clinical studies (Marsh, 2006; Filoche et al., 2010; Marsh et al., 2011; Wade, 2013). Poor management of oral hygiene can allow the accumulation of oral biofilm in the oral cavity. It is now becoming clear that many human illnesses, such as dental caries, periodontal disease and cardiovascular disease, are directly or indirectly related to a disruption in the equilibrium of the oral microbiota. This has lead to an increased interest in new ways to reduce disease causing microbial populations (Zarco et al., 2012).

Mechanical and chemical methods, such as tooth brushing and mouth rinses, have been traditionally used to prevent the accumulation of the oral biofilm. Effective manual or powered brushing and the use of interdental brushes are still the most important measures for oral hygiene control in patients (Brinkmann, 2005; Busscher et al., 2010; Kossack and Jost-Schatzle et al., 2010). Manual toothbrushes with a special head design, such as staged, v-shaped, or triple-headed, are more efficient than brushes with a conventional planar bristle field in patients with difficult to reach areas such as around brackets in those undergoing orthodontic treatment (Rafe et al., 2006). Some studies have found that powered toothbrushes are superior to manual toothbrushes in removing biofilm. However the comparison between different powered toothbrushes is difficult because of their different oscillation frequencies, types of vibration, areas or types of bristles, and study criteria or methods used in these experiments (Costa et al., 2007; Schatzle et al., 2010). Despite the fact that new designs of general toothbrushes come on the market regularly, longer brushing time and proper brushing techniques are still necessary for good oral hygiene.
There are a variety of measures that provide chemical control of oral biofilms. Some of these include the incorporation of antimicrobials into toothpastes, mouth rinses, varnishes and adhesives. Chlorhexidine still remains the most effective antimicrobial in reducing biofilm-induced iatrogenic side effects in patients (Gehlen et al., 2000a; Gehlen et al., 2000b). Unfortunately, long-term use of chlorhexidine is known to stain teeth and tongue and affect taste sensation (McCoy et al., 2008). The benefits of fluoride containing toothpastes and mouth rinses in preventing caries are widely known (Walsh et al., 2010). As well as aiding enamel remineralisation, fluoride acts as a buffer to neutralize acid-producing bacteria and suppresses their growth (Busscher et al., 2010). Stannous fluoride provides dual benefits with respect to caries and biofilm prevention by stannous ions (Wiegand et al., 2009). However, these measures of manipulating oral biofilms all have their limitations and with increasing bacterial resistance to chemical agents, the investigation of novel ways to manage biofilms is warranted (Zero, 2006).

Oral probiotics, which can improve oral health by affecting pathogenic bacteria without impacting negatively on the normal oral microbiota, are becoming popular (Reid et al., 2011). Probiotics are defined by the World Health Organization as ‘live organisms which, when administered in adequate amounts confer a health benefit on the host’ (Burton et al., 2013). Probiotics used for oral health present as a number of different strains, such as Lactobacillus, Bifidobacterium and Streptococcus (Burton et al., 2013). They have shown promise as a novel way to combat dental biofilms and strengthen natural micro-flora against pathogens that cause major oral diseases such as caries, periodontitis, oral candida and halitosis (Cagetti et al., 2013).

**Historical perspectives on oral probiotics and their mechanisms of action**

The use of probiotics to improve oral health is a relatively new concept, however the use of probiotics to improve health is not. In the early 20th Century, a Nobel Prize laureate, Elie Metchnikoff, reported that Bulgarians lived longer than other populations due to their consumption of fermented milk products containing good bacteria (Twetman and Stecksen-Blicks, 2008). The suggested mode of action was competition with pathogens, resulting in a favourable balance of ‘good’ bacteria to ‘bad’ bacteria (Cildir et al., 2009)
Most mechanisms of action are aimed at reducing biofilm accumulation and antagonising oral pathogens such as *Streptococcus mutans* and *Porphyromonas gingivalis*. They can be classified as direct or indirect actions. Direct interactions in biofilms, include the production of antimicrobial substances (organic acids, hydrogen peroxide, bacteriocins), secretion of toxins, enzymes and waste products, competition with pathogenic organisms for adhesion sites on mucosa, disruption of the binding of oral microorganisms to proteins, and interruption of bacteria to bacteria attachments (Meurman, 2005; Bonifait et al., 2009). The indirect actions in the oral cavity include the modification of the surrounding environment by modulating pH and oxidation-reduction potential which compromises the ability of pathogens to become established, stimulation of nonspecific humoral immunity, modulation of humoral and cellular immune responses, regulation of mucosal permeability and allowing the colonisation of less pathogenic bacterial species (Bonifait et al., 2009; Haukioja, 2010; Rastogi et al., 2011).

**Probiotic strains**

Traditionally, probiotics have been bacteria of intestinal origin, and their application has been in relieving problems of the gastrointestinal tract (Burton et al., 2013). Consequently, most studies investigating the potential of probiotics in preventing oral diseases have been based on intestinal probiotic strains. Although many strains have been investigated over the years, the most commonly used strains for oral health are those belonging to the genera *Lactobacillus*, *Bifidobacterium* and *Streptococcus*, such as *Lactobacillus rhamnosus* GG, *Lactobacillus reuteri* ATCC 55739, *Bifidobacterium* DN-173010 (Saxelin et al., 2005) and *Streptococcus salivarius* strains K12 and M18 (Burton et al., 2006; Burton et al., 2013). To a lesser extent, *Weissella cibaria* also plays a part in oral health, but mainly in the management of halitosis (Bjorkroth et al., 2002).

**Lactobacillus**

The ability of *Lactobacillus reuteri* SD 2112 and *Lactobacillus rhamnosus* GG to influence the colonisation of mutans streptococci has been studied (Haukioja et al., 2008). *L. reuteri* exists in the human gastrointestinal tract as an obligatory heterofermentative. Its exact mode of action is unclear but a number of suggestions have
been put forward. Firstly, *L. reuteri* produces anti-microbial substances called reuterin and reutericyclin (Talarico et al., 1988). These substances are water-soluble and broad-spectrum antimicrobials. They are effective in a wide range of environments and resistant to both proteolytic and lipolytic enzymes (el-Ziney and Debevere, 1998). Reuterin inhibits Gram-positive and Gram-negative microorganisms (El-Ziney et al., 1999). Secondly, *L. reuteri* strains have the ability to prevent the binding of pathogenic bacteria to host tissue. They also promote anti-inflammatory cytokine activity in the intestinal mucosa. Another possible mode of action is preventing the adherence of pathogenic bacteria and modifying the protein composition of the salivary pellicle (Haukioja et al., 2008).

**Weissella cibaria**

*Weissella cibaria*, originally classified in the genus *Lactobacillus*, is a Gram-positive facultative anaerobic lactic acid bacterium (Bjorkroth et al., 2002). *W. cibaria* secretes hydrogen peroxide and a bacteriocin that can act against other bacteria (Kang et al., 2006; Kang et al., 2006b). It can also coaggregate with *Fusobacterium nucleatum* and adhere to epithelial cells (Kang et al., 2006b). These properties potentially allow *W. cibaria* to colonise the oral cavity and prevent the proliferation of pathogenic bacteria.

**Bifidobacterium**

Strains of bifidobacteria exist in the human gastrointestinal tract. They exert similar effects to lactobacilli, such as the regulation of microbial homeostasis, the inhibition of harmful bacteria that colonise or infect the mucosa and the modulation of local and systemic immune responses (Cildir et al., 2009).

**Streptococcus**

Probiotic *Streptococcus salivarius* M18 and *Streptococcus salivarius* K12, both known to produce BLIS (bacteriocin-like inhibitory substances), are relatively new probiotic strains. Unlike *Lactobacillus* and *Bifidobacterium* probiotic strains, these two *S. salivarius* probiotic strains are sourced from the human oral cavity itself (Burton et al., 2013). They belong to commensal species that are known to have low pathogenic potential and were claimed to be the world’s first probiotics developed specifically for the mouth and throat (Burton et al., 2013).
*S. salivarius* probiotic strains (M18 and K12) have the potential to offer oral health benefits through the production of bacteriocins (Burton et al., 2013). These bacteriocins are antimicrobials, synthesised by ribosomes and have a narrow inhibitory spectrum against similar bacteria (Wescombe et al., 2009). The main difference between K12 and M18 is their inhibitory spectrum. K12 is mainly inhibitory towards *S. pyogenes* and upper respiratory tract pathogens (due to the production of salivaricin A and B); while M18 is less active on some of the upper respiratory tract pathogens but has strong activity against dental pathogens, in particular *Streptococcus mutans* (presumably due to the production of the bacteriocins salivaricins M, 9 and A) (Wescombe et al., 2009).

As well as producing bacteriocins that target the main decay causing species, *S. mutans*, these strains produce enzymes dextranase and urease, which have the potential to further limit the progression of dental caries by reducing dental plaque accumulation and acid levels in the mouth (Burton et al., 2013; Nascimento et al., 2009). They also work by competing with pathogenic organisms for adhesion sites and by modulating the host’s immune response (Saha et al., 2012).

**Clinical applications of oral probiotics**

Oral probiotics has been extensively studied and clinically used to reduce biofilm formation, inhibit pathogenic microorganisms, and prevent biofilm-related diseases such as dental caries, gingivitis, periodontitis, candidosis and halitosis (Meurman, 2005; Twetman and Stecksen-Blicks, 2008; Stamatova and Meurman, 2009; Teughels et al., 2011; Cagetti et al., 2013; Laleman et al., 2014a; Gruner et al., 2016)

**Dental caries**

Dental caries is still the most prevalent chronic disease worldwide, despite technological advancements and new initiatives introduced by the dental profession. The caries disease process is triggered by the interaction of cariogenic microorganisms, such as *mutans streptococci* and *lactobacilli*, a diet high in fermentable carbohydrates and host factors such as saliva secretion rate and buffering capacity (Selwitz et al., 2007).

Currently, the most commonly used and recommended products to combat caries are toothbrushes, floss, antimicrobial toothpastes and mouth rinses. Treatments using
conventional anti-streptococcal antimicrobials appear to be effective in reducing dental biofilm formation and levels of mutans streptococci in the short-term (Burton et al., 2013). However, their broad-spectrum antimicrobial activity destroys both commensal and harmful bacteria, which creates imbalances within the microflora. Antimicrobials are also often unpalatable to young children, which can result in poor compliance and compromise their intended effect (Burton et al., 2013).

Oral probiotics, on the other hand, have shown to be effective in managing cariogenic pathogens without impacting negatively on the normal oral microbiota. Many studies have assessed the effect of probiotics on the incidence of caries as well as the related main pathogenic microorganisms, such as mutans streptococci and lactobacilli.

Research designs vary greatly, with only a limited number of randomised controlled trials and cross-over studies on this subject present. The majority of these studies have examined the effect of certain probiotic strains on surrogate measures for caries incidence, such as \textit{S. mutans} levels. For obvious ethical and budget reasons, only a handful of studies have examined caries as an outcome measure. However, one has to question the value of studies that publish microbiological data over caries incidence as there is concern regarding the validity of using \textit{S. mutans} levels as an indicator of caries alone. This is mainly due to the discovery that caries can occur independently of \textit{S. mutans} (Griffen et al., 2011; Gross et al., 2012).

Nevertheless, although there are limitations with assessing surrogate measures for caries in the form of \textit{S. mutans} levels, this review will focus on publications reporting probiotic effects on both caries as an outcome measure as well as on levels of caries surrogates such as \textit{S. mutans}. This is mainly due to the majority of quality studies investigating probiotic effects on \textit{S. mutans} levels. To date, as at July 2016, close to 100 papers can be found in the literature examining the effect of probiotics on caries causing microorganisms, such as \textit{S. mutans}, and on caries incidence.

\textit{Effect of probiotics on caries incidence}

Currently, six randomised double-blind controlled trials demonstrate effects of probiotics, in which caries incidence is an outcome measure. Out of these, one study is on adult populations (Petersson et al., 2011), whereas the other five have investigated caries experience in children (Nase et al., 2001; Stecksen-Blicks et al., 2009; Taipale et al., 2013; Hasslof et al., 2013; Stensson et al., 2014). Of these, three studies reported
a beneficial effect of probiotics on childhood caries incidence (Nase et al., 2001; Stecksen-Blicks et al., 2009; Stensson et al., 2014) whereas two found that probiotics conferred no beneficial effect (Hasslof et al., 2013; Taipale et al., 2013).

**Evidence for a positive effect of probiotics on caries**

*Lactobacillus rhamnosus* and *Lactobacillus reuteri* strains have been shown to reduce the incidence of caries (Nase et al., 2001; Steckson-Blicks et al 2009; Stensson et al 2014; Petersson et al 2014), however effects on salivary levels of *S. mutans* have been unremarkable (Steckson-Blicks et al 2009; Petersson et al 2014; Stensson et al 2014).

The popular method of probiotic delivery in these population groups has been milk, possibly due to its ease of delivery in these subjects (Nase et al., 2001; Steckson-Blicks et al 2009; Petersson et al 2014), however equally positive results have been shown by probiotic delivery through oil drops (Stensson et al 2014).

**Evidence against a positive effect of probiotics on caries**

In contrast, two randomised, controlled trials in the literature show conflicting results to those above. This is possibly attributed to different probiotic strains used in these studies.

*Lactobacillus paracasei* F19 consumed in cereal form showed little difference in caries rates or *S. mutans* levels between the probiotic and control group (Hasslof et al., 2013). Similarly *Bifidobacterium animalis* subsp. *lactis* BB-12 taken in tablet form showed little difference in caries experience (Taipale et al., 2013). However, this study was limited in that it did not compare the probiotic to a placebo but rather control groups taking either xylitol or sorbitol. Conclusions on the true effect of probiotics on caries incidence therefore cannot be drawn, as sorbitol and xylitol groups were taking anti-cariogenic agents in their own right.

**Reasons for differences in the findings**

Studies on the effect of probiotics on caries incidence are difficult to compare due to the variety of study conditions such as:

*Sample size*—These vary greatly between studies.
Probiotic strain – Each study varies in its choice and concentration of probiotic strain. Some strains may be more effective in different areas of the body and in different doses and this may also translate to the mouth.

Age of populations – Studies vary in their selection of target populations. Different maturity in immune systems can influence microbial populations and should be taken into consideration when comparing ages of population groups.

Mode of delivery – There are a huge variation in the way of administering probiotics. These include oil, milk, lozenges, yoghurt and gum, to name a few. This may affect the concentration of probiotics that are administered or indeed how well they stay or are absorbed at their site of action.

Microbiological methods – Most studies estimate levels of S. mutans with culture-based methods that have limitations. Additionally, their accuracy and reliability over molecular methods using genetic analysis to identify the bacterial species present, is somewhat questionable.

Study duration – Study durations vary for weeks to months, which have obvious influences on outcome measures and persistence and concentration of intra-oral microbes.

Effect of probiotics on S. mutans

For obvious ethical and budgeting reasons, the majority of studies investigating the effects of probiotics use measures of S. mutans levels as an indicator of potential caries experience. Additionally, S. mutans levels allow researchers to speedily obtain significant microbiological changes (e.g. weeks) compared to the time required for clinical changes to occur (e.g. months or years).

To date, a combination of 15 relatively well designed randomised controlled trials and cross-over studies, all of which are included in a recent meta-analysis (Laleman et al., 2014b), showed that probiotics reduce the levels of S. mutans. Interestingly, type of probiotic strain administered, mode of delivery or length of time did not seem to have an effect on results.
Major conclusions from previous studies

Probiotic strains *L. reuteri* (especially *L. reuteri* ATCC 55730) and *L. rhamnosus* (especially *L. rhamnosus* GG) have a caries prevention effect in children.

Probiotics reduce levels of *S. mutans*. This may indicate that probiotics can influence the composition of the oral micro-ecology community and offer protection from pathogenic microorganism induced dental conditions such as caries, halitosis, gingivitis, periodontitis and candidosis.

Periodontitis

The main pathogens associated with periodontitis are *P. gingivalis*, *Treponema denticola*, *Tannerella forsythia* and *Aggregatibacter actinomycetemcomitans* (Ishikawa et al., 2003). These bacteria can colonise subgingivally, escape the host's defense system and cause tissue damage (Ishikawa et al., 2003).

Traditional mechanical and chemical methods of managing biofilms that cause periodontitis have their limitations. Some biofilms are difficult to disrupt by mechanical means, especially when they are subgingival or protected by tooth anatomy or restorations and some bacteria within these biofilms are resistant to certain chemical assaults (Darby, 2009). Probiotics have been shown to improve plaque and gingival indices, probing depths and bleeding on probing in subjects with gingivitis and periodontitis (Riccia et al., 2007; Kang et al., 2006a; Krasse et al., 2006). This has been demonstrated by a reduction in total anaerobic bacteria, especially the number of black-pigmented anaerobic rods, *P. gingivalis* and *Campylobacter rectus* (Teughels et al., 2011; Ishikawa et al., 2003). Additionally, probiotics like *Lactobacillus reuteri* have shown to exert positive treatment effects by promoting anti-inflammatory cytokine activity as it does in the intestinal mucosa (Krasse et al., 2006) and *L. brevis* has also demonstrated an anti-inflammatory mechanism of action through the prevention of nitric oxide production, the release of PGE2 and the activation of MMPs (Riccia et al., 2007). Furthermore, probiotics have been found to offer a long-term benefit to patients by promoting less recolonisation of bacteria in periodontal pockets (Teughels et al., 2007).
Candidosis

The elderly are often susceptible to oral candidosis, either due to prolonged antibiotic exposure or to accumulated deficiencies in their immune system. Probiotics could encourage faster *C. albicans* clearance (Elahi et al., 2005) and decrease yeast levels in patients (Hatakka et al., 2007). Similarly *Streptococcus salivarius* K12 has indicated a direct influence on *Candida* associated disease progression in mouse studies, that bodes well for its possible clinical application in human subjects (Burton et al., 2011).

Halitosis

Halitosis has many causes, including consumption of particular foods, metabolic disorders and respiratory tract infections; but in most cases is associated with an imbalance of the commensal microflora of the oral cavity (Scully and Greenman, 2008). The anaerobic bacteria in the mouth, such as *Porphyromonas gingivalis*, can break down salivary and food proteins and produce amino acids, which are then transformed into volatile sulphur compounds (VSCs), contributing to halitosis (Kang et al., 2006b).

A number of strategies have been used for the prevention of halitosis. For example, anti-bacterial toothpaste and mouth rinses are used to reduce the numbers of bacteria, or certain odour neutralising or masking agents are used (Scully and Greenman, 2008). However, they usually need to be used several times daily and provide only a short-term benefit (Scully and Greenman, 2008).

Probiotics provide a new treatment modality for halitosis by introducing a beneficial bacterial population to help counter the colonisation and multiplication of the odour causing microbes (Kang et al., 2006b). *Weissella cibaria* has been shown to prevent the production of volatile sulphur compounds by *Fusobacterium nucleatum*, possibly because the production of hydrogen peroxide by *W. cibaria* can prevent the proliferation of *F. nucleatum* (Kang et al., 2006b). Oral probiotic *S. salivarius* has also been found to decrease the VSC levels in the mouth and has shown potential in the prevention and treatment of halitosis by combating odour-causing, proteolytic anaerobes (Burton et al., 2005).
**Application in orthodontics**

The risk of biofilm formation in orthodontic patients is even higher than in the general population because micro niches and increased surface areas provided by orthodontic brackets and appliances not only promote biofilm formation but also impede daily oral hygiene.

The use of oral probiotics in orthodontic patients may be beneficial since they have shown effectiveness in managing caries and decreasing *S. mutans* level. However, little research has been done on the role probiotics can play in orthodontic patients. To date, there have only been five studies that have investigated the effect of probiotics in patients with fixed appliances (Cildir et al., 2009; Jose et al., 2013; Pinto et al., 2013; Ritthagol et al., 2013; Gizani et al., 2015).

*Lactobacillus paracasei* SD1 (Ritthagol et al., 2013), *Bifidobacterium animalis* subsp. *lactis* DN-173010 (Cildir et al., 2009) and a probiotic of unknown strain and concentration (Jose et al., 2013) have been shown to significantly decrease levels of mutans streptococci when delivered in milk, yoghurt, curd and toothpaste respectively. Only two of these five orthodontic studies have used the same strain. The strain used was Bifidobacterium but each study had different results (Pinto et al., 2013; Cildir et al., 2009). The first study found that there was a reduction in salivary mutans streptococci in orthodontic patients consuming *Bifidobacterium animalis* subsp. *lactis* DN-173010 yoghurt daily (Cildir et al., 2009). On the other hand, the second study found no change (Pinto et al., 2013). However the differing results, even though the two studies had similar designs and sample sizes, could be attributed to the yoghurt itself, which may influence the cariogenic potential and colonisation of the oral cavity (Pinto et al., 2013).

Since previous research on probiotics in dental patients has shown that over time *Bifidobacterium* has less consistent outcomes compared to *L. reuteri* and *L. rhamanosus*, their further investigation, along with the yet to be tested *Streptococcus* probiotic strains, is warranted in an orthodontic population.
Gaps in the current literature

Research on the effect of probiotics on oral health has become increasingly popular, and the number of systematic reviews on this topic has significantly increased (Meurman, 2005; Twetman and Stecksen-Blicks, 2008; Stamatova and Meurman, 2009; Teughels et al., 2011; Cagetti et al., 2013; Laleman et al., 2014a; Laleman et al., 2014b; Gruner et al., 2016). However, it is acknowledged that in order to gain a better understanding of the exact benefits of oral probiotics and draw accurate conclusions, studies need a variety of improvements such as:

Gold standard microbial profiling. Analysing the microbial profiles of the biofilm with molecular techniques such as by direct PCR-based amplification of the 16S ribosomal RNA gene, followed by sequencing of the PCR products using next-generation DNA sequencing (e.g. Ion Torrent or Illumina) technology. This method has the ability to provide a ‘gold standard’ for bacterial identification, identify known species and possibly identify microorganisms that cannot be cultured. It will equally provide evidence for the validity of using S. mutans as a surrogate measure for caries incidence.

Longitudinal and long-term follow-up studies. Longer observations would allow one to analyse the influence of probiotics on the oral micro-ecology at different time points and allow for the collection of data on clinical problems such as caries that take considerable time to develop. Long-term follow-up can provide information about the lasting effects of probiotics after administration has stopped, especially in orthodontic patients who usually receive several years of treatment. In addition, long-term studies provide the ability to assess whether permanent colonisation of the probiotic has occurred, thereby offering protective effects from bacterial induced consequences, without the need for continual probiotic consumption.

Administration and concentration of probiotics. Although probiotics administered by different modes of delivery have largely shown beneficial results, comparisons of the efficacy of delivery methods, such as yoghurt, milk, chewing-gum and lozenges are important. Equally, ideal concentrations of the probiotics and their relative substantivity would be worthwhile to assess their true benefits when present in the mouth at optimal concentrations.
Oral probiotic strains. The probiotic strains used in studies vary greatly from centre to centre, for example, *S. salivarius* K12 and M18 are commonly used in New Zealand, and they are the only probiotics that have been developed from the mouth, for the mouth. In European countries, the majority of research uses *L. reuteri*, *L. rhamnosus* and *Bifidobacterium* species (Nase et al., 2001; Nikawa et al., 2004; Caglar et al., 2005). Well-designed, high-powered studies allowing one to assess and compare the efficacy of these different probiotic strains on oral health are needed in order to find the most effective strain for use in dental and orthodontic patients.

Oral probiotics for orthodontic patients. Despite the increasing number of reports on the positive effects of oral probiotics decreasing *S. mutans* levels and preventing caries in the general dental population, studies in patients with orthodontic fixed appliances are still limited. Considering orthodontic patients are at an increased risk of caries, studies on the application of probiotics in orthodontic patients could provide an alternative way to combat white spot lesions, gingivitis and halitosis caused by biofilm formation around fixed appliances.

Conclusions and future directions

Probiotic use in oral health is gaining popularity due to the emergence of antibiotic-resistant bacteria and the limitations presented by current oral hygiene methods and products. The most commonly used probiotic strains for oral health research include *Lactobacillus*, *Bifidobacterium* and *Streptococcus*. These strains possess similar mechanisms of action that ultimately aim to promote healthy bacteria over bacteria prevalent in dental disease.

The effect of oral probiotics on managing dental biofilm formation and its consequences, such as caries, periodontal disease, candidosis and halitosis, seems encouraging. The probiotic approach to reducing biofilm accumulation makes good biological sense, provided the organisms can be delivered often enough to establish their presence over their commensal counterparts and offer patients a new beneficial oral micro-ecology (Burton et al., 2013). A commonly reported uncertain aspect of probiotic use is whether (and for how long) probiotic species are able to colonise the oral environment, especially since it is well known that probiotics in the gastrointestinal tract
usually colonise for a short time only (Rao et al., 2012). There are a number of publications that indicate long-term colonisation can occur after probiotic consumption ceases, although this is only seen in select participants.

It is therefore necessary for future studies to investigate why some probiotics colonise select patient’s mouths even after administration ceases, whereas in others, beneficial effects of probiotics are only received as long as the probiotics are being administered. Additionally, since there are many strains of probiotics on the market, it will be useful to investigate which probiotic strains can colonise the oral cavity most effectively and offer long-term oral health benefits. It has also been noted that the way in which probiotics are delivered can affect their success, yet few studies have compared modes of delivery. It is worthwhile investigating this area further to ascertain whether the effects seen by the probiotics are aided by their mode of delivery or strictly due to their individual mechanisms of action.

Overall, it seems that although probiotics have been known for their benefits in health, their application in oral health is still very much in its infancy and well conducted studies with long term follow up are scarce. Therefore, well-designed randomised controlled trials (RCTs), the ‘gold standard’ for evaluating the effectiveness of interventions (Akobeng, 2005), are needed to provide further scientific clinical evidence on this promising, yet poorly understood topic (Cagetti et al., 2013). Success in orthodontic patients could provide an alternative regime to limit the deleterious effects of biofilm accumulation, such as gingivitis, white spot lesions and halitosis.

**Aims of this thesis**

The aims of this thesis are to investigate the efficacy of oral probiotic *Streptococcus salivarius* M18 in managing biofilm formation in patients wearing fixed orthodontic appliances, and to assess its effects on the oral microbiome of these patients.

**Objectives**

To determine if oral probiotic *S. Salivarius* M18 is effective in:

1. Reducing biofilm formation
2. Establishing a balance between ‘good’ and ‘bad’ bacteria
3. Reducing halitosis
REFERENCES


Chapter 2

Efficacy of oral probiotic *Streptococcus salivarius* M18 in enhancing oral hygiene in patients wearing fixed appliances

– A triple-blind randomised placebo-controlled trial
ABSTRACT

Introduction: Orthodontic fixed appliances promote biofilm formation, leading to clinical problems such as enamel demineralisation, gingivitis and halitosis. Oral probiotics show promise in managing biofilms in general dental patients, but the efficacy of oral probiotics in enhancing oral hygiene in patients with fixed orthodontic appliances is still unclear.

Materials and Methods: The study was designed as a prospective, randomised, triple-blind, two-arm parallel-group, placebo-controlled trial with a 1-month treatment intervention and a 3-month treatment-free follow-up. A total of 64 patients undergoing orthodontic treatment at the Faculty of Dentistry, University of Otago, Dunedin, New Zealand, were selected for the study. Those eligible were 10 to 30 years of age, with at least 20 natural teeth, wearing stainless steel brackets, and using fluoride toothpaste not containing any supplementary antibacterial agents for daily oral hygiene. Participants were randomly assigned to two groups based on computer-generated random sequencing using balanced block randomization. In the oral probiotic group (n = 32), the patients consumed two probiotic lozenges containing the probiotic Streptococcus salivarius M18 each day for 1-month; in the placebo-control group (n = 32), the patients consumed two placebo-control lozenges without probiotic each day. The patients, investigators and statistician were blinded to the patient allocation, and the researcher managing the random sequence did not participate in allocation or measurement.

At each appointment (baseline, 1-month, 4-month), plaque index (PI) and gingival index (GI) scores were recorded. Volatile sulphur compounds (VSCs) levels were measured using a Halimeter. Results: Compared with the placebo-control group, the probiotics did not influence PI or GI scores (interaction time × treatment; p > 0.05). VSC levels decreased significantly in the probiotic group (VSC reduction = 17 ppb, p = 0.015) and control group (VSC reduction = 13 ppb, p = 0.039) after 1-month treatment. However, after the 3-month treatment-free follow-up, the VSC levels of the control group returned to a level (VSC = 203 ± 59 ppb) that was similar to the baseline (VSC = 204 ± 69 ppb) (p = 0.619), whereas the VSC levels of the probiotic group reduced further (VSC = 180 ± 47 ppb) and were significantly lower (p=0.005) than the baseline (VSC = 201 ± 71 ppb). No side effects were observed during the trial. Conclusions: Oral probiotic S. salivarius M18 reduced VSC levels in patients with fixed appliances but did not decrease their plaque or gingival indices.
INTRODUCTION

The placement of fixed orthodontic appliances can severely impede tooth brushing and provide areas of low salivary flow that promote dental biofilm (plaque) formation (Ren et al., 2014). Additionally, metabolites such as acids produced by biofilms cannot be effectively buffered and removed by saliva due to the placement of orthodontic appliances (Øgaard, 2008). If patients fail to maintain good oral hygiene during orthodontic treatment, the biofilm formed can cause a number of adverse events, including enamel demineralisation and gingival inflammation (Peng et al., 2014). These adverse events can occur in just a few weeks after the placement of fixed appliances, severely affecting the treatment results and patient’s quality of life (Øgaard, 2008).

Haltosis, more commonly known as bad breath, affects up to half the adult human population to various degrees (Yaegaki and Coil, 2000). Although generally not considered to be a medical concern, it can certainly confer a significant social stigma (Rosenberg, 2002). Fixed orthodontic appliances have been found to significantly increase the plaque and tongue coating indices. A positive association between halitosis and the use of fixed orthodontic appliances has been confirmed and halitosis has been considered an important indicator of oral health during orthodontic treatment (Zurfluh et al., 2013). The volatile sulphur compounds (VSCs) produced by oral biofilms, especially anaerobes located on the dorsum of the tongue, are considered to be one of the most common contributors to halitosis (Loesche and Kazor, 2002) and bacteria such as Leptotrichia and Prevotella are regarded as the main halitosis pathogens (Yang et al., 2013). However, no obvious association exists between oral malodour and any specific bacterial infection, suggesting that halitosis reflects complex interactions between several oral bacterial species (Porter and Scully, 2006).

The measures adopted to prevent and control biofilm formation in orthodontics usually include oral hygiene instruction, application of fluoride, (Benson et al., 2004), use of antimicrobial dentifrices, mouth rinses (Chen et al., 2011), and antimicrobial modifications of orthodontic materials (Shah et al., 2011). Antibiotics can decrease oral bacterial adhesion, however, their use can unbalance the normal oral microflora and lead to dysbacteriosis and drug resistance (Kouidhi et al., 2011). Although the prevention of biofilm formation in orthodontics has been studied extensively, and a variety of dental products for managing oral biofilms are commercially available, the prevalence of biofilm-related adverse events during orthodontic treatment, such as enamel demineralisation, still remain as high as 72.9%
The need for safe and efficacious management of biofilms is of great importance, especially in patients with fixed orthodontic appliances.

Probiotics, which are defined by the World Health Organisation as ‘live organisms which, when administered in adequate amounts confer a health benefit on the host’, have been shown to be effective in various approaches for biofilm management. These include reducing dental biofilm formation, preventing caries development and decreasing oral malodor (halitosis) (Rao et al., 2012; Saha et al., 2012; Cagetti et al., 2013). Traditionally, probiotics have been widely used to improve gut health. Probiotic mechanisms of action usually include competing with pathogenic organisms for adhesion sites, secreting antimicrobial substances such as bacteriocins and modulating the host’s immune response (Saha et al., 2012; Singh et al., 2013). During the last decade, an increasing number of products containing probiotics have entered the oral health market and show promise in the prevention and treatment of biofilm-related oral diseases (Haukioja, 2010; Gutkowski, 2013). According to recent studies, oral probiotics can be an effective and safe therapy for reducing dental biofilms and caries risk in children (Juneja and Kakade, 2012; Cannon et al., 2013; Burton et al., 2013a). In addition, the oral probiotic *Streptococcus salivarius* has been found to decrease the VSC levels in the mouth and has shown potential in the prevention and treatment of halitosis (Burton et al., 2005). Clinical research has confirmed the use of oral probiotic *S.salivarius* M18 as an effective measure in the treatment of dental decay and gingival inflammation without negatively impacting the normal oral microflora (Rastall et al., 2005; Burton et al., 2013a; Burton et al., 2013b). The effect of oral probiotics on managing dental biofilm formation seems encouraging and promising.

However, to date, there are few randomised controlled trials (RCTs), the ‘gold standard’ for evaluating the effectiveness of interventions (Akobeng, 2005), examining the efficacy of oral probiotics in enhancing oral hygiene; even fewer have investigated the role probiotics can play in orthodontic patients (Cagetti et al., 2013). To date, five RCTs have examined the effect of probiotics on orthodontic patients (Cildir et al., 2009; Jose et al., 2013; Pinto et al., 2013; Ritthagol et al., 2013; Gizani et al., 2015). However none of these have tested probiotic effect on halitosis or used *S. salivarius* M18. This oral probiotic strain is unique in that it has been isolated from the mouth, not the GI tract like others.

The aim of this trial was to determine the efficacy of oral probiotic *Streptococcus salivarius* M18 in enhancing oral hygiene in patients wearing fixed orthodontic appliances.
We aimed to assess clinical parameters including Plaque Index (PI), Gingival Index (GI), and VSC levels in patients at difference treatment timepoints.

MATERIALS AND METHODS

Study design

The study was designed as a prospective, randomised, triple-blind, two-arm parallel-group, placebo-controlled trial with a 1-month treatment intervention and a 3-month treatment-free follow-up. Data were collected between July 2015 and December 2015. An oral examination was taken at baseline, then after 1 and 4 months (Figure 2.1). The study examiners, orthodontic patients, and statisticians were blinded to the treatments given. The study was approved by the University of Otago Ethics Committee (H14/103) as well as the local Maori Research Consultation Committee. The trial was registered with the Australian New Zealand Clinical Trials Registry (ANZCTR) (ACTRN12615000341527).

Participants

A total of 64 orthodontic patients at the Faculty of Dentistry, University of Otago, Dunedin, New Zealand, (age range, 10-30 years) wearing fixed appliances were recruited into the study. The determination of sample size was based on previous estimates of plaque index (PI) variability (SD, 0.4) in adolescents requiring orthodontic treatment by setting type I error at 0.05 and type II error at 0.20 (80% power) (Acharya et al., 2011; Peng et al., 2014). We assumed that participants were treated with oral probiotics and found that about 27 patients (i.e. a total of 54 for 2 groups) were needed to detect a decrease in the PI of about 30%. To allow for possible dropout during the study, we aimed to recruit 64 patients (Figure 2.1, Table 2.1). Eligible participants were informed of the study design and gave their written consent.
Figure 2.1. Study flow chart showing patient flow during the trial.
Inclusion and exclusion criteria

Inclusion criteria were having at least 20 natural teeth; and, wearing stainless steel brackets (Victory Series; 3M Unitek, Monrovia, California, USA) in both arches.

Exclusion criteria were the presence of systemic disease (e.g. diabetes); living in a non-fluoridated area (e.g. Allanton, Waikouaiti and Mosgiel); periodontal disease; antibiotic therapy; wearing lingual braces; using a toothpaste with supplementary antibacterial agents; using a non-fluoride toothpaste; dental fluorosis; smoking; using powered toothbrushes; lactose intolerance; allergy to dairy products; and participants being physically unable to brush.

Randomisation and allocation concealment

Balanced block randomisation (with a block size of four and by blocking on sex) was used to ensure equal patient allocation to each treatment group. Allocation concealment was used to avoid selection bias and was conducted by a member of the research team (MF). The computer-generated random numbers were provided in opaque, sealed envelopes (MF) before the intervention was commenced (GB). The patients, investigators and statistician were all blinded to the allocation. BLIS Technologies only revealed the identities of the treatment groups to the investigators once the intention-to-treat (ITT) analysis was performed.

Probiotic intervention and placebo-control

Oral probiotic group: participants consumed two lozenges of BLIS M18 *S. salivarius* (live active) bacteria (BLIS Technologies Ltd, Dunedin, New Zealand) each day during the 1-month intervention. The dose administered was $3.6 \times 10^9 S. salivarius$ colony forming units (CFUs)/lozenge. Isomalt (sugar substitute), tableting aids and peppermint flavouring made up the rest of the lozenge.

Placebo-control group: participants consumed two placebo-control lozenges (BLIS Technologies Ltd, Dunedin, New Zealand) each day during the 1-month intervention. The packaging, appearance and taste of lozenges in both intervention and control groups were identical. Twenty milligrams of extra isomalt was used in place of the probiotic in the placebo-control lozenges.

The manufacturer did not reveal the identities of the treatment groups to the investigators until the intention-to-treat (ITT) analysis and statistical analyses were completed. The
participants were informed not to use any other probiotic-containing products during the study.

_Treatment protocol and follow up_

At the baseline appointment, an oral examination was conducted and both PI and GI were assessed. Participants were then asked to chew a plaque-disclosing tablet (Disclotabs, Colgate-Palmolive, Sydney, New South Wales, Australia) until dissolved and thoroughly remove the disclosed dental biofilm by brushing with a new toothbrush (Colgate® Ortho, Colgate-Palmolive, Sydney, New South Wales) and toothpaste (Colgate® Cavity Protection Toothpaste, Colgate-Palmolive, Sydney, New South Wales, Australia) under the supervision of an investigator (GB). The participants then rinsed their mouths with 30 ml chlorhexidine mouthwash (Savacol®, Colgate-Palmolive, Sydney, New South Wales, Australia) and water. They were then asked to suck the appropriate lozenge. A pack of the lozenges, toothpaste, toothbrush, compliance Lozenge Reminder Chart, pen and further instructions were given to the participants to take home. Each participant provided at least one email address and mobile telephone number to which morning and night email and text message reminders were regularly sent throughout the entire 1-month intervention period, to enhance compliance with taking the lozenges.

The protocol required the participants to suck two lozenges each day, one after brushing their teeth in the morning and one after brushing their teeth at night. The duration of the study was 4 months in total, including a 1-month treatment intervention followed by a 3-month treatment-free follow-up to assess recurrence rates.

_Study assessments_

The modified Plaque Index (PI) (Clerehugh et al., 1998) was used to assess the amount of biofilm formation on teeth. This PI system allows for the impact of brackets on biofilm distribution and has greater categorical discrimination than the original Silness and Løe index (Clerehugh et al., 1998). In this PI scoring system, the teeth were divided into mesial, distal, gingival, and incisal (occlusal) regions in relation to the bracket. Plaque was then scored for each of the four areas based on four plaque index codes as used by Løe, 1967. The four values were averaged for each tooth to give a mean PI tooth score. An overall final mean PI for all involved teeth was calculated for each participant.
Gingival Index (GI) was measured using a periodontal probe. Four areas around each tooth (mesial (labial/buccal), distal (labial/buccal), labial/buccal and lingual/palatal) were scored based on four gingival index codes as used by Löe, 1967. A tooth GI mean and a participant GI mean was calculated as described above for scoring PI.

Levels of volatile sulphur compounds (VSCs) were assessed in patients’ breath samples using a Halimeter (Interscan Corp., Chatsworth, USA) based on the average of three thirty second readings recorded as parts per billion (ppb) sulphide equivalents. The peak ppb values were displayed at the end of each sample period, as well as an average peak ppb value for all three samples. There was a three minute re-calibration period before each sample was taken. All of the measurements were performed in a standardised manner following the manufacturer’s instructions.

Side effects and patient compliance were also assessed. All remaining lozenges and Lozenge Reminder Charts were collected for the analysis of compliance with lozenge consumption. No harm to the participants was anticipated, however they were given clear directions on their information sheet on how to alert us if any adverse events did occur, so the study could be terminated immediately, if necessary.

**Statistical Analysis**

An intention-to-treat analysis was carried out using mixed-models and, where appropriate, nonparametric Kruskal-Wallis analyses was performed. Statistical analyses were performed using SPSS for Mac (version 20.0, IBM Corporation, Chicago, Illinois, USA). Alpha error was set at 0.05. The mixed model response variables were “PI”, “GI” and “VSC”. “Sex”, “treatment group” and “time” were entered as fixed factors. Due to data clustering within participants, the term “patient” was entered as a random term. Where appropriate, Bonferroni corrected post-hoc multiple comparisons were run. The efficacy of treatment interventions were tested by the interaction of treatment × time.
RESULTS

A total of 188 patients were enrolled and assessed for eligibility and 64 recruited for randomisation (oral probiotic group n=32 and placebo-control group n=32) (Figure 2.1). After the 4-month follow-up, no participants were lost to the study and all participant data were included for analysis. No adverse events were recorded during the trial.

The baseline characteristics of the participants are summarised in Table 2.1. Age and sex distribution, as well as PI, GI and VSC values at baseline were very similar (Table 2.1).

| Table 2.1. Baseline characteristics of participants in the two treatment groups. |
|-----------------------------------|------------------|------------------|
|                                   | Oral probiotics (N=32) | Placebo control (N=32) |
| Age (N, %)                        |                   |                   |
| <14 years                         | 14 (22%)          | 22 (34%)          |
| ≥14 years                         | 18 (28%)          | 10 (16%)          |
| Sex (N, %)                        |                   |                   |
| Male                              | 12 (19%)          | 11 (17%)          |
| Female                            | 20 (31%)          | 21 (33%)          |
| Plaque Index (mean ± SD)          | 1.1 ± 0.4         | 1.1 ± 0.2         |
| Gingival Index (mean ± SD)        | 0.8 ± 0.4         | 0.8 ± 0.3         |
| VSCs (ppm) (mean ± SD)            | 204 ± 69          | 201 ± 85          |
**Plaque Index and Gingival Index**

PI scores were not significantly influenced by gender (F=0.2; p=0.685), nor by treatment group (F=0.3; p=0.569), or by time (F=1.6; p=0.201). There was no significant interaction between treatment group and time (F=1.0; p =0.353) indicating that PI scores did not differ between the probiotic and placebo-control group over time (Figure 2.2A).

GI scores were not significantly influenced by gender (F = 0.6; p = 0.455), nor by treatment group (F = 0.3; p = 0.867). The interaction between treatment group and time was not significant (F = 0.2; p = 0.844) (Figure 2.2B).

![Figure 2.2. Box-and-whisker plots of the PI (A) and GI (B) scores of the two groups at baseline, after 1-month intervention and 3-month treatment-free follow-up.](image)
VSC levels

VSC levels scores were not significantly influenced by gender (F=0.3; p=0.561) and treatment group (F=0.2; p=0.619), but they changed significantly over time (F=7.9; p=0.001).

There was a significant interaction between treatment group and time (F=6.4; p=0.01), indicating that the changes of VSC over time differed between treatment groups.

Indeed, the level of VSCs decreased significantly in the probiotic group (VSC reduction = 17 ppb, p = 0.015) and placebo-control group (VSC reduction = 13 ppb, p = 0.039) after 1-month of treatment. However, 3-months after the 1-month intervention, the VSC levels of the placebo-control group returned to a level (VSC = 203 ± 59 ppb) that was similar to baseline (VSC = 204 ± 69 ppb) (p = 0.619), whereas the VSC levels of the probiotic group decreased even more (VSC = 180 ± 47 ppb) compared with the baseline readings (VSC = 201 ± 71 ppb) (p=0.005) (Figure 2.3 and Table 2.1)

![Figure 2.3. Mean VSC levels of the two groups at baseline, after 1-month intervention and 3-month treatment-free follow-up. (Data represent means and standard errors)](image-url)
DISCUSSION

This trial assessed the efficacy of oral probiotic *S. salivarius* M18 at managing biofilm-related problems in patients with fixed orthodontic appliances by investigating changes in clinical parameters (gingival index (GI), plaque index (PI), volatile sulphur compounds (VSCs)). PI scores did not differ between the probiotic and placebo-control groups over time. GI scores were also not significantly different between probiotic and placebo-control groups, however with time, the GI of both groups worsened.

With regard to VSC levels, there was a significantly decrease in both probiotic group and placebo-control group after 1-month of treatment. However, 3-months after the 1-month intervention, the VSC levels of the placebo-control group returned to a level similar to baseline, whereas the VSC levels of the probiotic group decreased further when compared to baseline readings.

This study had several strengths, including a randomised placebo-controlled trial, triple blinded, adequate sample size of 64 patients, zero drop-outs, and objective assessment of plaque, gingivitis and halitosis. Additionally the compliance with taking the lozenges was good. This may have been helped by the provision of a Lozenge Reminder Chart, as well as daily email and text message reminders (Paterson et al., 2016). This study was adequately powered so the lack of effect from the probiotic on the GI and PI is unlikely to be due to the lack of power.

This study had a number of limitations. The most notable is that the 1-month intervention was relatively short, which could have contributed to the lack of a positive effect of *S. salivarius* M18 on the PI and GI. The relatively short follow-up period (3 months) was also a shortcoming as this time period may have been too short to see a significant change in PI and GI scores (Teughels et al., 2008).

The traditional PI and GI indices were initially designed for normal patients without fixed orthodontic appliances (Löe, 1967). The modified PI and GI that were used in this study accounted for the presence of brackets, making it more fitting for assessing an orthodontic population (Clerehugh et al., 1998). Still, PI and GI are somewhat subjective measures and difficult to compare between studies.

Operating a Halimeter also has limitations, despite its regular use to measure VSC levels in other studies (Rosenberg, 1996; Salako and Philip, 2011). The Halimeter cannot
discriminate among the sulphur gases, and its sensitivity is relatively lower for methyl mercaptan than for hydrogen sulfide. To minimise any potential influence on the VSC measurements, we instructed our participants not to eat prior to their visit, as per the manufacturer’s guidelines, and performed the measurements at a similar time of the day (Shaw, n.d). Although the Halimeter lacks ideal accuracy, it still provides useful data when regularly calibrated (Furne et al., 2002). The issue remains with how to interpret the VSC readings and discriminate when a reduction can be deemed clinically significant.

To our knowledge, this is the sixth study to assess probiotic effects on the biofilm of patients with fixed orthodontic appliances. Previous studies (Cildir et al., 2009; Jose et al., 2013; Pinto et al., 2013; Ritthagol et al., 2013; Gizani et al., 2015), mainly focused on the effect of probiotics on levels of mutans streptococci and lactobacilli, with only one recently published long-term RCT (17-month mean intervention), having investigated clinical parameters (Gizani et al., 2015). Our study differs from others in that PI, GI and VSC levels were assessed. It seems that this is the first time that probiotic effect on VSC levels have been evaluated, and the first time probiotic S. salivarius M18 has been tested in an orthodontic population. S. salivarius M18 was the probiotic of choice, as it has strong activity against dental pathogens, in particular cariogenic S. mutans (due to the production of salivaricins M, 9 and A) (Wescombe et al., 2009). Other commonly used strains for oral health include those from the genera Lactobacillus, Bifidobacterium and Streptococcus, such as L. rhamnosus GG, L. reuteri ATCC55739, Bifidobacterium DN-173010, but these are all derived from the gastrointestinal tract (Saxelin et al., 2005; Cagetti et al., 2013; Laleman et al., 2014; Gruner et al., 2016).

When compared with general dental patients, orthodontic patients have a higher risk of caries and biofilm formation, due to the impact of fixed orthodontic appliances on oral hygiene (Ogaard, 1989; Sudjalim et al., 2007), therefore comparable studies to this one, should include an orthodontic population. However due to the scarcity of probiotic studies on orthodontic patients and the different study designs and output measures, meaningful comparisons are not possible. Like this study, only Gizani and colleagues have assessed clinical parameters, namely incidence of white spot lesions. With findings that there was no difference in WSL incidence over time, their conclusion that probiotics had little effect in adolescent orthodontic patients, was similar to ours (Gizani et al., 2015). Although using non-orthodontic dental populations, the influence of oral probiotics on PI and GI was compared in a recent systematic review and meta-analysis (Gruner et al., 2016). Similarly, this group
found that overall there were no significant reductions in PI, but they did find that there was a small yet significant benefit of probiotic therapy on GI.

The mean level of VSCs in patients with fixed orthodontic appliances has been found to be significantly higher than the normal population, possibly due to the poorer oral hygiene caused by braces (Ogaard, 1989; Sudjalim et al., 2007). The effects on halitosis in orthodontic patients have not been investigated before this study. However in a general dental population, *S. salivarius* K12 has been shown to reduce VSC levels in 85% of participants when delivered by lozenge (Burton et al., 2006). Besides *S. salivarius* strains, *Weissella cibaria* has been found to produce similar effects on VSC levels (Kang et al., 2006).

Generally the literature on the effects of probiotics on PI and GI in the general dental population shows more significant benefits in patients experiencing oral disease, such as gingivitis. Since GI is a marker of inflammation, the more likely effect of probiotics on gingival disease could be their effect on host responses rather than on oral pathogens at disease sites (Yanine et al., 2013).

A pretreatment using antimicrobial mouth rinse such as chlorhexidine before the consumption of oral probiotics was used in this study. We deemed it beneficial to have a pretreatment mouth rinse to rid the mouth of its bacterial load thereby allowing the ‘good’ probiotic bacteria to dominate the oral microbial community after administration (Aminabadi et al., 2011). The lasting effect of chlorhexidine on the oral microbiota should be minimal, thereby allowing microbial populations to recolonise to pretreatment levels within 24 hours (Rindom Schiøtt et al., 1970). However, the composition of the biofilm post-mouth rinse could have been affected by the chlorhexidine thereby influencing the PI, GI and VSCs. It is likely, early colonising bacteria would have dominated the mouth though the 1-month intervention due to the clear scaffold provided by the rinse. In contrast, a mixture of both early and late colonisers in a more mature biofilm are likely to have been present at the baseline and 4-month time points. Nevertheless, as both probiotic and placebo-control groups received the mouth rinse, the choice to use a pre-treatment shouldn’t have confounded results between the groups.

The influence of the chlorhexidine pre-treatment (Roldan et al., 2004) on the biofilm composition or the Hawthorne Effect (McCambridge et al., 2014), may have contributed to the control group also having a decrease in the VSC levels after 1-month placebo treatment. At commencement of the study, the participants of the placebo-control group and the oral
probiotic group were blinded to their treatment and informed that their oral hygiene would be examined during the trial, therefore, if they paid more attention to their oral hygiene during this period, aware that they were being observed, the results could have been influenced further. After the 3-month treatment-free follow-up, the VSC levels in the control group returned to baseline levels, which may suggest that the microflora had returned to its original state. In contrast, the oral probiotic group continued to see a decline in the VSC levels. This could be suggestive of *S. salivarius* M18 possibly colonising the mouth, thereby establishing a new and less pathogenic oral micro-ecology, that produced lower levels of VSCs (Burton et al., 2013a).

There have been extensive experimental studies and review articles on the use of probiotics in general dental patients (Meurman, 2005; Twetman and Stecksen-Blicks, 2008; Stamatova and Meurman, 2009; Teughels et al., 2011; Cagetti et al., 2013; Laleman et al., 2014; Gruner et al., 2016). Although probiotics have been shown to provide benefits in general health, their application in oral health, especially orthodontic patients, is still very much up for debate as well-conducted comparable studies are limited. In general, there is great variation in probiotic strains used, concentrations, dosages, administering methods, intervention times, methods for the analysis of bacteria, and different age and makeup of the study populations. This makes comparisons of findings difficult.

Considering orthodontic treatment duration is usually about two years, oral probiotics could be used as a supplemental practice but not as a substitution for a preventive dental program, which includes the use of fluoride, hygiene regimes and regular dental visits. Future well-designed randomised controlled trials with long-term follow-up are needed. Although ethically questionable, clinical outputs such as white spot lesion incidence or gingivitis would be more beneficial that the subjective and difficult to compare assessments of the past, such as GI and PI. Additionally, studies utilising molecular techniques such as microbiological gene sequencing, are needed for a better understanding of the detailed changes in the oral microflora after oral probiotic consumption.
CONCLUSION

Oral probiotic *S. salivarius* M18 could reduce VSC levels in patients with fixed appliances but did not decrease their plaque index or gingival index. However studies of much longer duration are needed to more accurately assess probiotic benefits. Although the reductions in VSC levels were statistically significant, the clinical significance of these reductions needs to be further investigated.

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CONFLICT OF INTEREST

The authors all declare no conflict of interest.
REFERENCES


Chapter 3

Oral microbiome changes with use of oral probiotic

*Streptococcus salivarius* M18 in orthodontic patients
ABSTRACT

Placement of fixed appliances in orthodontic patients can promote the formation of dental biofilms. Biofilms can lead to problems such as tooth demineralisation, gingivitis and halitosis. Commercially available dental products to prevent biofilm buildup appear to have little effect. Probiotics show promise in inhibiting biofilm growth in general dental patients, but little probiotic research has been carried out with the orthodontic population.

In this randomised, triple-blind, placebo-controlled trial, we tested the hypothesis that regular consumption of the oral probiotic *Streptococcus salivarius* M18, by patients wearing fixed appliances, will reduce the proportion of pathogenic bacteria in an individual’s biofilm. Sixty-four patients aged 12-30 years, undergoing fixed orthodontic treatment, consumed two lozenges daily, of either probiotic (n=32) or placebo (n=32) for a 1-month period.

We investigated the changes to the orthodontic patient’s oral ecosystem after probiotic or placebo lozenge consumption, by utilising next-generation sequencing of the bacterial 16S rRNA genes amplified from oral plaque samples.

The effects of the probiotic on the microbial composition were minor, suggesting that short-term probiotic use may not be effective in reducing the prevalence of potentially pathogenic microbiota in biofilms of orthodontic patients. However, the true clinical significance of these effects still deserves further long-term research with more attention given to standardised study designs, molecular techniques and clinical output measures.
INTRODUCTION

Orthodontic treatment can improve aesthetics and dental function. The placement of fixed orthodontic appliances, however, severely impedes tooth brushing and provide areas of low salivary flow that promote dental biofilm (plaque) formation. Additionally, metabolites produced by biofilms (such as acids) cannot be effectively buffered and removed by saliva due to the placement of orthodontic appliances (Øgaard, 2008). If patients fail to maintain good oral hygiene during orthodontic treatment, the biofilm formed can cause many adverse events, including enamel demineralization and gingival inflammation (Peng et al., 2014). These adverse events can occur within just a few weeks after the placement of fixed appliances, severely affecting treatment results and the patient’s quality of life (Øgaard, 2008).

Halitosis, more commonly known as bad breath, affects up to half the adult human population to various degrees (Yaegaki and Coil, 2000). Although generally not considered to be a medical concern, it certainly can confer a significant social stigma (Rosenberg, 2002). Fixed orthodontic appliances have been found to increase the plaque and tongue-coating indices significantly. A positive correlation between halitosis and the wearing of fixed orthodontic appliances has been confirmed and halitosis has been considered an important indicator of oral health during orthodontic treatment (Zurfluh et al., 2013). Some of the most common contributors to halitosis are the volatile sulphur compounds (VSCs) produced by anaerobic bacteria located on the dorsum of the tongue (Loesche and Kazor, 2002). Bacteria such as Porphyromonas gingivalis and other members of the phylum Bacteroidetes are regarded as the main producers of VSCs (Yang et al., 2013).

The measures adopted to prevent and control biofilm formation in orthodontics usually include oral hygiene instruction, application of fluoride (Benson et al., 2004), the use of antimicrobial dentifrices and mouth rinses (Chen et al., 2011), and antimicrobial modifications of orthodontic materials (Shah et al., 2011). Whilst antibiotics show effectiveness in decreasing oral bacterial adhesion, their use could lead to an imbalance of the normal oral microflora, leading to dysbiosis and drug resistance (Kouidhi et al., 2011). Although the prevention of biofilm formation in orthodontics has been studied extensively, and a variety of dental products are commercially available, the prevalence of biofilm-related adverse events such as
enamel demineralization still remain as high as 72.9% (Richter et al., 2011). The need for safe and efficacious management of biofilms is of great importance, especially in patients wearing fixed orthodontic appliances.

Probiotics, which are defined by the World Health Organization as ‘live organisms which, when administered in adequate amounts, confer a health benefit on the host’ (Burton et al., 2013a) have been shown to be effective in various approaches to biofilm management. These include reducing dental biofilm formation, preventing caries development and decreasing oral malodor (halitosis) (Rao et al., 2012; Saha et al., 2012; Cagetti et al., 2013). Traditionally, probiotics have been widely used for gut health. Probiotic mechanisms of action usually include competing with pathogenic organisms for adhesion sites, secreting antimicrobial substances (e.g. antimicrobial peptides or bacteriocins) and modulating the host’s immune response (Saha et al., 2012; Singh et al., 2013). During the last decade, an increasing number of products containing probiotic organisms have entered the market for oral health purposes and show promise in the prevention and treatment of biofilm-related oral diseases (Haukioja, 2010; Gutkowski, 2013). According to recent studies, oral probiotics can be an effective and safe therapy option for reducing dental biofilm and caries risk in children (Juneja and Kakade, 2012; Cannon et al., 2013; Burton et al., 2013a). It is important to note that the most effective oral probiotics should ideally be derived from oral inhabitants rather than microbes of intestinal origin (Wescombe et al., 2012). Several orally-derived probiotic products exist in the market today with a variety of claims including reduction of malodor. For example, the commercially-available oral probiotic *Streptococcus salivarius* strain K12 was found to decrease the VSC levels in the mouth and showed potential in the prevention and treatment of halitosis (Burton et al., 2005). Clinical research has confirmed the use of another oral probiotic strain *S. salivarius* M18 as an effective measure in the treatment of dental decay and gingival inflammation without negative impact on the normal oral microflora (Rastall et al., 2005; Burton et al., 2013a; Burton et al., 2013b).

Although many bacterial strains have been investigated over the years, the most commonly used strains for improving oral health are those belonging to the genera *Lactobacillus, Bifidobacterium* and *Streptococcus*, such as *Lactobacillus rhamnosus* GG, *Lactobacillus reuteri* ATCC 55739, *Bifidobacterium* DN-173010 (Saxelin et al., 2005) and *S. salivarius* strains K12 and M18 (Burton et al., 2013a). To a lesser extent,
Weissella cibaria also plays a part in oral health, but mainly in halitosis management (Bjorkroth et al., 2002). There have only been a few clinical studies that have found probiotic strains or products to be efficacious in the treatment of halitosis, with the most studied including S. salivarius K12 and W. cibaria (Burton et al., 2006; Kang et al., 2006).

S. salivarius K12 is known to produce two lantibiotic bacteriocins (salivaricins A and B) that are inhibitory to strains of several species of Gram-positive bacteria implicated in halitosis. The main difference between strains K12 and M18 is their inhibitory spectrum. K12 is mainly inhibitory towards Streptococcus pyogenes and upper respiratory tract pathogens (due to the production of salivaricins A and B) while M18 is less active on some of the upper respiratory tract pathogens but has strong activity against dental pathogens, in particular Streptococcus mutans (due to the production of salivaricins A, 9 and M) (Wescombe et al., 2009). Therefore, S. salivarius M18 would be an appropriate probiotic strain to assess with regard to any beneficial effects on the microbiome of orthodontic patients.

However, to date, randomised controlled trials (RCTs), the ‘gold standard’ for evaluating the effectiveness of interventions (Akobeng, 2005) have not been carried out in sufficient numbers to provide convincing scientific clinical evidence on the efficacy of oral probiotics in enhancing oral hygiene (Cagetti et al., 2013). Even less is known of the role probiotics can play in orthodontic patients. To date, only five RCTs have examined the effect of probiotics on orthodontic patients (Cildir et al., 2009; Jose et al., 2013; Pinto et al., 2013; Ritthagol et al., 2013; Gizani et al., 2015).

The study of the biofilm composition in orthodontic patients itself is scarce, with the majority of studies having investigated the changes in the microbiome of patients by using culture-based or targeted molecular methods (Choi et al., 2009; Thornberg et al., 2009; Kim et al., 2012; Torlakovic et al., 2013). These methods are limited by the variety of pathogenic species they can detect and so the composition of a typical orthodontic patient’s microbiome is still largely unknown. Only recently have researchers attempted to address this gap in the literature, with studies using open-ended molecular methods to investigate the dynamics of the oral microbiome of adolescents during orthodontic treatment (Tanner et al., 2012; Koopman et al., 2015). Using next-generation, namely Ion Torrent (semiconductor), DNA sequencing
technology to sequence 16S rRNA genes amplified from oral samples, not only enables identification of known bacterial species but also the identification of as yet unculturable microorganisms.

In the present study, the aim was to conduct a prospective, randomised, triple-blind, two-arm parallel-group, placebo-controlled study, in order to: (i) investigate if any changes to the oral microbiota are introduced by probiotic lozenges containing *S. salivarius* M18; and (ii) to determine if the regular consumption of these probiotics is effective in reducing biofilm formation, establishing a favourable balance between ‘good’ commensal bacteria and ‘bad’ potentially pathogenic bacteria in patients wearing fixed orthodontic appliances.

**MATERIALS AND METHODS**

**Participants**

Orthodontic patients aged 10-30 years wearing fixed appliances at the Faculty of Dentistry, University of Otago, New Zealand, were assessed for eligibility by the principal investigator G.B.

The inclusion criteria for this study were: at least 20 natural teeth (extraction and non-extraction patients), wearing conventional stainless steel brackets (3M Unitek) in both arches, using fluoride toothpaste not containing any supplementary antibacterial agents for daily oral hygiene. The exclusion criteria were: the presence of systemic disease (i.e. diabetes), living in a non-fluoridated area (e.g. Allanton, Waikouaiti and Mosgiel), periodontal disease, antibiotic therapy or prophylaxis, wearing lingual braces, using a toothpaste with supplementary antibacterial agents (e.g. triclosan), using a non-fluoride toothpaste, dental fluorosis, smoking, using powered toothbrushes, lactose intolerance, allergy to dairy products, and participants being physically unable to brush their teeth.

Eligible participants were informed of the study design through an information sheet and a total of 64 participants gave their written informed consent. The study protocol was approved by the University of Otago Ethics Committee (approval number H14/103) as well as the Maori Research Consultation Committee. The trial
was accepted for inclusion in the Australian New Zealand Clinical Trials Registry (ANZCTR; trial no. ACTRN12615000341527). Whilst no harm to the participants was anticipated, they were given clear instructions on their information sheet on how to alert the research team if any adverse events did occur so that their participation in the study could be terminated immediately, if necessary.

Sample size

Calculation of the sample size and statistical power were based on previous randomised, controlled trials. Type I error was set at 0.05 and type II error at 0.20 (80% power). At least 27 patients in each group (i.e. a total of 54 for two groups) were needed to detect a decrease in biofilm formation of about 30%. To account for possible withdrawals during the study, the decision was made to recruit a total of 64 patients (32 per group).

Provision of the probiotic-containing product

Lozenges containing *Streptococcus salivarius* M18 were provided by BLIS Technologies Ltd, Dunedin, New Zealand, and were manufactured to contain more than one billion viable colony-forming units (CFU) per lozenge at time of manufacture. BLIS Technologies has determined that an efficacious dose consists of at least $1 \times 10^8$ (100 million) CFU and states that their probiotic is stable enough to achieve this with one lozenge even at the end of the stipulated shelf life.

Study protocol and intervention

This prospective, randomised, triple-blind, two-arm parallel-group, placebo-controlled study was carried out between July 2015 and December 2015. Figure 3.1 shows the flowchart of the clinical trial.

Each participant was randomly allocated to the probiotic group or the control group, according to a computer-generated balanced block randomisation method, to ensure equal patient allocation to each treatment group. A block size of four was used, and it was stratified according to gender. Allocation concealment was used to avoid selection bias. Researcher M.F., who managed the random sequence, did not participate in the allocation and measurement. The dental investigators, orthodontic
patients, laboratory assistants and statisticians were all blinded to the treatments given.

The duration of the study was 4 months in total, which included a 1-month treatment intervention followed by a 3-month treatment-free follow-up to assess recurrence rates. Plaque samples were taken from the participant’s mouths at baseline (before treatment), and after one and four months post-treatment.

During the 1-month intervention, the participants in the probiotic group (n=32) consumed two probiotic lozenges containing probiotic strain, *S. salivarius* M18 (BLIS Technologies Ltd, Dunedin, New Zealand) daily. The participants in the placebo group (n=32) consumed two control (bacteria-free) lozenges daily. For blinding purposes, the packaging, appearance and taste of both lozenges was identical. The manufacturer did not reveal the identities of the treatment groups to the investigators until the intention-to-treat (ITT) analysis was performed. The use of other probiotic-containing products during the study was forbidden.

**Clinical Examination**

Plaque samples were collected by a researcher (G.B) at all time points (baseline, 1-month and 4-months post-treatment). Using sterilized dental probes, supragingival dental plaque samples were collected and pooled from buccal/labial/palatal and buccal/labial/lingual surfaces of all teeth mesial to, and including, the first molars in both upper and lower arches. The samples were transferred to a centrifuge tube containing 400 µL of Digestion Buffer (25 mM Tris-HCl, pH 8.0, 2.5 mM EDTA and 1% [vol/vol] Triton X-100)) and temporarily stored on ice within 30 minutes of sampling. Samples were then stored at -80°C until ready for isolation and purification of DNA (Appendix 14 and 18).

At the baseline appointment, following the collection of plaque samples, participants were asked to chew a plaque-disclosing tablet (Disclotabs, Colgate-Palmolive, Sydney, New South Wales, Australia) and then thoroughly remove the disclosed dental biofilm in their mouths by brushing with a new toothbrush (Colgate® Ortho, Colgate-Palmolive, Sydney, New South Wales) and toothpaste (Colgate® Cavity Protection Toothpaste, Colgate-Palmolive, Sydney, New South Wales, Australia) under the supervision of an investigator (GB). The participants then
rinsed their mouths with water and a 30-mL cup of chlorhexidine-containing mouthwash (Savacol®, Colgate-Palmolive, Sydney, New South Wales, Australia). They were then asked to suck the appropriate lozenge. A take-home pack comprising the appropriate lozenges, toothpaste, toothbrush, compliance lozenge reminder chart, pen and further instructions were given for home use. The protocol required the participants to suck two lozenges each day, one after brushing their teeth in the morning and one after brushing their teeth at night for 1 month.

Each participant provided at least one e-mail address and mobile telephone number where morning and night email and text message reminders were sent to enhance compliance with taking the lozenges. At the 1-month time point, plaque samples were collected in a similar manner. In addition, all remaining lozenges and Lozenge Reminder Charts were collected for the analysis of compliance with lozenge consumption. At the 4-month time point (3-months post-intervention), plaque samples were collected as on the previous two occasions.
Figure 3.1. CONSORT flow chart showing patient flow during the trial
Bacterial DNA extraction and purification

DNA was extracted and purified from the supragingival plaque samples using PureLink Genomic DNA Mini Kits (ThermoFisher Life Technologies) in two separate batches. Each batch contained 65 samples prepared for an Illumina Miseq sequencing run (64 samples and 1 control). The first batch were baseline samples and the second batch represented samples taken at the end of the 1-month intervention. Although samples were taken at the 4-month time point, these were not prepared for sequencing due to funding constraints. (Appendix 15 and 16).

The genomic DNA was quantified spectrophotometrically (NanoVue, GE Healthcare) and its quality assessed (by agarose gel electrophoresis on 1-2% [w/v] agarose gels) prior to sending the samples for library preparation.

Each sample, which contained at least 20 ng of purified genomic DNA at a concentration of at least 5 ng/µl was aliquoted into one of two discrete ABI Fast 96-well real-time polymerase chain reaction (PCR) plates, sealed, and packaged in dry ice. The first plate contained the baseline samples and the second plate, contained the 1-month post-treatment genomic DNA samples. Both plates were then sent for next-generation sequencing (New Zealand Genomics Limited [NZGL], Massey Genome Centre, Palmerston North, New Zealand).

16S library preparation and Illumina MiSeq DNA sequencing

The V3-V4 regions of the 16S rRNA were used by NZGL to prepare barcoded amplicon libraries for each sample. The 16S rDNA in each patient sample was PCR amplified with primers that included a unique ‘barcode’ sequence to identify the nature of the sample. This allowed all PCR products from all samples to be pooled and sequenced at once as the barcode sequence (within individual sequence reads) would identify that DNA’s source. The libraries were then pooled by equal molarity before being loaded onto the Illumina MiSeq runs. Quality control checks were carried out at each step.

Sequencing data analysis

The software Quantitative Insights Into Microbial Ecology (QIIME) version 1.5.0 was used to analyze the sequences (Caporaso et al., 2010). QIIME was used to generate a
taxonomic overview of the samples and then taxonomic profiles of the individual samples were compared using functions in QIIME to look for similarities and differences between samples via a variety of output methodologies. The overall aim in this thesis was to compare bacterial populations of the control and intervention groups at two different time points, prior to treatment and after the 1-month intervention. Later, molecular findings will be compared with the clinical findings, and detected operational taxonomic units (OTU) will be further analysed further. Widely used in large-scale characterisations of microbial communities, Operational Taxonomic Units (OTUs), by definition, are clusters of similar 16S rRNA sequences representing basic diversity units (Schmidt et al., 2014).
RESULTS

The baseline characteristics of the participants are summarised in Table 3.1. Age and sex distribution were very similar (Table 3.1).

Table 3.1. Baseline characteristics of the two groups of participants.

<table>
<thead>
<tr>
<th></th>
<th>Oral probiotics (N=32)*</th>
<th>Placebo control (N=32)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Age (N, %)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;14 years</td>
<td>14 (22%)</td>
<td>22 (34%)</td>
</tr>
<tr>
<td>≥14 years</td>
<td>18 (28%)</td>
<td>10 (16%)</td>
</tr>
<tr>
<td><strong>Sex (N, %)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>12 (19%)</td>
<td>11 (17%)</td>
</tr>
<tr>
<td>Female</td>
<td>20 (31%)</td>
<td>21 (33%)</td>
</tr>
</tbody>
</table>

* Sample B38_S38 (14-year old female) in the oral probiotic group was later excluded (see below).

Data pre-processing initial read analysis

There were 130 samples sent for processing (64 from participants and one negative control at each time point). The numbers of processed sequences per sample are shown in Table 3.2 below. The samples are presented in two columns. In the first column are ‘A’ samples and the second column are ‘B’ samples. The ‘A’ samples represent those taken at baseline (T=0) and the ‘B’ samples represent those taken 1-month post-treatment (T=1). Each number following the ‘A’ or ‘B’ represents the individual participant the sample is from (1-64 for participants, 65 for controls). It can be seen that there were approximately 9.47 million and 9.44 million total reads for the baseline (T=0) and 1-month post-treatment (T=1) samples, respectively.

Two samples, A65_S65 and B65_S65, were excluded from further analysis as they were no template (i.e. negative) controls. One patient sample (B38_S38) was excluded due to a very low number of sequence reads with poor quality, and thus this patient’s corresponding T=0 sample A38_S38 was also excluded, to ensure consistency in the comparisons of our data. This patient was a 14-year-old female in the probiotic-consuming group. The remaining 126 samples (two from each of 63 patients) were then analysed with QIIME and Phyloseq software packages.

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The number of sequence reads per sample – excluding the negative control samples A65_S65 and B65_S65 – ranged from 16,972 to 342,881 with an average of 147,651, a standard deviation of 50,060, and a coefficient of variation of 0.339. The average fraction that individual patient samples contributed to the total sequences was approximately 1.56% with a standard deviation 0.53%.

Table 3.2. Sequences per sample at two time points.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Baseline</th>
<th>After 1-month intervention</th>
</tr>
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<tbody>
<tr>
<td>A1</td>
<td>200,124</td>
<td>21.1%</td>
</tr>
<tr>
<td>A2</td>
<td>187,756</td>
<td>19.9%</td>
</tr>
<tr>
<td>A3</td>
<td>281,182</td>
<td>29.7%</td>
</tr>
<tr>
<td>A4</td>
<td>212,821</td>
<td>22.5%</td>
</tr>
<tr>
<td>A5</td>
<td>198,712</td>
<td>21.0%</td>
</tr>
<tr>
<td>A6</td>
<td>163,812</td>
<td>17.4%</td>
</tr>
<tr>
<td>A7</td>
<td>173,476</td>
<td>18.2%</td>
</tr>
<tr>
<td>A8</td>
<td>119,784</td>
<td>12.1%</td>
</tr>
<tr>
<td>A9</td>
<td>165,450</td>
<td>17.2%</td>
</tr>
<tr>
<td>A10</td>
<td>235,209</td>
<td>24.9%</td>
</tr>
<tr>
<td>A11</td>
<td>158,127</td>
<td>16.7%</td>
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<tr>
<td>A12</td>
<td>108,750</td>
<td>11.2%</td>
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<tr>
<td>A13</td>
<td>77,459</td>
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</tr>
<tr>
<td>A14</td>
<td>108,748</td>
<td>11.5%</td>
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<td>165,876</td>
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<td>80,659</td>
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<td>A17</td>
<td>112,964</td>
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<tr>
<td>A18</td>
<td>75,028</td>
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<td>108,070</td>
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<td>A21</td>
<td>77,001</td>
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<td>A22</td>
<td>81,338</td>
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<tr>
<td>A23</td>
<td>91,021</td>
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<td>A24</td>
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<td>228,361</td>
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<td>A32</td>
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<td>A41</td>
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<td>17.6%</td>
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<td>A42</td>
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<tr>
<td>A43</td>
<td>159,352</td>
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<td>A44</td>
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<td>A57</td>
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<td>15.1%</td>
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<td>13.9%</td>
</tr>
<tr>
<td>A59</td>
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<td>13.0%</td>
</tr>
<tr>
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<td>116,287</td>
<td>12.5%</td>
</tr>
<tr>
<td>A65</td>
<td>216</td>
<td>0.01%</td>
</tr>
</tbody>
</table>

(Green: high number of sequences; red: low number of sequences.)
Thus, the initial range of sequence reads for the full samples was very large. We therefore trimmed the data at 3,500 reads per sample and used these subsampled reads for subsequent analyses. An initial comparison of these subsamples with their original samples revealed that the proportions of species were comparable and thus the subsampled data were representative and used for subsequent analyses.

**Taxonomic representation of oral microbiota**

QIIME provided a detailed analysis of the oral samples and a large amount of data. Due to the complexity of the analyses, this thesis will provide a general observational overview of the findings from the sequencing procedures to provide early insight into whether the probiotics effected any change in the participants’ microbiomes. It is anticipated that the data will be further analysed in due course. As mentioned previously, the authors intend to subject the data to statistical analysis and compare the molecular findings with the clinical findings, as well as statistically analyse the operational taxonomic units (OTU) detected.

In this thesis, data representing the taxonomy of the oral microbiota of the 126 samples can be viewed at a variety of taxonomic levels ranging from kingdom down to species. In order to reduce the data complexity and for convenience, the results will be presented progressively from phylum to species level (Figures 3.2 to 3.5) at a relative abundance cut-off value of 0.5%. This means that for any OTU, (i.e. species) to be included in the analysis, 5 in 1,000 of the total number of sequence reads for that particular oral sample is required. In other words, an OTU that is present at less than 0.5% of the total number of OTUs will not be included in the analysis.

Figure 3.2 shows the distribution of phyla across the 126 oral samples with the different colours representing different phyla. Despite the obvious complexity of Figure 3.2, five phyla are common to all samples (from most to least abundant): Firmicutes (i.e. Gram-positive bacteria) > Bacteroidetes > Fusobacteria > Proteobacteria > TM7. These five phyla, are known to be abundant in the oral cavity as evidenced by earlier diversity studies using next-generation DNA sequencing (Keijser et al., 2008; Zaura et al., 2009). These results confirmed that samples obtained from orthodontic patients were representative of the oral cavity and there appeared to be little environmental (i.e. non-oral) contamination.
Fig 3.2. Distribution of phyla (Y-axis) from all 126 oral samples (X-axis) sequenced in this study.
Figures 3.3 to 3.5 show the distribution of phyla, genera and species, respectively, between the probiotic and control groups at baseline and 1-month time points. The upper left bar chart represents probiotic group samples at baseline and the upper right bar chart represents the placebo-control group samples at baseline. The lower left bar chart represents probiotic group samples taken after the 1-month intervention and the lower right bar chart represents the placebo (control) group samples taken after the 1-month intervention. Horizontal comparisons reveal differences between the two treatment groups, and vertical comparisons show the differences between baseline and 1-month post-treatment. Note that in Figures 3.3 and 3.4, the left-most bar of each of these bar charts represents accumulated unassigned genera or species, respectively.

1. Phylum level

The distribution of phyla is shown in Figure 3.3 for each study group (probiotic and control) at baseline and 1-month after intervention. There are few differences between the groups at baseline, suggesting randomisation was adequate. Five main phyla (Firmicutes, Fusobacteria, Proteobacteria, Bacteriodetes and Actinobacteria) were present in both groups in large abundances, while five other phyla (Spirochetes, TM7, Synergistetes, GN02 and SR1) were only found in relatively low abundance (Figure 3.3). Firmicutes were the most dominant phylum found among the two study groups at both time points. In the probiotic group, there was a decrease in the level of Firmicutes, relative to baseline, with a concomitant increase in the Proteobacteria between the two time points (Figure 3.3). On the other hand, the relative abundance of Bacteroidetes increased with a corresponding drop in Actinobacteria levels (Figure 3.3).
Fig 3.3. Phylum-level taxonomic profiles of probiotic and control groups at baseline and post-treatment, 0.5% abundance threshold
2. Genus level

Results at the genus level are shown for each study group (probiotic and control) at baseline and 1-month after the intervention in Figure 3.4. They are representative of species known to inhabit supragingival sites in the mouth. At baseline, both probiotic and control groups were comparable, although the probiotic group had slightly less *Veillonella*, *Selenomonas*, *Rothia* and *Neisseria* than the probiotic group and slightly more *Fusobacteria*, *Prevotella* and *Corynebacteria* (Figure 3.4).

After the 1-month intervention, the probiotic group shows a decrease in several genera, namely *Veillonella*, *Selenomonas*, *Prevotella*, *Leptotrichia*, *Corynebacterium*, *Capnocytophaga* and an increase in *Aggregatibacter*, *Cardiobacterium*, *Fusobacterium*, *Haemophilus*, *Rothia* and TG-5.

After 1-month, the control group also shows a decrease in *Veillonella*, *Selenomonas*, and *Streptococcus* like the probiotic group, but also shows a decrease in *Actinomyces*, *Neisseria* and *Rothia*. This group shows an increase in *Haemophilus*, *Fusobacterium*, and *Cardiobacterium*, like the probiotic group, but also an increase in *Capnocytophaga* and *Lautropia*.
Fig 3.4: Genus-level taxonomic profiles of probiotic and control groups at baseline and post-treatment, 0.5% abundance threshold.
3. Species level

The distribution of species with a relative abundance of >0.5% (i.e. >5 in every 1,000 sequences) for each study group at baseline and at 1 month post-intervention is shown in Figure 3.5. The administration of the probiotic strain appeared to have had little effect on the microbiota (Figure 3.5). Interestingly, most of the sequence reads were included in the “unassigned” group (see leftmost bar of each chart). At baseline, both groups had comparable levels of each species although the probiotic group had slightly elevated levels of Streptococcus sobrinus, Selenomonas noxia and Prevotella melaninogenica when compared to the control group. It had decreased levels of Rothia dentocariosa. Any differences were not deemed to be statistically significant.

After 1 month, the probiotic group had a slight increase in Rothia dentocariosa and Haemophilus parainfluenzae and a reduction in Veillonella dispar, Selenomonas noxia and Prevotella melaninogenica. The control group also had an increase in Haemophilus parainfluenzae but a decrease in Rothia dentocariosa and a decrease in Veillonella dispar and Selenomonas noxia as in the probiotic group.
Fig 3.5: Species-level taxonomic profiles of probiotic and control groups at baseline and post-treatment, 0.5% abundance threshold.
DISCUSSION

A number of reviews have been published over the past decade to demonstrate the effects of probiotics on general oral health of dental patients (Meurman, 2005; Twetman and Stecksen-Blicks, 2008; Stamatova and Meurman, 2009; Teughels et al., 2011; Cagetti et al., 2013; Laleman et al., 2014; Gruner et al., 2016). However there is a great variation within these studies that make comparisons difficult and unreliable. Some of these variations include differences in the probiotic strains, the concentrations of bacteria per dose, the, dosage regimen, intervention times, sampling sites, delivery methods, and ages and composition of the study populations. Moreover, there have been different molecular methods for the analysis of bacterial microbiota. Extrapolating findings from a general dental population to an orthodontic population is unwise considering the different oral environment in orthodontic patients with intra-oral appliances. Hence, there is a need to investigate the use of probiotics in an orthodontic patient cohort as these patients experience a much higher level of plaque accumulation resulting from orthodontic appliances. Due to the variances in these studies, this discussion will focus mostly on comparisons of results obtained in this study with the 5 studies published on orthodontic patients to date (Cildir et al., 2009; Jose et al., 2013; Pinto et al., 2013; Ritthagol et al., 2013; Gizani et al., 2015).

The aim of this study was to assess the effects of an orally-administered probiotic, *Streptococcus salivarius* M18, on the microbiome of orthodontic patients. To the best of our knowledge, it is the first clinical trial to utilize a probiotic of oral (rather than intestinal) origin in orthodontic patients, and to assess the microbes using next-generation DNA sequencing technology.

**Participation rates and compliance**

A strength of this study was the zero withdrawal rate and the good overall compliance of participants. The zero withdrawal rate could have been attributed to the utilisation of e-mail and text message reminders (provided 24 hours prior to scheduled appointments), as well as the mailing of an appointment card. Good compliance could be due to daily morning and night text message reminders during the period of the intervention, as well as the provision of a Lozenge Reminder Chart.
Bias

The random assignment of participants into the two groups protected against selection bias as the randomisation was computer-generated. Participants in both treatment groups were blinded as to whether their lozenge was the placebo or probiotic due to identical manufacturing protocols for both sets of lozenges. The clinician was blinded to the group of each patient, thereby preventing differential treatment of the two groups. The statistician and personnel carrying out the laboratory-based work and bioinformatics were all blinded to ensure objective analysis of the results. Only once the raw data had been processed and the results of analyses produced were all research team members informed (by BLIS Technologies) as to which group was receiving the probiotic lozenge. Collectively, these factors minimised any bias and allowed a much greater confidence to be placed in the results of this trial.

Molecular methods

Oral biofilms, like any biofilms in nature, are very complex microbial communities. Traditional culture-based microbiological techniques have uncovered many species, some of which are found in high abundance in the oral cavity. The major benefits of next-generation sequencing (NGS) techniques over traditional microbiology is the ability to detect non-cultur able microorganisms and that these low-abundance microorganisms can be detected by their molecular signatures (Muyzer and Smalla, 1998). Given the complexity of the oral microbiota implicated in biofilm-related diseases such as halitosis, gingivitis and white spot lesions (Socransky and Haffajee, 2002; Kazor et al., 2003; Socransky and Haffajee 2005; Taipale et al., 2012; Tanner et al., 2012), next-generation molecular methods are useful in providing enough sensitivity to detect some of the critical bacterial changes in patients and can provide a more in-depth microbial profile analysis of biofilm samples.

This study is the first to use NGS with an orthodontic population. The data analysed and the associations made, will be a valuable addition to the microbiological, dental and orthodontic literature. Due to the complexity of the data generated by QIIME and Phyloseq, and also due to time constraints, this thesis will focus only on a small proportion of the overall findings collected in this study.

The Phyloseq package employed was useful in its ability to generate a variety of
graphical outputs for the dataset. It does, however, have some issues in the way in which the data are presented, such as the single taxonomy plots reordering the taxonomic assignments from the most abundant to the least, which is something QIIME does not do. This can make identification a little complex. However, the ability to generate plots such as such as the bar charts representing the taxonomy in this study is a strength.

Effects of probiotic consumption on the oral microbiome

The results from the trial presented in this thesis showed that consumption of probiotic lozenges for 1 month has little effect on the patient’s microbiome.

From a microbiological perspective there is interest in the changes in pathogens already known to dentists as well as in new, unculturatable species. The main pathogens associated with gum diseases such as gingivitis and periodontitis are *P. gingivalis, Treponema denticola, Tannerella forsythia* and *Aggregatibacter actinomycetemcomitans* (Ishikawa et al., 2003). Many bacterial species have been suggested as the main causes of halitosis, with volatile sulphur compounds (VSCs) produced by these bacteria contributing to the malodour (Kang et al., 2006). *Fusobacterium nucleatum* and other proteolytic anaerobes are the main suspects (Burton et al., 2005). The most common cariogenic bacteria are the mutans streptococci (mainly *Streptococcus mutans*), which initiate the caries process and lower the pH to below the critical pH of 5.5 (at which enamel begins to demineralise), and the lactobacilli, which then establish as the oral pH drops to ~4.5-5.0.

Most microbiological studies have used culturing techniques rather than molecular techniques, and so the identity of all the bacteria in plaque (or saliva) is largely unknown. Past studies on probiotic effects have mainly assessed levels of *S. mutans* and lactobacilli, thereby providing a better understanding of the probiotic role in caries rather than gingival health and halitosis.

In three of the five probiotic studies performed on an orthodontic population, the strains *Lactobacillus paracasei* SD1 (Ritthagol et al., 2013), *Bifidobacterium animalis* subsp. *lactis* DN-173010 (Cildir et al., 2009) and a probiotic of unknown strain and concentration (Jose et al., 2013) significantly decreased the levels of mutans streptococci when delivered in milk, yoghurt, curd and toothpaste respectively.
Analysis of similar studies using *Bifidobacterium* probiotic strains in patients without orthodontic braces, indicates that it is likely there would be a similar reduction in salivary mutans streptococci in orthodontic patients consuming yoghurt containing the probiotic *Bifidobacterium animalis* subsp. *lactis* DN-173010 daily (Cildir et al., 2009) rather than no change as found by Pinto and colleagues who used the same strain (Pinto et al., 2013).

The contrasting results of these two studies above, despite their similar designs and sample sizes, could be attributed to the yoghurt used to deliver the probiotic. The yoghurt may influence the cariogenic potential of the microbiota and colonisation of the oral cavity by the probiotic strain (Pinto et al., 2013). Studies using dairy products to deliver their probiotic are more commonly associated with a reduction in mutans streptococci. However, it should be noted that studies using *Bifidobacterium* have shown less consistent outcomes over time compared to those employing *Lactobacillus reuteri* and *Lactobacillus rhamnosus* strains in recent reviews on dental patients without fixed appliances.

On the other hand, Ginzani and colleagues, using a strain of *L. reuteri*, found no difference in the levels of mutans streptococci and lactobacilli in saliva, or any difference in the incidence of white spot lesions in orthodontic patients (Gizani et al., 2015). This study used lozenges as the mode of delivery, had a good sample size and intervention period of 17 months. It was also the first study to report white spot lesion incidence as an outcome, rather than the traditional reporting of surrogate measures of caries such as mutans streptococci and lactobacillus levels. The shortcomings of using such surrogate measures is discussed below.

As previously mentioned, the detection of specific bacteria is limited by the molecular techniques used. The majority of studies have opted for chairside kits or microbial culture, possibly due to financial constraints and possibly the unavailability of next-generation sequencing services. Jose and colleagues (Jose et al., 2013), represents one of the few groups to have used the sensitive and reproducible quantitative real-time polymerase chain reaction (qPCR) technique to examine the levels of *S. mutans* in a cohort of 60 orthodontic patients who consumed a probiotic for 30 days. In that study, a significant decrease in *S. mutans* levels was observed. However, a dairy product was used as the mode of delivery and the probiotic strain
was not disclosed.

The lack of significant changes in the microbiota of our participants in both groups is contrary to the results of other researches who showed a significant decrease in mutans streptococci when probiotics were tested in non-orthodontic patients. The consumption of *L. rhamnosus* GG seems to decrease mutans streptococci counts when consumed for 3 weeks in the form of cheese or for 7 months when delivered in milk (Nase et al., 2001; Ahola et al., 2002). Similarly, *L. reuteri*-containing products have shown this reduction in mutans streptococci levels (Nikawa et al., 2004; Caglar et al., 2005; Çaglar et al., 2006; Caglar et al., 2007; Caglar et al., 2008).

These differences may be explained by the differences in study designs. Using the study by Calgar and colleagues (Caglar et al., 2007) as an example, saliva samples were assessed rather than plaque samples, participants were asked to chew gum three times daily after meals for three weeks and used a different probiotic strain. It is known that saliva is abundant in *S. mutans*, whereas plaque from teeth is less likely to have high concentrations of *S. mutans* unless the participant is experiencing early caries and the pH is decreasing thereby providing an environment in which *S. mutans* flourishes (Marsh, 2009). These factors alone could have contributed to the difference in results independent of the difference in the oral conditions of a non-orthodontic dental population compared to an orthodontic one.

DNA fragments representing *S. salivarius* were present in samples from both groups, albeit in small numbers, this was to be expected as *S. salivarius* is more commonly found in saliva samples and on the tongue. Its detection in plaque samples is possibly due to saliva coating the oral surface. It would be interesting to assess the abundance of *F. nucleatum*, *W. cibaria* and proteolytic anaerobes as these microbes play a role in halitosis, which is a common issue in orthodontic patients, although samples from the tongue would be more appropriate for this.

Masdea and colleagues found that *S. salivarius* K12 has antimicrobial activity against bacteria involved in halitosis. They found that *S. salivarius* K12 suppressed the growth of all Gram-positive bacteria they tested, but the bacteria were inhibited to varying degrees (Masdea et al., 2012. *Eubacterium sulci* ATCC35585 was the most sensitive strain, while *Solobacterium moorei* was inhibited less (Masdea et al., 2012).
**Surrogate measures of caries**

Although the majority of probiotic studies have examined the effect of certain probiotic strains on surrogate measures of caries incidence, such as *S. mutans* levels, for obvious ethical and budget reasons, only a handful of studies have examined, more usefully, caries as an outcome measure. In our study we took clinical photos at each time point of each participant so that assessment of white spot lesions (early caries) as an end point can be investigated in this population after their orthodontic treatment.

One may question the value of studies that publish microbiological data over caries incidence as there is concern regarding the validity of using *S. mutans* levels alone as an indicator of caries. This is mainly due to the discovery that caries can occur independently of *S. mutans* (Gross et al., 2012; Tanner et al., 2012).

**Microbial sampling method**

Rather than sampling plaque, we could have sampled saliva. An earlier study has shown that the composition of salivary microbiota is representative of that found on dorsal and lateral surfaces of the tongue (Mager et al., 2003), which suggests that this method would have been appropriate to detect halitosis-causing microorganisms. Saliva samples for microbiological studies on gingival health and caries have also been used with success. However, a limitation may be that the saliva composition represents the entire oral microbiota, so changes on tooth surfaces and gingiva could be missed. Therefore we deemed our method of pooling the plaque of all surfaces of all teeth with braces as the most suitable to provide an accurate representation of the biofilm profile of each patient at the site most likely to experience the most deleterious effects of biofilm accumulation (white spot lesions around brackets, and gingivitis).

**Individual variability**

It is thought that the participant’s response to probiotic treatment could be influenced by their disease stage and the microbes occupying the diseased site (Devine and Marsh, 2009).
In this study, the numbers of bacteria in each sample were shown as a proportion of each sample’s total bacterial count. It would be beneficial to have quantifiable bacterial numbers to make a statistical rather than an observational comparison between subjects at different time points and between the two groups. Additionally, understanding the proportion and type of bacteria present is useful, but we are unaware of the relevance of this by not knowing how these bacteria interact within their host environment.

**Influence of gender on results**

In the present study, the female: male participant ratio was 2:1. There was no difference between genders in their compliance with consuming the lozenges. Furthermore, the probiotic did not perform better in either gender, although the female participants generally had better oral hygiene status than the male participants which may result in less deleterious effects from the accumulation of biofilm during orthodontic treatment.

**Pre-treatment regime**

A pre-treatment regime using the broad-spectrum antimicrobial chlorhexidine before the consumption of the probiotic lozenge was used in this study. The rationale for this regime was to rid the mouth of its bacterial load thereby allowing the ‘good’ probiotic bacteria to colonise the oral microbial surfaces during administration (Aminabadi et al., 2011). The pre-treatment was deemed to have little lasting effects as a previous study on the effect of chlorhexidine on the oral microbiota showed that microbial populations could recover to pretreatment levels within 24 hours (Rindom Schiøtt et al., 1970). The mouth rinse was used by both groups so any changes caused by the chlorhexidine should have been similar.

**Duration of the study**

There has been a huge variation in the duration of treatments reported in previous studies using probiotic strains. Since plaque builds up around orthodontic braces within 24 hours, we were confident that a 4-week intervention with a 3-month follow-up was sufficient to provide a glimpse as to whether probiotics could provide a beneficial effect on plaque biofilm. However, based on the results obtained in this study, if the intervention period had been extended more meaningful conclusions may
have been obtained. This notion is supported by some authors who have seen a significant reduction in plaque of children only after three months and suggest that an intervention period longer than 1 month is necessary for an effect to be obvious (Burton et al., 2013a). However, studies such as this were in patients without braces, where the oral environment is quite different and plaque build-up is a lot slower.

Studies of probiotic use in non-orthodontic dental populations are usually focused on whether consumption of the probiotic has any beneficial effect on the oral health of the host. There is also interest in whether these beneficial effects persist after cessation of consumption, i.e. whether the probiotic strain colonises the oral cavity for long periods (Burton et al., 2013a).

Since orthodontic treatment is usually of 1-1.5 years in duration, and the larger levels of biofilm assault only last this long, we were more interested in the benefit provided to the patient with the regular consumption of the probiotic for the duration of orthodontic treatment. This is fitting, as the current evidence suggests that when the consumption of probiotics ceases, the probiotic bacteria are slowly eliminated from the mouth (Burton et al., 2013a) through natural shedding via salivary flow. Although it would be beneficial to see a persisting positive action of probiotics in biofilms after use has stopped, it is not imperative to argue for continuing probiotic use in the orthodontic population.

**Confounders**

We did our utmost to limit confounders in this study. While we believe confounders were minimised, it was impossible to monitor each participant’s every move. There are a number of factors that may have influenced plaque build-up and the presence of pathogenic bacteria. Some of these include: the diet of participants; home access to additional antibacterial products; stimulation of saliva by chewing sugar-free gum; changes in brushing and flossing technique; and swallowing or chewing the lozenge rather than sucking it, thereby washing away the probiotic from the mouth. The type of orthodontic auxiliaries used by the respective postgraduate orthodontic students during participants’ orthodontic treatment may have also had an effect on the results.
**Future work**

Further analysis of the data by a biostatistician could well provide interesting insight into the composition of the orthodontic biofilm. Our molecular methods enabled a highly sensitive technique to identify species that are difficult to discover with traditional methods. Although a full analyses of the data could not be completed, there should be a number of interesting observations when the data is analysed further. It should be stated that due to resource constraints, only the oral samples from baseline and 1-month time points were processed and subjected to next-generation DNA sequencing. Since we obtained plaque samples from the participants 3 months after the 1-month intervention, it would be interesting in the future to assess these adequately stored samples, to see whether the probiotic strain used in our study persisted in the oral cavity well after its consumption.

**CONCLUSION**

Sucking probiotic-containing lozenges twice daily for one month did not change the microorganism compositions in the plaque samples from our orthodontic cohort significantly. Therefore, it is not clear if sucking probiotic lozenges over a short term would reduce caries or gingivitis risk in orthodontic patients.

Probiotic lozenge consumption, even as a supplemental practise, should not be a substitute for a comprehensive preventive dental programme that includes the application of fluoride, hygiene practices and regular dental visits. The evidence regarding the benefits of the use of probiotics in the prevention of dental disease is limited and studies of longer duration, ideally with clinical output measures, are needed before their routine use can be justified for oral disease prevention.

Oral diseases are difficult to treat due to their polymicrobial causes, non-specific aetiology, high cost of adjunctive microbiological testing, and the required skills for data interpretation. Before probiotic treatment is dismissed, future molecular methods that do not just determine the microbial taxa present in oral biofilms, but also help us to understand the functions and activities of each microbial sub-community within the biofilm, would be most beneficial.
In conclusion, although probiotics have been known for their benefits in general (intestinal) health, their application in oral health, especially with orthodontic patients, is still very much under development and well-conducted comparable studies are scarce. Therefore, well-designed randomised controlled (preferably longitudinal) trials and the utilisation of advanced molecular techniques are needed to provide further scientific clinical evidence on this promising, yet poorly understood topic (Cagetti et al., 2013).

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CONFLICT OF INTEREST

The authors all declare no conflict of interest.
REFERENCE


Chapter 4

General discussion
Introduction

Biofilm formation around fixed orthodontic appliances causes a number of clinical consequences, such as enamel demineralisation (white spot lesions), gingival inflammation and halitosis (Kang et al., 2006; Srivastava et al., 2013; Zurfluh et al., 2013; Ren et al., 2014). Patients undergoing orthodontic treatment have a higher risk of biofilm-related complications due to impeded oral hygiene, compromised natural oral cleansing and extra retentive surfaces provided by fixed appliances (Lee et al., 2001; Ogaard, 2008). Traditional methods of biofilm removal include mechanical cleaning (e.g. tooth brushing and flossing) and chemical therapy (e.g. fluoride toothpaste, antibacterial mouthwashes). However these methods are not entirely reliable at reducing biofilm formation and so the prevalence of biofilm-induced clinical issues such as white spot lesions remain high (Srivastava et al., 2013; Ren et al., 2014).

Oral probiotics, on the other hand, can exert their effects on the biofilm by shifting the micro-ecological balance to favour beneficial non-pathogenic bacteria (Reid et al., 2011). It is apparent that the role of probiotics in oral health is gaining acceptance (Meurman, 2005; Twetman and Stecksen-Blicks, 2008; Stamatova and Meurman, 2009; Teughels et al., 2011; Laleman et al., 2014; Gruner et al., 2016). Oral probiotics may be beneficial for managing biofilm formation in patients with fixed appliances. In this triple-blind randomised controlled trial, we investigated the efficacy of oral probiotic Streptococcus salivarius M18 on plaque index (PI), gingival index (GI) and halitosis measurements in patients with fixed appliances.

The effect of oral probiotics on PI and GI

After a 1-month intervention, the PI and GI did not change significantly in the oral probiotic group or placebo-control group, at all time points (baseline, 1-month and 3-month) (Chapter 2). This may be due to the relatively short intervention period (only 1-month active treatment and 3-month treatment-free follow-up) or the inability for S. salivarius M18 to exert its effects on the biofilm (Chapter 3). Similarly the taxonomic profiles showed little changes between probiotic and placebo-control group after the 1-month intervention. The similar taxonomic profiles between groups at baseline, suggest that the sampling and randomisation was carried out well (Chapter 3).
The effect of oral probiotics on halitosis

The volatile sulphur compounds (VSCs) produced by oral biofilms, especially anaerobes located on the dorsum of the tongue, are considered one of the most common contributors to halitosis (Loesche and Kazor, 2002). The VSC levels in orthodontic patients have been found to be relatively higher than populations without fixed appliances (Babacan et al., 2011; Sokucu et al., 2016).

In the study, the VSC levels of the probiotic group significantly decreased after 1 month of consuming two oral probiotic lozenges daily, and decreased the levels even further after a 3-month treatment-free follow-up (Chapter 2). This may be due to the probiotic being able to effectively neutralise the pathogens responsible for halitosis (e.g., many Gram-negative bacteria), or perhaps that *S. salivarius* M18 is able to remain longer at the site where these halitosis-causing pathogens are found—the tongue (Chapter 3). Since we did not sequence our 3-month post-intervention samples, we were unable to analyse the changes in the microbiota over this time period to see if there were any distinct changes that correlated with these clinical findings. However, the similar reduction in VSC levels of both probiotic and placebo-control groups after 1-month was supported by a similar distribution of species in both groups with a relative abundance of >0.5% at this time point.

Probiotics have been used as a treatment modality in dentistry, with early studies using a probiotic such as *S. Salivarius* K-12 showing promise and warranting further investigation. Analysing the microbial profiles of the biofilm with molecular techniques such as by direct PCR-based amplification of the 16S ribosomal RNA gene, followed by sequencing of the PCR products using next-generation DNA sequencing (Ion Torrent) technology is not done routinely. This technique provides a ‘gold standard’ for bacterial identification and ultimately allows one to identify the known species in an oral biofilm and possibly identify microorganisms that cannot be cultured. These methods improve our understanding of microbiota-host interactions, and will impact the future development of diagnostic tests. So far, all efforts have been focused on identifying members of the oral microbiome with a taxonomic survey.
Clinical implications and future research

Oral probiotic *S. salivarius* M18 was found to improve halitosis in patients with fixed orthodontic appliances in this study, but it did not reduce plaque or gingival indices. The probiotic had little effect on taxonomic profiles and ultimately the long-term effectiveness of *S. salivarius* M18 is still unclear.

Future studies should be of longer duration, with interventions longer than 3-months. Previous studies evaluating caries surrogates are not helpful as some patients that have an abundance of these bacteria, have little experience of adverse effects. Similarly, although molecular methods such as next generation sequencing enable us to provide evidence of bacteria unexposed by culture methods, they merely show us taxonomic profiles and abundances of certain bacteria. This is useful for identifying new species, but since it is becoming clearer that probiotics have different effects on different individuals, it is perhaps the interactions within the individual biofilms that are of more importance when concluding on their effects. Once we are able to discern these, we should have a better understanding of their potential. Additionally, conducting long-term studies that assess clinical outcomes would be of huge benefit, although ethical considerations around this may be a hindrance.

Although probiotics administered by different modes of delivery have largely shown beneficial results, comparisons of delivery methods, concentrations and assessing how long they remain in the oral cavity, would be worthwhile. So far, observation of the current literature suggests dairy based delivery methods show the most promise at having an effect in the oral cavity.

*Streptococcus salivarius* K12 and M18 are the only probiotics to have been developed from the mouth, for the mouth. Albeit a small number, the majority of studies investigating these probiotic strains have shown positive results, however the strain was developed and patented in New Zealand, which has possibly made its obtainability for research more difficult. The commonly available *L. reuteri, L. rhamnosus* and Bifidobacterium species in European countries are used in the majority of research on this topic. Therefore it would be worthwhile conducting a well-designed, high-powered study to compare strains of probiotics and their effects on disease incidence.
The combination of a probiotic such as *S. salivarius* M18 with a Lactobacillus probiotic may have synergistic effects. Utilising an antibacterial sugar substitute such as Xylitol or supplementation with fluoride or Casein phosphopeptide-amorphous calcium phosphate (CPP-ACP), could hold potential too. It seems that a single probiotic strain may be lacking the broad-spectrum effect needed to positively influence the majority of the bacterial population in an individual host’s microbiome.

Most importantly however, future trials should begin to follow a systematic method of conducting and reporting their studies so that research around the globe can be compared adequately and ‘apples’ can be compared with ‘apples’ so quicker progress can be made on the clinical effectiveness of probiotic use in dental and orthodontic populations.
REFERENCES


Summary
Summary

Oral biofilms cause several clinical problems in general dental patients and orthodontic patients. Fixed appliances make it difficult to maintain good oral hygiene, compromise natural oral cleansing and provide additional biofilm retention areas. This contributes to an increased risk of biofilm-related problems such as enamel demineralisation, gingivitis and halitosis. Traditional mechanical and chemical biofilm management approaches all have limitations.

Probiotics pose a promising method for managing biofilm formation. Chapter 1 reviews the historical perspectives and proposed mechanisms of action of probiotics and discusses the various strains used in general oral health and orthodontics. It also scrutinises past trials that report their effects. From this review, we outline the limitations of past studies and provide guidance on possible future directions and considerations for further studies on oral probiotics. In this trial we intended to address many of the shortcomings that are apparent in past studies to get a more accurate idea of the effects of probiotics in patients undergoing orthodontic treatment.

Using a triple-blind randomised placebo-controlled clinical trial, we assessed the effects of oral probiotics on clinical parameters such as plaque index (PI), gingival index (GI) and volatile sulphur compounds (VSCs) in Chapter 2. We found no significant difference in GI and PI between the two groups after the 1-month intervention or at the 3-month treatment free follow-up. There was a statistically significant reduction in VSC levels in the oral probiotic group at all time points. This suggests that the oral probiotic may improve bad breath in orthodontic patients.

In order to better understand the effects of probiotic therapy on the oral microbiome of orthodontic patients, Chapter 3 investigated the changes of oral microflora using Next Generation Gene Sequencing. Taxonomic profiles of probiotic and place-controlled groups were similar at baseline and after intervention, suggesting S. salivarius M18 had little effect on microbiota. The 1-month intervention period was very short. Future longer duration, well-designed studies, assessing clinical outputs are needed before orthodontists can justify biofilm management with probiotics in patients undergoing fixed appliance treatment.

Clinical relevance: Probiotic use may help with combating halitosis in orthodontic patients but can’t be a substitute for standard oral care.
Appendices
## Appendices

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Appendix 1. Independent peer review and correspondence

23rd June 2014

Dear Dr Mei,

Re: Efficacy of the oral probiotic *Streptococcus salivarius* in managing biofilm formation in patients wearing fixed orthodontic appliances: A double-blind randomised placebo-controlled trial.

Thank you for asking me to critically evaluate your research protocol. Your project looks interesting and clinically significant to the field of Orthodontics. The study design seems appropriate and the method is well constructed.

However I encourage you to consider the following points:

1) Exclusion criteria
   a) Will you consider participants who live in both fluoridated and non-fluoridated areas in Dunedin as fluoride can act to reduce biofilm levels?
   b) Will you consider participants who are unable to brush their teeth themselves (e.g. special needs, physical disabilities, etc.). These participants could be disadvantaged in their ability to remove plaque from their teeth (without external assistance).

2) 1-month treatment intervention

   Will this be a long enough period to gain meaningful results? The minimal time period required to detect a clinically significant effect does not seem clear in your proposal.

3) “If any serious adverse events are observed in any of the participants, the study will be terminated immediately”.

Can you please elaborate on the nature and likelihood of these potential risks?

I hope this feedback is helpful, and I wish you all the best in your proposed research.

Kind Regards

Joseph Antoun

Senior Lecturer
Discipline of Orthodontics
University of Otago, New Zealand
E: joseph.antoun@otago.ac.nz
P: +64 3 479 7071
F: +64 3 479 7070
10th July 2014

Dear Dr Antoun,

Many thanks for your feedback with regards to our research protocol. I will address each issue under the heading you provided.

1) Exclusion criteria.

a) Will you consider participants who live in both fluoridated and non-fluoridated areas in Dunedin as fluoride can act to reduce biofilm levels?

This is a valid point. We will exclude any participant living in Allanton, Waikouaiti and Mosgiel as these areas are non-fluoridated and the participants could provide biased results.

b) Will you consider participants who are unable to brush their teeth themselves (e.g. special needs, physical disabilities, etc.). These participants could be disadvantaged in their ability to remove plaque from their teeth (without external assistance).

Participants who are unable to physically brush their teeth will be excluded from the study as they could affect the reliability of results.

2) 1-month treatment intervention.

Will this be a long enough period to gain meaningful results? The minimal time period required to detect a clinically significant effect does not seem clear in your proposal.

Studies carried out by other authors (Burton et al., 2013), suggest that a 1-month intervention will be sufficient to obtain reliable results on the effect of Streptococcus salivarius on biofilms.

3) “If any serious adverse events are observed in any of the participants, the study will be terminated immediately”. Can you please elaborate on the nature and likelihood of these potential risks?

The lozenges we intend to use, will contain probiotic strain Streptococcus salivarius M18. All humans have Streptococcus salivarius in their oral cavity. Streptococcus salivarius are common in the mouth and make up to 40% of all the bacteria in the normal healthy mouth.

However the probiotic product provided by BLIS technologies Ltd, is made on equipment that also processes milk, soy and wheat. Any adverse events are likely to be associated with milk, soy, wheat protein allergies. The prevalence of these allergies is in the range of 1-2% (Kattan et al., 2011), which suggests the chances of them occurring is extremely low.

Once again, many thanks for your feedback.

Kind Regards

Dr Peter Li Mei

Appendix 2. Ethics approval

Dr L Mei
Department of Oral Sciences
Faculty of Dentistry

19 September 2014

Dear Dr Mei,

I am again writing to you concerning your proposal entitled “Efficacy of the oral probiotic Streptococcus salivarius in managing biofilm formation in patients wearing fixed orthodontic appliances: A double-blind randomized placebo-controlled trial.”, Ethics Committee reference number H14/103.

Thank you for your e-mail of 16th September 2014 with attached modified application addressing the issues raised by the Committee.

On the basis of this response, I am pleased to confirm that the proposal now has full ethical approval to proceed.

The standard conditions of approval for all human research projects reviewed and approved by the Committee are the following:

Conduct the research project strictly in accordance with the research proposal submitted and granted ethics approval, including any amendments required to be made to the proposal by the Human Research Ethics Committee.

Inform the Human Research Ethics Committee immediately of anything which may warrant review of ethics approval of the research project, including: serious or unexpected adverse effects on participants; unforeseen events that might affect continued ethical acceptability of the project; and a written report about these matters must be submitted to the Academic Committees Office by no later than the next working day after recognition of an adverse occurrence/event. Please note that in cases of adverse events an incident report should also be made to the Health and Safety Office:

http://www.otago.ac.nz/healthandsafety/index.html

Advise the Committee in writing as soon as practicable if the research project is discontinued.
Appendix 3. Maori consultation

Ngāi Tahu Research Consultation Committee
Te Komiti Rakahau ki Kai Tahu

Tuesday, 19 August 2014.
Dr Li Mei,
Faculty of Dentistry - Department of Oral Science,
DUNEDIN.

Tēnā koe Dr Li Mei,

Efficacy of the oral probiotic Streptococcus salivarius in managing biofilm formation in patients wearing fixed orthodontic appliance: A double-blind randomized placebo-controlled trial

The Ngāi Tahu Research Consultation Committee (the committee) met on Tuesday, 19 August 2014 to discuss your research proposal.

By way of introduction, this response from the Committee is provided as part of the Memorandum of Understanding between Te Rūnanga o Ngāi Tahu and the University. In the statement of principles of the memorandum it states “Ngāi Tahu acknowledges that the consultation process entailed in this policy provides no power of veto by Ngāi Tahu to research undertaken at the University of Otago”. As such, this response is not “approval” or “mandate” for the research, rather it is a mandated response from a Ngāi Tahu appointed committee. This process is part of a number of requirements for researchers to undertake and does not cover other issues relating to ethics, including methodology that are separate requirements with other committees, for example the Human Ethics Committee, etc.

Within the context of the Policy for Research Consultation with Māori, the Committee base consultation on that defined by Justice McEchlan:

“Consultation does not mean negotiation or agreement. It means: setting out a proposal not fully decided upon, adequately informing a party about relevant information upon which the proposal is based; listening to what the others have to say with an open mind (in that there is room to be persuaded against the proposal); undertaking that task in a genuine and not cosmetic manner. Reaching a decision that may or may not alter the original proposal.”

The Committee considers the research to be of importance to Māori health.

As this study involves human participants, the Committee strongly encourage that ethnicity data be collected as part of the research project. That is, the questions on self-identified ethnicity and descent, these questions are contained in the latest census.

The Committee suggests dissemination of the findings to relevant Māori health organisations, for example the National Māori Organisation for Dental Health, Oranga Nōhe and to Professor John Broughton, who is involved in Māori Dental Health, University of Otago.

The Ngāi Tahu Research Consultation Committee has membership:

Te Rūnanga O Otago Incorporated
Kahina Kaitahi H Pakairangi
Ti Rūnanga o Māori
NGAI TAHU RESEARCH CONSULTATION COMMITTEE
TE KOMITI RAKAHU Ki KAI TAHU

We wish you every success in your research and the committee also requests a copy of the research findings.

This letter of suggestion, recommendation and advice is current for an 18 month period from Tuesday, 19 August 2014 to 19 February 2016.

Nihaua ton, nā

Mark Brunton
Kaiwhak hapere Nga Pā ora Māori
Research Manager Māori
Research Division
Te Whare Wānanga o Otago
Ph: +64 3 479 3728
Email: mark.brunton@otago.ac.nz
Web: www.otago.ac.nz

The Ngai Tahu Research Consultation Committee has membership from:

Te Rūnanga o Ōtautahi Incorporated
Kāi Māraua Rūnanga ki Puketawhau
Te Rūnanga o Moaaki
Appendix 4. Information sheet mailed to potential participants

As a current patient under fixed orthodontic treatment by Azza, Catherine, Yana or Gareth, you/your child is being considered for inclusion in a randomised controlled trial.

The study is being conducted at the Orthodontic Department, Dental School.

It is in a new and exciting area of research and we are excited to be carrying it out from July this year. We have enclosed details on the study.

We ask that you please read the attached forms:

Green-information sheet for child participants
Grey-information sheet for parents
Blue-consent form for parents/caregivers (to be signed please)
Yellow-consent form for child participants (to be signed and ethnicity stated please)

We ask that you please fill in the completed forms in the self addressed envelope as soon as you are able.

Should you have any questions, please feel free to ask your treating Orthodontic Student (Azza, Catherine, Yana or Gareth) for more information at your next appointment or alternatively I can be reached at: benga221@otago.ac.nz.

Many thanks in advance.

Kind Regards,

Gareth Benic and the Research Team
Appendix 5. Information sheets and informed consent forms

**Efficacy of the oral probiotic *Streptococcus salivarius* in managing biofilm formation in patients wearing fixed orthodontic appliances: A double-blind randomised placebo-controlled trial**

**INFORMATION SHEET FOR PARTICIPANTS or PARENTS/GUARDIANSETC.**

Thank you for showing an interest in this study. Please read this information sheet carefully before deciding whether or not to take part. It would be great if you decided to take part, however if you decide not to, your braces treatment will not be affected by your decision.

**What is the aim of the project?**

Braces make it more difficult to brush teeth properly and can increase the build-up of plaque (which houses bad dental bugs) in these hard-to-brush areas. A large amount of plaque can lead to problems like white spots on teeth, bleeding gums and bad breath.

Toothbrushes, mouth rinses and toothpastes can help stop plaque build up on teeth, but they do not always work.

We are looking at a new way to stop bad bugs growing in mouths and on teeth. We are using lozenges that have good friendly bugs which we hope will fight and replace the bad bugs in mouths. These good bugs are called probiotics.

These good bugs have shown to help fight bad bugs in all parts of the body, but few dentists and scientists have tried using them in the mouth.

If these bugs work like we hope they will, they could stop white spots from developing on teeth, improve gum health and freshen breath in patients undergoing treatment with braces.

This study is being done by Gareth Benic, who is a Dentist studying to be an Orthodontist at the School of Dentistry, University of Otago.

**Who are we looking for?**

Patients wearing braces and being treated at the Dental School. We want patients who are 10-30 years old, have at least 20 real teeth and are wearing braces.
We cannot include patients who:
- wear braces on the inside of their teeth (lingual braces)
- use a non-fluoride toothpaste
- use a toothpaste with supplementary antibacterial agents (i.e. Colgate Total)
- have gum disease
- have had lots of dental treatment
- have a health condition (i.e. diabetes)
- taking antibiotics
- have dental fluorosis
- live in non-fluoridated areas (Allanton, Waikouaiti and Mosgiel)
- use an electric toothbrush
- are physically unable to brush their teeth themselves
- smoke cigarettes
- lactose intolerant
- allergic to dairy products

Please note that the probiotic product provided by BLIS technologies Ltd, is made on equipment that also processes milk, soy and wheat. Any adverse events are likely to be associated with milk, soy, wheat protein allergies. The prevalence of these allergies is in the range of 1-2%, which suggests the chances of them occurring is extremely low.

What will Participants Be Asked to Do?

First Visit
Gareth Benic will brush dye onto the participant’s teeth to show the plaque that is on each tooth surface. He will then take some quick measurements. The participant will then be asked to remove the plaque (that is shown by the dye) by brushing and flossing. Gareth Benic will help with this. He will then let the participant rinse with a mouthwash and will ask them to suck one of the two lozenges. One will have the good bugs in it and the other will have none, but the participants won’t know which lozenge they are getting.

A months worth of lozenges (plus toothpaste and toothbrushes) will be given to the participants to eat at home. We will also give them a sticker chart to help as a reminder to take the lozenges. The participant will be asked to suck one lozenge after brushing their teeth in the morning and one after brushing their teeth at night.

One month later
After one month they will come back to the Orthodontic Department to have measurements taken again. They can then stop taking the lozenges.

Three months later
After another three months they will come back to the Orthodontic Department to have measurements taken again for one last time.

The study will be for 4 months in total, and consist of three lots of 10-20 minute appointments.
What will we do?
• See how much plaque is on each tooth surface as shown by the dye
• Take a small sample of plaque from the upper front tooth with a little stick.
• Measure the breath.

Is it safe?
The good bug (probiotic strain) we are using is S. Salivarius M18 (BLIS Technologies Ltd, Dunedin, New Zealand). This product can be bought from shops and has been trialed and tested to show that it is safe and will not negatively affect health or well-being. More information can be found at: www.blis.co.nz

What information and data will be collected?
Age, gender, amount and type of bugs in the plaque, and breath measurements.

Privacy protection
Information will only be used by Gareth Benic and kept completely private. Data will be stored in a password-protected database and only Gareth Benic and his supervisors (Dr. Li Mei, Dr Nick Heng and Prof. Mauro Farella) will have access to the collected information. Furthermore, all the collected information will be destroyed, ten years after the research is finished.

Data will be used to:
Compare plaque, gum and breath measurements with other participants in the study. We may use the results of the study for other similar research and publish them in healthcare journals or use them in healthcare presentations.

Can participants change their mind and withdraw from the project?
Participants may withdraw from the study at any time and without any disadvantage of any kind.

What if participants have any questions?
If you have any questions about the project, please feel free to contact us:

Gareth Benic: 027 305 2448  benga221@student.otago.ac.nz
Dr. Li (Peter) Mei: 03 479 7480  li.mei@otago.ac.nz
Prof. Mauro Farella: 03 479 5852  mauro.farella@otago.ac.nz

This study has been approved by the University of Otago Human Ethics Committee. If you have any concerns about the ethical conduct of the research you may contact the Committee through the Human Ethics Committee Administrator (ph. 03 479 8256 or email gary.witte@otago.ac.nz). Any issues you raise will be treated in confidence and investigated and you will be informed of the outcome.

Compensation
In the unlikely event of a physical injury as a result of your participation in this study, you may be covered by ACC under the Accident Compensation Act 2001. ACC cover is not automatic, and your case will need to be assessed by ACC according to the provisions of the Accident Compensation Act 2001. If your claim is accepted by ACC, you still might not get any compensation. This depends on a number of factors, such as whether you are an earner or non-earner. ACC usually provides only partial reimbursement of costs and expenses, and there may be no lump sum compensation payable. There is no cover for mental injury unless it is a result of physical injury. If you have ACC cover, generally this will affect your right to sue the investigators. If you have any questions about ACC, contact your nearest ACC office or the investigator. You are also advised to check whether participation in this study would affect any indemnity cover you have or are considering, such as medical insurance, life insurance and superannuation.
Efficacy of the oral probiotic *Streptococcus salivarius* in managing biofilm formation in patients wearing fixed orthodontic appliances: A double-blind randomised placebo-controlled trial

INFORMATION SHEET FOR CHILD PARTICIPANTS

Thank you for showing an interest in this study. Please read this information sheet carefully before deciding whether or not to take part. It would be great if you decided to take part, however if you decide not to, your braces treatment will not be affected by your decision.

**What is the aim of the project?**

Braces make it more difficult to brush teeth properly and can increase the build-up of plaque (which houses bad dental bugs) in these hard-to-brush areas. A large amount of plaque can lead to problems like white spots on your teeth, bleeding gums and bad breath.

Toothbrushes, mouth rinses and toothpastes can help stop plaque build up on your teeth, but they do not always work.

We are looking at a new way to stop bad bugs growing in your mouth and on your teeth. We are using lozenges that have good friendly bugs which we hope will fight and replace the bad bugs in your mouth. These good bugs are called probiotics.

These good bugs have shown to help fight bad bugs in all parts of the body, but few dentists and scientists have tried using them in the mouth.

If these bugs work like we hope they will, they could stop white spots on your teeth, give you healthy gums and fresh breath while you are undergoing treatment with braces.

This study is being done by Gareth Benic, who is a Dentist studying to be an Orthodontist at the School of Dentistry, University of Otago.

**Who are we looking for?**

Patients wearing braces and being treated at the Dental School. We want patients who are 10-30 years old, have at least 20 real teeth and are wearing braces.
**We cannot include patients who:**

- wear braces on the inside of their teeth (lingual braces)
- use a non-fluoride toothpaste
- use a toothpaste with supplementary antibacterial agents (i.e. Colgate Total)
- have gum disease
- have had lots of dental treatment
- have a health condition (i.e. diabetes)
- taking antibiotics
- have dental fluorosis
- live in non-fluoridated areas (Allanton, Waikouaiti and Mosgiel)
- use an electric toothbrush
- are physically unable to brush their teeth themselves
- smoke cigarettes
- lactose intolerant
- allergic to dairy products

*Please note that the probiotic product provided by BLIS technologies Ltd, is made on equipment that also processes milk, soy and wheat. Any adverse events are likely to be associated with milk, soy, wheat protein allergies. The prevalence of these allergies is in the range of 1-2%, which suggests the chances of them occurring is extremely low.*

**What will Participants be Asked to Do?**

**First Visit**
On your first visit, Gareth Benic will brush dye onto your teeth to show the plaque that is on the tooth surfaces. He will then take some quick measurements. You will then be asked to remove the plaque (that is shown by the dye) in your mouth by brushing and flossing. Gareth Benic will help you to do this. He will then let you rinse with a mouthwash and you will be asked to suck one of the two lozenges. One will have the good bugs in it and the other will have none, but you won’t know which lozenge you are getting.

A month's worth of lozenges (plus toothpaste and toothbrushes) will be given to you to eat at home. We will also give you a sticker chart to help you remember to take the lozenges. You will be asked to suck one lozenge after brushing your teeth in the morning and one after brushing your teeth at night.

**One month later**
After one month you will come back to the Orthodontic Department to have measurements taken again. You can then stop taking the lozenges.

**Three months later**
After another three months you will come back to the Orthodontic Department to have measurements taken again for one last time.

The study will be for 4 months in total, and consist of three lots of 10-20 minute appointments.
What will we do?
• See how much plaque is on the tooth surfaces as shown by the dye
• Take a small sample of plaque from your upper front tooth with a little stick.
• Measure your breath.

Is it safe?
The good bug (probiotic strain) we are using is S. Salivarius M18 (BLIS Technologies Ltd, Dunedin, New Zealand). This product can be bought from shops and has been trialed and tested to show that it is safe and will not negatively affect health or well-being. More information can be found at: www.blis.co.nz

What information and data will be collected?
Your age, gender, amount and type of bugs in your plaque and breath measurements.

Privacy protection
Your information will only be used by Gareth Benic and kept completely private. Data will be stored in a password-protected database and only Gareth Benic and his supervisors (Dr. Li Mei, Dr Nick Heng and Prof. Mauro Farella) will have access to the collected information. Furthermore, all the collected information will be destroyed, ten years after the research is finished.

Data will be used to:
Compare plaque, gum and breath measurements with other participants in the study. We may use the results of the study for other similar research and publish them in healthcare journals or use them in healthcare presentations.

Can participants change their mind and withdraw from the project?
Participants may withdraw from the study at any time and without any disadvantage of any kind.

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If you have any questions about the project, please feel free to contact us:

Gareth Benic: 027 305 2448 benga221@student.otago.ac.nz
Dr. Li (Peter) Mei: 03 479 7480 li.mei@otago.ac.nz
Prof. Mauro Farella: 03 479 5852 mauro.farella@otago.ac.nz

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In the unlikely event of a physical injury as a result of your participation in this study, you may be covered by ACC under the Accident Compensation Act 2001. ACC cover is not automatic, and your case will need to be assessed by ACC according to the provisions of the Accident Compensation Act 2001. If your claim is accepted by ACC, you still might not get any compensation. This depends on a number of factors, such as whether you are an earner or non-earner. ACC usually provides only partial reimbursement of costs and expenses, and there may be no lump sum compensation payable. There is no cover for mental injury unless it is a result of physical injury. If you have ACC cover, generally this will affect your right to sue the investigators. If you have any questions about ACC, contact your nearest ACC office or the investigator. You are also advised to check whether participation in this study would affect any indemnity cover you have or are considering, such as medical insurance, life insurance and superannuation.
Efficacy of the oral probiotic *Streptococcus salivarius* in managing biofilm formation in patients wearing fixed orthodontic appliances: A double-blind randomised placebo-controlled trial

CONSENT FORM FOR PARTICIPANTS

I have read the Information Sheet concerning this project and understand what it is about. All my questions have been answered to my satisfaction. I understand that I am free to request further information at any stage.

I know that:
1. My participation in the project is entirely voluntary;
2. I am free to withdraw from the project at any time without any disadvantage;
3. Personal identifying information will be destroyed at the conclusion of the project but any raw data on which the results of the project depend will be retained in secure storage for ten years;
4. I will be given free instructions on oral hygiene, free toothbrushes, toothpastes and lozenges during the study;
5. I understand that I will take two tablets a day for one month
6. The researchers will write up the results from this study for their University work. The results may also be written up in journals and talked about at conferences. My name will not be on anything the researchers write up about this study.

I agree to take part in this study.

.......................................................................................................................... ........................................
(Signature of participant) ............................................................... (Date)

..........................................................................................................................
(Printed Name)

This study has been approved by the University of Otago Human Ethics Committee. If you have any concerns about the ethical conduct of the research you may contact the Committee through the Human Ethics Committee Administrator (ph 03 479 8256 or email gary.witte@otago.ac.nz). Any issues you raise will be treated in confidence and investigated and you will be informed of the outcome.
Efficacy of the oral probiotic *Streptococcus salivarius* in managing biofilm formation in patients wearing fixed orthodontic appliances: A double-blind randomised placebo-controlled trial

CONSENT FORM FOR CHILD PARTICIPANTS

I have been told about this study and understand what it is about. All my questions have been answered in a way that makes sense.

I know that:
1. Participation in this study is voluntary, which means that I do not have to take part if I don’t want to and nothing will happen to me.
2. I can stop taking part at any time and don’t have to give a reason.
3. If I have any worries or if I have any other questions, I can talk to the researchers about them.
4. Papers and computer files with my information will only be seen by the researchers and the people they are working with. They will keep whatever I say private.
5. I will receive free toothbrushes and toothpastes at every orthodontic visit during the study, as thanks for helping out.
6. I understand that I will take two tablets a day for one month
7. The researchers will write up the results from this study for their University work. The results may also be written up in journals and talked about at conferences. My name will not be on anything the researchers write up about this study.

I agree to take part in this study.

............................................................................. .................................................. ...............................
(Signature of participant) (Date)

...................................................................................................................(Printed Name)

This study has been approved by the University of Otago Human Ethics Committee. If you have any concerns about the ethical conduct of the research you may contact the Committee through the Human Ethics Committee Administrator (ph. 03 479 8256 or email gary.witte@otago.ac.nz). Any issues you raise will be treated in confidence and investigated and you will be informed of the outcome.
Efficacy of the oral probiotic *Streptococcus salivarius* in managing biofilm formation in patients wearing fixed orthodontic appliances:
A double-blind randomised placebo-controlled trial

**CONSENT FORM FOR PARENTS/GUARDIANS**

I have read the Information Sheet concerning this project and understand what it is about. All my questions have been answered to my satisfaction. I understand that I am free to request further information at any stage.

I know that:-  
1. My child’s participation in the project is entirely voluntary;
2. I am free to withdraw my child from the project at any time without any disadvantage;
3. Personal identifying information will be destroyed at the conclusion of the project but any raw data on which the results of the project depend will be retained in secure storage for ten years;
4. If I have any worries or if I have any other questions, then I can talk about these with the researchers.
5. My child will receive free advice on oral hygiene, free toothbrushes and toothpastes during the study as thanks for taking part.
6. I understand that my child will take two tablets a day for one month
6. The researchers will write up the results from this study for their University work. The results may also be written up in journals and talked about at conferences. My child’s name will not be identified on anything the researchers write up about this study.

I agree for my child to take part in this study

............................................................................................................. .............................................
(Signature of parent/guardian) (Date)

.............................................................................................................
(Name of child)

This study has been approved by the University of Otago Human Ethics Committee. If you have any concerns about the ethical conduct of the research you may contact the Committee through the Human Ethics Committee Administrator (ph. 03 479 8256 or email gary.witte@otago.ac.nz). Any issues you raise will be treated in confidence and investigated and you will be informed of the outcome.
Appendix 6. Clinical exam kit checklist

**Clinical exam kit**

- Toothpaste
- Toothbrush
- Sterile Blunt probe, mirror, tweezers, triple-x
- Vial for plaque
- Plaque disclosing tablet
- 30ml Mouthwash
Appendix 7. Take home pack checklist

**Take Home Package**

- Tube of toothpaste
- Toothbrush
- Months supply of lozenges
- Instructions
- Lozenge reminder chart
Appendix 8. Information for trial participants

Thank you once again for agreeing to take part in this study.

Please use the toothbrushes and toothpastes provided by us. The lozenges don’t require any special storage instructions, maybe just keep them next to your toothbrush so it is easy to remember to take them.

What to do:

- **Morning**
  - Brush your teeth
  - Suck 1 lozenge
  - Record on chart

- **Evening**
  - Brush your teeth
  - Suck 1 lozenge
  - Record on chart

Please continue to do this right up until your next appointment (about 30 days from your first appointment).

Problems?
The success of our study relies heavily on our participants, so if you have any problems please feel free to get in touch at benga221@otago.ac.nz or 0273052448. If you could please let us know if anything changes during the trial period (such as starting a course of antibiotics), that would be great!

What else?
We will send out text and email reminders to take the lozenges once in the morning and evening. We will also use this system to remind you about your next appointment.

Thanks again and see you soon!

Regards,

Gareth, Peter, Nick and Mauro
Appendix 9. Lozenge reminder chart

**LOZENGE REMINDER CHART**

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Please tick each time you take one of your lozenges.

Please remember to:

**BRING THIS FORM TO YOUR SECOND TRIAL APPOINTMENT**

**AND**

**BRING ALL OF YOUR UNUSED LOZENGES**

THANKS!
Appendix 10. Further information and appointment template

Thank you for agreeing to take part in the BLIS Study.

You were initially selected because you are undergoing orthodontic treatment at the Dental School with Azza, Yana, Catherine or Gareth. You have opted in to the Study either via return mail, after receiving a phone call, or at your last orthodontic appointment with Azza, Yana, Catherine or Gareth.

You should have already received all information documents outlining details of the study and been given appropriate consent forms to sign (if you have not, please email us at benga221@otago.ac.nz, so they can be sent to you).

Please find below your appointments for the BLIS Study (and any orthodontic treatment appointments you already have scheduled in our appointment system). The BLJS Study appointments have been carefully allocated in 0 week, 4 week and 12 week intervals, starting the weeks of the 27th July, 24th August and 16th November.

Each appointment will be 30 minutes long and we will try our very best to keep on time. If you could arrive 10 minutes before your appointment, that would be great! We have endeavoured to make these appointments as convenient for you as possible and have tried to balance them around your orthodontic treatment appointments the best we can. However, with over 200 appointments to schedule, it has not been an easy task, and we have unfortunately been left with very little wriggle room! Hopefully they are suitable.

We are extremely grateful to you for giving up your time to be a part of this study-the first of its kind in the orthodontic world! Your attendance at all appointments will certainly determine its success, so if you have any questions that haven’t yet been answered or if we can help in any way, please feel free to call or email us (benga221@otago.ac.nz or 0273052448). Otherwise, we look forward to seeing you at your first appointment.

**What will happen at appointment one?**
Firstly, Gareth will check that the informed consent form has been filled out, all inclusion criteria has been met and that you have no further questions. He will then take 3 breath measurements by having you breathe onto a straw. He will then take measurements of the plaque on your teeth and assess gum health. This will be followed by taking a sample of your mouth plaque with a blunt probe. He will then brush dye onto the your teeth to show you
the plaque that is on each tooth surface. You will then be asked to remove the plaque (that is shown by the dye) by brushing. Gareth will then give you mouthwash to rinse with and give you your first lozenge to **suck** on. You will **suck** a second lozenge at night time and then one every morning and night after tooth brushing for the next ~28 days, until your next appointment.

**What else?**
Further instructions and information will be given at your first appointment. We will send out text and email reminders to take the lozenges once in the morning and once in the evening. We will also use this system to remind you about your next appointment.

**Your appointments**

*Insert patient’s appointment here*

If you can’t make your appointments for any reason please contact us so we can try reschedule you.

*We really appreciate your involvement, thanks once again and see you soon!*
### PLAQUE INDEX / GINGIVAL INDEX MEASUREMENTS

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</table>

Baseline measurement: Patient Unique Identifier #: Age: Sex: Group: Date: Appendix 11. Data recording documents
BEFORE WE BEGIN:

Participant happy all questions regarding study have been answered
Consent form returned

BEFORE LET PATIENT GO:

Consent form returned
Participant happy all questions regarding study have been answered

NEW APPOINTMENT MADE

Patient given take home package
Plaque sample taken and added to 200 µl of PBS, put on ice
Sample had 200 µl of digestion buffer added and stored in lab freezer

LAB CHECKLIST:

| Mean VSC | VSC Reading 1 | VSC Reading 2 | VSC Reading 3 |

BASELINE VOLATILE SULPHUR COMPOUND MEASUREMENTS
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<th>Tooth Number</th>
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**PLAQUE INDEX / GINGIVAL INDEX MEASUREMENTS**

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**Patient Unique Identifier:**

**Date:**

**Group:**

**Sex:**

**Age:**
**LAB CHECKLIST:**

- New appointment made
- Unused pills collected
- No. remaining
- Sticker chart collected
- Sample had 200µl of digestion buffer added and stored in lab freezer
- New toothpaste and toothbrush given
- Plaque sample taken and added to 200µl of PBS put on ice
- Sample had 200µl of digestion buffer added and stored in lab freezer

### Mean VSC

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<th>VSC Reading 1</th>
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**1 MONTH VOLATILE SULPHUR COMPOUND MEASUREMENTS**
**Patient Unique Identifier #:**

**Age:**

**Sex:**

**Group:**

**Date:**

**4-Month Measurements**

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**PLAQUE INDEX / GINGIVAL INDEX MEASUREMENTS**

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**LAB CHECKLIST:**

- Unused pills collected
- Sticker chart collected
- Discrepancy with sticker chart
- New appointment made
- New toothpaste and toothbrush given
- Plaque sample taken and added to 200µl of PBS, put on ice

**BEFORE WE LET PATIENT GO**

- Plaque sample had 200µl of digestion buffer added and stored in lab freezer
- Sample had 200µl of PBS, put on ice
- Sample had 200µl of digestion buffer added and stored in lab freezer

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<thead>
<tr>
<th>Mean VSC</th>
<th>VSC Reading 1</th>
<th>VSC Reading 2</th>
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4-MONTH VOLATILE SULPHUR COMPOUND MEASUREMENTS
Appendix 12. Clinical trial detailed procedure flow charts

**Clinical Trial Procedure (Baseline Appointment)**

In dental chair, confirm consent form has been signed and patient satisfies all inclusion criteria and has no questions. If no problems, trial can begin. Fill out details at top of Data Recording Form. Give explanation on trial proceedings

1. VSC recorded 3 times with Halimeter
2. Plaque sampling
   - Supra-gingival dental plaque collected from all teeth with an explorer, pooled and transferred to centrifuge tube

3. Temporarily store samples in a bucket of ice (ice obtained from 1st floor of Theatre, or 3rd floor lab ice box), within 30 minutes of sampling. Samples will be then stored (-80°C) in the Molecular Biosciences Laboratory (Department of Oral Sciences)

4. GI recorded
5. PI recorded
6. Plaque disclosure
7. Plaque removed by patient (brushing teeth and tongue)
8. Investigator ensures all plaque removed
9. Patient to rinse for 30 seconds with 30ml antibacterial Mouth rinse
10. Patient to suck first lozenge (remind patient to suck not chew)
11. New appointment made and Take Home Package given
Clinical Trial Procedure (1-month Appointment)

Compliance Assessment. Collect Sticker chart and collect and record remaining lozenges

↓

VSC recorded 3 times with Halimeter

↓

Plaque sampling

Supra-gingival dental plaque collected from all teeth with an explorer, pooled and transferred to centrifuge tube

↓

Temporarily store samples in a bucket of ice (ice obtained from 1st floor of Theatre, or 3rd floor lab ice box), within 30 minutes of sampling. Samples will be then stored (-80°C) in the Molecular Biosciences Laboratory (Department of Oral Sciences)

↓

GI recorded

↓

PI recorded

↓

New appointment made and new toothbrush and toothpaste given

*Choose 5 patients with poor oral hygiene and plaque disclose for photographic records at each appointment!
Clinical Trial Procedure (4-month Appointment)

VSC recorded 3 times with Halimeter

Plaque sampling
Supra-gingival dental plaque collected from all teeth with an explorer, pooled and transferred to centrifuge tube

Temporarily store samples in a bucket of ice (ice obtained from 1st floor of Theatre, or 3rd floor lab ice box), within 30 minutes of sampling. Samples will be then stored (-80°C) in the Molecular Biosciences Laboratory (Department of OralSciences)

GI recorded

PI recorded

New toothbrush and toothpaste given. Trial over
Plaque sampling protocol

Patient

↓

Plaque sampling (blunt explorer)

↓

Add to vial containing 400 µL digestion buffer
(Tris-HCL+EDTA+Triton X-100)

↓

Put vial on ice in clinic until able to go to lab

↓

Store in -80°C until ready for purification
II. DNA purification from samples 1a-65a, T=0

PureLink Genomic DNA kit-Gram Positive Protocol

Step I-Lysis (production of Gram Positive Bacterial Cell Lysate)

Set two water baths at 37°C and 55°C, respectively

Defrost samples at room temperature

Dissolve 200 mg of lysozyme in 1 mL of Digestion Buffer (this gives a stock solution of 200 mg/mL). Add 50 µl of this stock solution to the microcentrifuge containing the sample (this gives a final lysozyme concentration of 10 mg/ml).

Add 20 µl of RNAse to the sample and mix

Incubate at 37°C for 30 minutes (mix every 10 minutes by tube inversion)

Add 20 µl of proteinase K. Mix well by brief vortex

Add 500 µl PureLink Genomic Lysis/Binding Buffer and mix well by brief vortex

Incubate at 55°C for 30 minutes – no need to invert but swirl every 10 minutes

Add 500 µl of 100% ethanol and mix well by vortex for 5 seconds to yield a homogenous solution

Proceed to Binding DNA
Step II-Binding

Transfer first 500 µl of Lysate sample to a Purelink Genomic Spin Column that is positioned in a 2-ml collection tube

↓

Centrifuge at 10,000-x g for 60 seconds

↓

Discard the 2-ml collection tube containing flow through and place the column in a new 2-ml collection tube

↓

Transfer second 500 µl of Lysate sample to the Purelink Genomic Spin Column

↓

Centrifuge at 10,000-x g for 60 seconds

↓

Discard the 2-ml collection tube containing flow through and place the column in a new 2-ml collection tube

↓

Transfer final third 500 µl of Lysate sample to the Purelink Genomic Spin Column

↓

Centrifuge at 10,000-x g for 60 seconds

↓

Discard the 2-ml collection tube containing flow through and place the column in a new 2-ml collection tube

↓

DNA Bound
Step III-Washing DNA

Add 500 µl of Wash buffer 1 (with added ethanol) to the Purelink column and centrifuge at 10,000 x g for 60 seconds

Discard the flow through that presents in the collection tube. Place the Purelink column back into the 2-ml collection tube

Add 500 µl of Wash Buffer 2 (prepared with Ethanol) to the Purelink column and centrifuge at 10,000 x g for 60 seconds. Repeat this step.

Discard the flow through that presents in the collection tube. Place the Purelink column back into the 2-ml collection tube and centrifuge again for 3 minutes at maximum speed (14-16,000 x g) to dry the column matrix. Discard the collection tube.

DNA washed and dried

Step IV-Eluting DNA

Transfer the dried Purelink column to a sterile 1.5-ml centrifuge tube (use sterile swabs to remove excess ethanol)

Add 70 µl of PureLink Genomic Elution Buffer to the centre of the column matrix

Leave it for 15 minutes at room temperature

Centrifuge at maximum speed for 1 minute to elute the purified DNA

The microcentrifuge tube will now contain purified genomic DNA
IIb. DNA purification from samples 1b-65b, T=1

**PureLink Genomic DNA kit**

**Step I-Lysis (production of Gram Positive Bacterial Cell Lysate)**

Set two water baths at 37°C and 55°C, respectively
---
Defrost samples at room temperature
---
Dissolve 200 mg of lysozyme in 1 mL of Digestion Buffer
(this gives a stock solution of 200 mg/mL).
---
Centrifuge tubes containing samples at maximum speed for 1 min to harvest the bacterial cells
---
Aspirate off digestion buffer, leave pellet completely submerged
---
Re-suspend pellet in 100 µl of Lysozyme Digestion Buffer containing Lysozyme from Step 2.
This gives a final lysozyme concentration of 20 mg/ml.
---
Incubate at 37°C for 30 minutes (every 10 minutes invert)
---
Add 20 µl of proteinase K. Mix well by brief vortex
---
Add 200 µl PureLink Genomic Lysis/Binding Buffer and mix well by brief vortex
---
Incubate at 55°C for 30 minutes – no need to invert but swirl every 10 mins
---
Add 200 µl of 100% Ethanol and mix well by vortex for 5 seconds to yield a homogenous solution
---
Proceed to Binding DNA
Step II-Binding

Transfer all Lysate sample (~500 µl) to a Purelink Genomic Spin Column that is positioned in a 2ml collection tube

- Centrifuge at 10,000-x g for 60 seconds
- Discard the 2-ml collection tube containing flow through and place the column in a new 2-ml collection tube

DNA Bound

Step III-Washing DNA

Add 200 µl of Wash buffer 1 (prepared with ethanol) to the purelink column and centrifuge at 10,000-x g for 60 seconds

- Discard the flow through that presents in the collection tube. Place the Purelink column back into the 2ml collection tube

- Add 200 µl of Wash Buffer 2 (prepared with Ethanol) to the purelink column and centrifuge at 10,000-x g for 60 seconds. Repeat this step.

- Discard the flow through that presents in the collection tube. Place the Purelink column back into the 2ml collection tube and centrifuge again for 3 minutes at maximum speed (14-16,000xg) to dry the column matrix. Discard the collection tube.

DNA washed and dried
Step IV - Eluting DNA

Transfer the dried purelink column to a sterile 1.5-ml centrifuge tube 
(use sterile swabs to remove excess Ethanol) 
↓
Add 70µl of PureLink Genomic Elution Buffer to the centre of the column matrix 
↓
Leave it for 15 minutes at room temperature 
↓
Centrifuge at maximum speed for 1 minute to elute the purified DNA 
↓
The centrifuge tube will now contain purified genomic DNA

III. PCR Quantification – NanoVue Spectrometer

Clean NanoVue spectrophotometer with distilled H2O and Kimwipes 
↓
Add 2 µl of elution buffer (twice) to the spectrometer machine for calibration 
↓
Press OA/100% T to zero (to blank the detector) 
↓
Remove elution buffer 
↓
Add another 2 µl of elution buffer and machine will automatically do the second reading 
↓
Remove elution buffer 
↓
Vortex sample centrifuge tube. Add 2 µl of sample 
↓
NanoVue will measure the quantity of the DNA
IV. Preparing master mix for PCR

T=0 month 18 samples + 1 negative = 19 reactions
T=1 month 18 samples + 1 negative = 19 reactions
à38 reactions + 2 reactions (to account for pipetting error) = 40 reactions

**Master mix**

<table>
<thead>
<tr>
<th></th>
<th>One reaction</th>
<th>Ready to load (40 reactions)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer</td>
<td>2.5 µl</td>
<td>100 µl</td>
</tr>
<tr>
<td>Primer 1 (forward)</td>
<td>0.5 µl</td>
<td>1 µl</td>
</tr>
<tr>
<td>Primer 2 (Reverse)</td>
<td>0.5 µl</td>
<td>1 µl</td>
</tr>
<tr>
<td>dNTP mix</td>
<td>0.5 µl</td>
<td>20 µl</td>
</tr>
<tr>
<td>MgSo4</td>
<td>2 µl</td>
<td>80 µl</td>
</tr>
<tr>
<td>Taq enzyme</td>
<td>0.25 µl</td>
<td>10 µl</td>
</tr>
<tr>
<td>Water</td>
<td>17.75 µl</td>
<td>710 µl</td>
</tr>
<tr>
<td>Genomic DNA</td>
<td>1 µl</td>
<td></td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>25 µl</strong></td>
<td></td>
</tr>
</tbody>
</table>

IV. PCR RUN

- PCR Machine
- Place the tubes in the wells
- Set programme to Unnamed C
- Select 0.2 µl tube, select 50 µl
- Press start
V. Running a gel

Making a gel

Mix 0.80 grams of Agarose and 80 ml of 1 x TAE buffer and boil in a microwave oven until all agarose has melted

Cool to room temperature (5 minutes)

Pour into a gel tray and leave it to set

Once gel is set, remove comb and place inside a gel electrophoresis tank containing 1 x TAE buffer

Mix 5 µl of PCR product with 1 µl of 6X loading dye and dispense into allocated wells in the gel. Load 5 µl of 1 kb DNA ladder

Run gel at 100 volts for approximately 90 minutes (until the purple dye front has run two thirds down the gel)

VIII. Sequencing

The samples, each containing at least 20 ng of purified genomic DNA at a concentration of at least 5 ng/µl, were placed in ABI Fast 96-well real-time PCR plates, with seals, and packaged in dry ice. The 130 purified and quantified samples of genomic DNA templates were then sent to New Zealand Genomics Limited (NZGL) for sequencing. However, since NZGL had the 16S V3 - V4 rRNA primers to amplify and generate the libraries for the project, no primers were supplied by the principal investigator.
Appendix 14. Laboratory tasks undertaken by our team prior to sample delivery to NZGL for sequencing

**Laboratory tasks undertaken by our team-mock samples**

A preliminary PCR experiment with the QC primers (V3-V4) was performed as specified by NZGL.

The first three lanes contained three different oral samples. The fourth lane contained digestion buffer only, to act as the non-oral, negative control sample. The fifth lane was a PCR without template DNA (water only), and the final lane was a DNA marker. The template DNA used was at concentrations of 12-15 ng/ul, and one microlitre (12-15 ng) was used in each PCR.

The preliminary results showed considerable amounts of bacterial DNA in the oral samples but also a lot of contaminating human DNA, as indicated by extra bands (“ladder”) on the PCR gel. These unfortunate results were similar to a previous project undertaken by some of the research team in the past.

These results may have rendered certain sequences useless as the contaminating human DNA could dramatically reduce the bacteria-specific sequences.

Consequently an attempt was made to optimise the conditions for the subsequent PCR with the 130 samples. Polymerisation times were changed from 35 seconds to 10 seconds. Annealing temperatures were changed from 53°C to 55-60°C. Although the unfavourable results could also have been due to the enzyme used, this was unlikely.

The team also performed trial purifications of pure bacterial cultures with existing columns to be familiar with procedures and ensure systems were optimised before proceeding with the 130 oral samples. The trial purification included quantification and gel electrophoresis for template quality control. The trial oral genomic DNA procedures worked very well which ensured the preparation of the 130 samples would be suitable for NZGL, however they were informed of the concern regarding the initial sample contamination and appropriate guidance was obtained.

**Laboratory tasks-actual samples**

The 128 human mouth-swab samples were extracted and purified (half placebo, half probiotic), as well as two controls, giving a total of 130 samples.

The QC V3-V4 PCR primers as specified by NZGL for the quality control PCR as below.
They were long primers with most of the primer being the Illumina recognition sequence:

- V3-Fwd
  5’TCGTCGCGCAGCGTCAGATGTATAAGAGACACAGCCTACGGGNGGCWGC G-3’
- V3-Rev
  5’-GTCTCGTGGGCTCGGAGATGTATAAGAGACAGGACTACHVGGGTATCT ATCC-3’

At least 20 ng of purified genomic DNA was provided for each sample at a concentration of at least 5 ng/ul using the ‘Purelink’ extraction kit from Life-Technologies.

The quality of the genomic DNA was quantitated and assessed, by carrying out NanoVue analyses and by running a small portion of samples on a 2% agarose gel with a high molecular weight ladder. A preliminary gel image of a small portion of ‘good’ and ‘bad’ sample PCR products generated using the V3-V4 primers and their corresponding NanoVue readings, were sent to NZGL. NZGL were to ensure that the genomic DNA samples were of high molecular weight, of good quantity and quality prior to the full set of samples being sent for sequencing.

The gel image appeared to show some non-specific binding as indicated by a faint higher molecular weight band, but changes that were made to the cycling protocol, following the preliminary experiments, reduced the concentration of the unwanted products significantly. It is known that high quality genomic DNA should run as a high molecular weight band on a 1-2% agarose gel, with the majority of DNA greater than 10 kb in size and with minimal
lower molecular weight smearing. It is suggested that if the majority of the DNA is below 10 kb or smearing is visible, this suggests that the DNA is degraded or indicative of the presence of RNA contamination, which could influence the success of sequencing results.

The Massey Genome Service uses tailed PCR primers which flank the 16S V3-V4 rRNA hypervariable region to generate the libraries for their projects. Therefore, it was ensured that the products sent (i.e. the stronger ~500-bp bands on the gel) contained the 16S V3-V4 rRNA hypervariable region by confirming with NZGL the PCR primer sequences that we used to generate these amplicon products before delivering the samples to their facility.

Regarding the OD 260/280 readings. There were some samples where the reads were low (below 1.8), which could indicate the samples had some protein or other organic contaminants present. However, NZGL believed that so long as they could get amplified PCR products off these samples then the quality should be adequate.

NZGL checked our sequences against their 16S V3-V4 target sequences as a final check to make sure the ~500-bp products actually represented this region. They confirmed that these products were indeed amplified 16S V3-V4 regions and proceeded with the sequencing phase.

Samples were prepared in two separate plates, each containing 65 samples (including a template-free water [negative] control) for each Illumina MiSeq sequencing run. Each plate represented the timeline when the samples were collected: Baseline (T=0) and 1-month following lozenge use (T=1). Template Genomic DNA samples were sent to NZGL in dry ice via overnight courier, for library preparation and MiSeq sequencing.

**Work by NZGL-Illumina MiSeq/16s V3-V4 amplicon sequencing**

New Zealand Genomic Laboratories (NZGL) was contracted to generate 16S rRNA amplicon libraries, containing a set of core genes, using an Illumina 16S V3-V4 metagenomics protocol or custom amplicon single-step PCR. The overall aim was to produce a microbial diversity survey whereby we were provided with genomic and bioinformatics deliverables.

**Library Preparation method**

The 130 libraries (baseline and 1-month samples including two controls) were prepared using the Illumina 16S V3-V4 rRNA library preparation method split into two batches of 65 samples for each Illumina MiSeq run. The Massey Genome Service had dual index PCR primers which flank the V3-V4 hypervariable region of 16S rRNA and uses a “Single Step
PCR Library preparation method” to prepare the libraries. The libraries were pooled by equal molarity before being loaded onto the Illumina MiSeq runs.

**Two Illumina MiSeq 2X 250 base PE runs**
The 130 libraries were run on two Illumina MiSeq 2X 250 base PE runs, version 2 chemistry, ~ 65 libraries for each run. These two runs generated approximately 19.2-24 million reads, ~147,692–184,615 reads per sample and 9.6-12.0 Gb of total data output. It was noted that the sequencing data output estimation included the 20% PhiX loaded as an internal sequencing control.

**Genomic tasks**
- Sample QC check 130 genomic DNA samples
- Library preparation of 130 samples (custom single-step PCR library preparation method)
- Library QC check 130 amplicons libraries
- Two Illumina MiSeq 2X 250 base PE runs
- Data QC check of two Illumina MiSeq runs
- Data delivery via BIO-IT

**Genomics deliverables**
3 data outputs:
- Unprocessed data: Demultiplexed fastq sequence reads
- Processed data: PhiX control library reads and adapter sequences removed, using FASTQ-MCF¹
  - Processed_Trimmed data: sequence reads from “Processed data” trimmed at an error probability of 0.01 (Phred score of Q20)

All data generated was QC checked using the following processes:
- FastQC and FastQscreen
- SolexaQA²
- Map back to the PhiX control library to identify the known SNPs, with BWA, samtools and Varscan. This QC data, along with a “Data Quality Report”, “Sequencing Run Report” and “Bioanalyzer QC Reports” was provided to us within a project directory via NZGL BIO-IT
**Bioinformatics tasks**

The NZGL bioinformatics team was instructed to complete a number of tasks. The overall intention was to perform an analysis of the generated 16S rRNA reads. This was done through the use of mothur, QIIME or similar programs and/or environment to provide a phylogenetic profile of the organisms present in the sample in a tabulated form for further analysis by our team.

1. For each sample:
   a. Detailed quality assessment at various error probabilities.
   b. Assessment of overlapping using tools such as Flash to maximise the output.

2. For all samples, the following work was performed in QIIME:
   a. Processing of the reads to generate an index file required for QIIME analysis.
   b. The protocols supplied with QIIME were followed to generate a taxonomic overview of the samples.
   c. Taxonomic profiles of the individual samples were compared using functions in QIIME to look for similarities and differences between samples via a variety of output methodologies.

3. A pair of reports on the work carried out:
   a. An interim report on the first batch of data.
   b. A final report covering the analysis of the second batch, a comparison with the first batch.

**Bioinformatics deliverables**

- Data processed at a variety of quality thresholds and subsequently analysed with Flash.
- Metafiles and associated files for working with the QIIME workflow.
- Summary data from the QIIME pipeline mostly viewable as html files in a web browser.
- Other summary outputs from other programs as other ways to visualise the data results.
- Two reports (one interim and one final) summarising the work performed.

**References**


Hot FIREPol DNA polymerase 1000 units for QCs
Appendix 15. Clinical examination details

PI and GI

The plaque index, gingival index and volatile sulphur compound levels of the participants were recorded by researcher G.B, a dentist, at baseline, 1-month and 4-month time intervals.

We used an adaptation of the modified Silness and Løe Plaque Index as described by Clerehugh et al., to measure the amount of biofilm formation (Clerehugh et al., 1998). The teeth were divided into mesial, distal, gingival, and incisal (occlusal) regions in relation to the bracket. Plaque was then scored for each of the four areas based on four plaque index codes (Table 4.1) as used by Løe (Løe, 1967).

The four values were averaged for each tooth to give a mean PI tooth score. An overall final mean PI for all involved teeth was calculated for each participant. The plaque index measurement involved recording soft and hard deposits on all teeth including and mesial to the first molars in both arches.

Diagram showing the modified Silness and Løe index as described by Clerehugh and colleagues. The tooth is divided into mesial (M), distal (D), gingival (G), and incisal/occlusal (I) regions for plaque measurement.

Table 4.1: Plaque Index four code scoring system of Løe (Løe, 1967)

<table>
<thead>
<tr>
<th>Score</th>
<th>Criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>No plaque.</td>
</tr>
<tr>
<td>1</td>
<td>A film of plaque adhering to the free gingival margin and adjacent area of the tooth. The plaque may be seen in situ only after application of disclosing solution or by using the probe on the tooth surface.</td>
</tr>
<tr>
<td>2</td>
<td>Moderate accumulation of soft deposits within the gingival pocket, or the tooth and gingival margin which can be seen with the naked eye.</td>
</tr>
<tr>
<td>3</td>
<td>Abundance of soft matter within the gingival pocket and/or on the tooth and gingival margin.</td>
</tr>
</tbody>
</table>

GI measurements were made with a blunt probe. The same teeth as recorded in the plaque index were used. Four areas around each tooth (mesial (labial/buccal), distal (labial/buccal), labial/buccal and lingual/palatal) were scored based on four gingival index codes (Table 4.2), as used by Løe (Løe, 1967). A tooth GI mean and a participant GI mean was calculated as was done above for the PI.
Table 4.2: Gingival Index System (Löe, 1967)

<table>
<thead>
<tr>
<th>Score</th>
<th>Criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>No inflammation.</td>
</tr>
<tr>
<td>1</td>
<td>Mild inflammation, slight change in colour, slight oedema, no bleeding on probing.</td>
</tr>
<tr>
<td>2</td>
<td>Moderate inflammation, moderate glazing, redness, bleeding on probing.</td>
</tr>
<tr>
<td>3</td>
<td>Severe inflammation, marked redness and hypertrophy, ulceration, tendency to spontaneous bleeding.</td>
</tr>
</tbody>
</table>

**VSCs**

Levels of volatile sulphur compounds (VSCs) were assessed with patient’s breath samples using a Halimeter (Interscan Corp., Chatsworth, USA) based on the average of three readings. This was done in a standardized manner and in compliance with the manufacturers instructions, for best consistency of results.

At the baseline appointment, following the clinical data collection, participants were asked to chew a plaque-disclosing tablet (Disclotabs, Colgate®) and then thoroughly remove the disclosed dental biofilm in their mouths by brushing with a new toothbrush (Colgate® Ortho, Colgate-Palmolive) and toothpaste (Colgate® Cavity Protection Toothpaste, Colgate-Palmolive) under the supervision of the principal investigator (G.B.). The participants then rinsed their mouths with water and a 30ml cup of Chlorhexidine mouthwash (Savacol®, Colgate®). They were then asked to suck the appropriate lozenge. A take home pack of the appropriate lozenges, toothpaste, toothbrush, compliance lozenge reminder chart, pen and further instructions were given for home use. The protocol required the participants to suck two lozenges each day, one after brushing the teeth in the morning and one after brushing the teeth at night.

Each participant provided at least one email address and mobile telephone number where morning and night email and text message reminders were sent to enhance compliance with taking the lozenges.

At 1 month, clinical data was collected in a similar manner. In addition, all remaining lozenges and lozenge reminder charts were collected for the analysis of compliance with lozenge consumption. At 4 months, clinical data was collected as in the previous two occasions (see Appendix 13) for further information on our clinical protocol.

**References**

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