Effectiveness of the Lorodent Probiotic Lozenge in Reducing Plaque and S. Mutans Levels in Orthodontic Patients: a Randomized, Double-Blind, Placebo-Controlled Trial

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science
Department of Orthodontics, Faculty of Dentistry
University of Toronto

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ABSTRACT

Background/Objectives: To determine the efficacy of the Lorodent probiotic complex at reducing plaque accumulation and Streptococcus mutans levels in adolescent orthodontic patients. Methods: 60 adolescent orthodontic patients were randomized in a double-blind, parallel-group, placebo-controlled trial to receive either the Lorodent probiotic or placebo lozenge orally every day for a 28-day administration period. Plaque scores, supragingival plaque samples and saliva samples were assessed over a total of 56 days. Results: Participants receiving the placebo lozenge had a greater improvement in plaque scores compared to those receiving the Lorodent probiotic lozenge. There were no significant changes in S. mutans and total lactobacilli levels between the probiotic and placebo groups. Lozenge consumption compliance was over 90% and no adverse events were observed in the probiotic group. Conclusions: The Lorodent probiotic lozenge did not decrease plaque or S. mutans levels in adolescent patients undergoing fixed orthodontic appliance treatment. Funding: Ontario Centres of Excellence.
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Lastly, I wish to dedicate my thesis to my husband, Dr. Saleel Jivraj, who has believed in me and supported me every step of the way. You have been my pillar through one of the busiest periods of my life and you make me happier than I ever dreamt possible. I would also like to dedicate my thesis to my baby bro, Dr. Fouad-Hassan Ebrahim, who has been by my side through what seems like a lifetime of post-secondary education – I simply could not have done it without you.
# TABLE OF CONTENTS

ABSTRACT .................................................................................................................. II

ACKNOWLEDGMENTS ................................................................................................. III

TABLE OF CONTENTS ................................................................................................. IV

LIST OF TABLES .......................................................................................................... VIII

LIST OF FIGURES ...................................................................................................... XI

LIST OF ACRONYMS .................................................................................................... XIV

LIST OF APPENDICES ............................................................................................... XVI

1. INTRODUCTION .................................................................................................... 1

1.1 Significance of the Problem .................................................................................. 1

1.2 Enamel Formation & Mineralization .................................................................... 2

1.3 Dental Caries & Enamel Demineralization ............................................................. 3

1.3.1 Etiology & Disease Process .............................................................................. 3

1.3.2 Pathophysiology & Disease States ................................................................. 5

1.3.3 Detailed Description of Contributing Factors ............................................... 7

1.4 White Spot Lesions in the Orthodontic Population ............................................. 12

1.4.1 Prevalence ........................................................................................................ 13

1.4.2 Prevention ......................................................................................................... 16

1.5 Oral Probiotic Therapy ......................................................................................... 19

1.5.1 Introduction to Probiotics ................................................................................ 19

1.5.2 Proposed Mechanism of Action ..................................................................... 20

1.5.3 Probiotic Strains for the Oral Cavity ............................................................... 22

1.5.4 Clinical Oral Probiotic Studies ....................................................................... 25

1.6 Study Considerations ............................................................................................ 35

1.6.1 Lorodent Probiotic Complex ........................................................................... 35

1.6.2 Duration of Probiotic Therapy .......................................................................... 35

1.6.3 Surrogate vs. Clinical Endpoints ..................................................................... 36

1.6.4 Validity of Assessing Plaque & S. Mutans Levels as Surrogate Endpoints for Caries & White Spot Lesions ................................................................. 37

1.6.5 Plaque Assessment .......................................................................................... 38
# LIST OF TABLES

Table 1. Studies assessing oral probiotic effectiveness at reducing caries experience, plaque levels and/or microbial levels in all dental populations. ................................................................. 29

Table 2. Studies assessing oral probiotic effectiveness at reducing caries experience or plaque levels as the primary outcome in all dental populations. ................................................................. 33

Table 3. Studies assessing oral probiotic effectiveness in only orthodontic populations. .... 34

Table 4. Plaque Index (PI) scoring criteria.\textsuperscript{153} ................................................................. 53

Table 5. Forward (F) and reverse (R) qPCR primer sequences for various bacterial species... 55

Table 6. Tests of normality for various qPCR datasets at T1 and T3. ................................. 63

Table 7. Landis and Koch interpretation of the kappa statistic for rater reliability.\textsuperscript{178} ........... 64

Table 8. Demographic data. ................................................................................................. 67

Table 9. Comparison of the frequency distributions of the baseline PI scores in each lozenge group.......................................................................................................................... 69

Table 10. Comparison of the frequency distributions of the baseline PI scores in each lozenge group subgrouped by tooth type. ............................................................................ 70

Table 11. Comparison of the frequency distributions of the baseline PI scores in each lozenge group subgrouped by surface. .................................................................................. 70

Table 12. Comparison of the baseline cPI scores between lozenge groups and between sexes. ............................................................................................................................... 72

Table 13. Comparison of the baseline cPI subscores between lozenge groups subgrouped by tooth type. .................................................................................................................. 73

Table 14. Comparison of the baseline cPI subscores between lozenge groups subgrouped by surface. .................................................................................................................. 74
Table 15. Comparison of the proportions of *S. mutans* DNA and lactobacilli DNA between lozenge groups at baseline................................................................. 75

Table 16. Frequency distribution of the PI scores from T1-T4 for the probiotic and placebo groups.......................................................................................................................... 78

Table 17. Comparison of the improvement, worsening or ties in PI scores and cPI scores from baseline to each time point within each lozenge group................................................................. 79

Table 18. Comparison of the improvements in PI and cPI scores from baseline to each time point between the probiotic and placebo groups................................................................. 79

Table 19. Comparison of the improvements in PI and cPI scores subgrouped by tooth type from baseline to each time point between the lozenge groups........................................ 81

Table 20. Comparison of the improvements in PI and cPI scores subgrouped by surface at each time point to baseline between the lozenge groups........................................................... 82

Table 21. Comparison of the improvements in PI scores subgrouped by tooth type and by surface at each time point to baseline between the lozenge groups........................................ 84

Table 22. Comparison of the improvements in PI and cPI scores from baseline to each time point between sexes................................................................. 84

Table 23. Changes in the proportions of *S. mutans* DNA within the probiotic and placebo groups................................................................. 90

Table 24. Changes in the proportions of total lactobacilli DNA within the probiotic and placebo groups................................................................. 90

Table 25. Comparison of the amount of change experienced by the probiotic and placebo groups in their proportions of *S. mutans* and lactobacilli DNA from T1 to T3......................... 91

Table 26. Inter-rater reliability for the Plaque Index................................................................. 92

Table 27. Intra-rater reliability for the Plaque Index................................................................. 92
Table 28. Comparison of number of lozenges taken between lozenge groups and between sexes. ................................................................. 96

Table 29. Compliance by lozenge group................................................................. 96

Table 30. Compliance by sex. .............................................................................. 97

Table 31. Compliance by examiner..................................................................... 97

Table 32. Comparison of the proportion of dental studies that show significant decreases in *S. mutans* using a dairy based delivery vehicle for probiotic therapy........................................ 107
**LIST OF FIGURES**

Figure 1. The caries process results from an imbalance favoring the demineralization of enamel. .............................................................................................................................. 5

Figure 2. White spot lesions affecting the anterior maxillary teeth are a significant esthetic concern in the orthodontic population. White spot lesions (yellow arrows) commonly develop adjacent to where the orthodontic brackets used to be (black arrows) during treatment. .......... 13

Figure 3. Study design................................................................................................................................................................................. 42

Figure 4. Compliance calendar................................................................................................................................................................. 43

Figure 5. Stainless steel Gracey curette (A). Supragingival plaque collection around the orthodontic bracket on #23 (B). Placement of supragingival plaque into a 1.5 mL Eppendorf tube (C). Participant expectorating unstimulated saliva into a 15 mL Falcon tube (D). Labeling of Eppendorf and Falcon tubes with participant’s subject ID, lozenge group, appointment number, and sample type (E)........................................................................................................... 54

Figure 6. Probiotic and placebo lozenges were identical in color, shape and size (A). The packaging for each bottle of lozenges was identical except for a marking on each bottle of “A” or “B to indicate the lozenge group (B). .............................................................................................................................. 57

Figure 7. A priori sample size determination with G*Power 3.1 for a difference between proportions of 0.25, a power of 0.8 and an alpha of 0.05. .................................................................................................................. 60

Figure 8. Participant flow......................................................................................................................................................................... 65

Figure 9. Male and female distributions in the probiotic and placebo groups................................. 67

Figure 10. Comparison of the frequency distributions of the baseline PI scores between lozenge groups and between sexes (*p < 0.05). ..................................................................................................................... 69

Figure 11. Comparison of the frequency distributions of the baseline PI scores in each lozenge group subgrouped by tooth type........................................................................................................................................ 71
Figure 12. Comparison of the frequency distributions of the baseline PI scores in each lozenge group subgrouped by surface. ................................................................. 71

Figure 13. Comparison of the baseline cPI scores between lozenge groups and between sexes. Box plots show median values (solid vertical line), interquartile range (box outline), highest and lowest values within the upper and lower limits (1.5 IQR) (whiskers), and any outliers (circles). ........................................................................................................ 72

Figure 14. Comparison of the baseline cPI subscores between lozenge groups subgrouped by tooth type. Box plots show median values (solid vertical line), interquartile range (box outline), highest and lowest values within the upper and lower limits (1.5 IQR) (whiskers), and any outliers (circles). ........................................................................................................ 73

Figure 15. Box-and-whisker plots comparing the baseline cPI subscores between lozenge groups subgrouped by surface. Box plots show median values (solid vertical line), interquartile range (box outline), highest and lowest values within the upper and lower limits (1.5 IQR) (whiskers), and any outliers (circles). ........................................................................................................ 74

Figure 16. Frequency distributions of the PI scores at each time point for the probiotic and placebo groups. ........................................................................................................ 78

Figure 17. Comparison of the improvement, worsening or ties in PI scores and cPI scores from baseline to each time point within each lozenge group (*p < 0.05). ......................................................... 80

Figure 18. Comparison of the PI scores from baseline to each time point between lozenge groups (*p < 0.05). ........................................................................................................ 80

Figure 19. Comparison of the PI scores from baseline to each time point between lozenge groups subdivided by tooth type (*p < 0.05). ........................................................................................................ 83

Figure 20. Comparison of the PI scores from baseline to each time point between lozenge groups subdivided by surface (*p < 0.05). ........................................................................................................ 83
Figure 21. Expression of supragingival *S. mutans* (A), salivary *S. mutans* (B), supragingival total lactobacilli (C), and salivary total lactobacilli (D) for each participant at T1 and T3. In the majority of samples, *S. mutans* DNA levels were not detectable, unlike lactobacilli............. 85

Figure 22. Frequency distributions of the proportion of *S. mutans* DNA and total lactobacilli DNA at T1, T3 and the differences from T1 to T3 in both supragingival plaque and saliva. Note the non-normal distribution of frequencies in each histogram and the high number of frequencies around 0%........................................................................................................ 88

Figure 23. Inter-rater reliability for the Plaque Index.......................................................... 93

Figure 24. Intra-rater reliability for the Plaque Index.......................................................... 93

Figure 25. Lozenge consumption of all participants at 14 days (T2)................................... 95

Figure 26. Lozenge consumption of all participants at 28 days (T3)................................. 95

Figure 27. Comparison of the proportion of participants with ‘perfect compliance’ to ‘less than perfect compliance’ in probiotic vs. placebo, male vs. female, and examined by examiner 1 vs. examiner 2 subgroups (*p < 0.05)............................................................................................... 98
# LIST OF ACRONYMS

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>BLIS</td>
<td>Bacteriocin-like inhibitory substances</td>
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<td>CFU</td>
<td>Colony forming units</td>
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<td>CPP-ACP</td>
<td>Casein phosphopeptide amorphous calcium phosphate</td>
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<td>Cycle threshold</td>
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<tr>
<td>DIPA</td>
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<td>qPCR</td>
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<td>rRNA</td>
<td>Ribosomal ribonucleic acid</td>
</tr>
<tr>
<td>WSL</td>
<td>White spot lesions</td>
</tr>
</tbody>
</table>
LIST OF APPENDICES

Appendix 1. University of Toronto Health Sciences Research Ethics Board Approval...... 135
Appendix 2. University of Western Ontario Ethics Approval................................. 136
Appendix 3. Health Canada Approval .................................................................. 137
Appendix 4. Ontario Centres for Excellence Funding Agreement ......................... 140
Appendix 5. Patient Information & Consent Form.................................................. 145
Appendix 6. Faculty of Dentistry, University of Toronto Privacy & Treatment Consent
Forms ......................................................................................................................... 150
Appendix 7. Lorodent Probiotic & Placebo Lozenges Product Information.............. 156
Appendix 8. PowerSoil®-htp 96 Well Soil DNA Isolation Kit Protocol ................. 157
Appendix 9. Satisfaction Questionnaire .................................................................. 159
Appendix 10. Randomization Schedule .................................................................. 160
1. INTRODUCTION

1.1 SIGNIFICANCE OF THE PROBLEM

Fixed orthodontic appliances have allowed for more efficient and precise orthodontic tooth movement. One of the negative effects of these appliances, however, is the resulting difficulty with oral hygiene and the introduction of new plaque retentive sites on the dentition. The presence of smooth surface plaque and the levels of *Streptococcus mutans* within the plaque have been shown to increase significantly in patients with fixed orthodontic appliances;\(^1\text{-}^4\) factors leading to increased enamel demineralization and caries.\(^5,6\) If enamel demineralization develops and then progresses, an early carious lesion – known as a white spot lesion – may form on these smooth surfaces posing both an esthetic and biologic concern to the patient.\(^7\) As well, such lesions could progress to the formation of fully developed caries lesions.

The prevalence of white spot lesions during orthodontic treatment has been reported to range from 26% to 89%.\(^8\) Various preventive approaches have been studied thoroughly including the use of fluoride, sugar substitutes, remineralizing agents, and antimicrobial chemical rinses. Regardless, the evidence reported in the literature has not yet pointed to any particularly effective way to prevent the development of white spot lesions during orthodontic treatment.\(^1\) There has been some evidence showing that patient motivation, professional debridement and fluoride-enriched orthodontic bonding agents might effectively control or reduce the incidence of white spot lesions, but despite the widespread use of these modalities, the incidence of white spot lesions still remains quite high. Thus, for the at-risk orthodontic population, there remains a need for a simple, adjunctive aid that can be used in addition to existing oral care regimes to reduce plaque accumulation and the levels of cariogenic oral pathogens. A novel and safe oral probiotic complex called Lorodent has been recently developed as a natural means to treat dental disease, but has yet to be investigated within the orthodontic context.

This current study was focused on investigating the effectiveness of the Lorodent probiotic complex in reducing plaque and *S. mutans* levels in adolescent participants undergoing fixed orthodontic appliance therapy using a randomized, double-blind, parallel-group, placebo-
controlled trial. The following sections will include a review of the normal formation of enamel, the caries disease process including the formation of white spot lesions, the prevalence and prevention of white spot lesions within the orthodontic context, the use of oral probiotic therapy as a novel adjunctive treatment for white spot lesions, and key considerations pertaining to the design of study.

1.2 ENAMEL FORMATION & MINERALIZATION

Enamel is formed through the process of amelogenesis during normal tooth development. Amelogenesis has three distinct phases: the inductive phase, the secretory phase and the maturation phase. During the inductive phase, the newly formed predentin matrix of the dental papilla induces cells of the inner enamel epithelium to elongate and differentiate into ameloblasts or enamel producing cells. In the secretory phase, enamel proteins are secreted by the ameloblasts to form the enamel matrix and the ameloblasts travel away from the dentinoenamel junction towards the surface of the tooth as enamel matrix is progressively laid down. In the maturation stage, the ameloblasts actively transport substances used in enamel formation including the proteins amelogenenins, enamelins and tuftelins both into and out of the enamel matrix. Importantly, calcium ions are also transported both actively and passively to the matrix allowing mineralization of the tissue. As the enamel mineralizes, it becomes denser and less porous, and the amelogenins and ameloblastins are removed after use so the protein content of enamel is much lower after maturation. Ameloblasts are then broken down before eruption of the tooth into the oral cavity.

Mature enamel is organized in densely packed enamel rods, which originate at the dentinoenamel junction and are oriented parallel to the long axis of the tooth. Enamel is the most highly mineralized tissue in the human body. It consists of a highly organized mass of approximately 95% hydroxyapatite crystals or crystalline calcium phosphate (Ca$_{10}$(PO$_4$)$_6$(OH)$_2$), 4% water and 1-2% proteins. This unique mineral composition and crystalline structure also makes enamel the hardest substance in the human body and translucent to light transmission. Notably, enamel structure is unique in that it does not have
any cells within its structure that can actively repair or regenerate damaged or demineralized enamel. Rather, enamel is in a state of flux between episodic cycles of demineralization and remineralization, and any imbalance favoring the demineralization of enamel can result in the development of caries.

1.3 DENTAL CARIES & ENAMEL DEMINERALIZATION

1.3.1 ETIOLOGY & DISEASE PROCESS

Dental caries is a multifactorial disease process that occurs slowly over time as acid produced by bacteria demineralize and eventually destroy enamel and tooth structure allowing bacterial ingress into the tooth. Caries is the one of the most widespread chronic infectious diseases.\textsuperscript{13} Although the incidence of caries has dropped dramatically in the past decades, caries is still very common and according to data from the Third National Health and Nutrition Examination Survey (NHANES III) survey approximately 80% of the children in the United States experience caries by age 17.\textsuperscript{14} The distribution of caries is disproportionate within the population and approximately 20% of the population accounts for 60% of all dental caries.\textsuperscript{15} Caries incidence and risk is increased in the orthodontic population due to the food and plaque retentive nature of fixed orthodontic appliances with a greater proportion of buccal smooth surface carious lesions in orthodontically treated patients compared to untreated controls.\textsuperscript{16,17}

The dental caries process begins at the surface of the tooth where microorganisms accumulate creating a biofilm known as dental plaque. Within the plaque, acidogenic bacteria produce organic acids from fermentable carbohydrates which decrease the pH of the biofilm and saliva resulting in the demineralization of enamel structure.\textsuperscript{18} These acid by-products are a prerequisite to caries development, and this increase in acidity also favors the growth of bacteria that are able to thrive in acidic conditions (i.e., aciduric bacteria) over other potentially innocuous bacteria that are adversely affected by this change in pH.\textsuperscript{19} Since many acidogenic bacteria are also aciduric, acid production favors a local environment in which both these bacteria and their acid byproducts proliferate further. Thus, under prolonged acidic
conditions, there is an ecological shift in the microbial flora of the plaque to more aciduric and acidogenic species, thereby increasing further the risk of demineralization and caries.

Caries develops through an interrupted and imbalanced series of demineralization and remineralization episodes on the hard tooth structures – enamel and dentin.\textsuperscript{19,20} The dynamics of mineral loss and mineral gain – simply put, destruction and repair – vary according to fluctuations in the pH and mineral content of the biofilm and greater oral environment. The process of acid dissolution of tooth structure occurs when the critical pH of 5.5 for enamel is reached thus upsetting the homeostatic balance between demineralization and remineralization in favor of the former, leading to the loss of calcium ions from the crystalline structure of hydroxyapatite.\textsuperscript{21} This reaction is spontaneously driven by the hydrogen ion gradient produced by the organic acids in the dental plaque. Conversely, if the source of the local acid production – for instance, the acidogenic bacteria and fermentable carbohydrates – is removed and the pH in the vicinity of the demineralized tooth is allowed to increase, a process of remineralization can occur spontaneously. Remineralization occurs when calcium and phosphate ions in the saliva crystalize to repair damaged hydroxyapatite crystals. Notably, if fluoride is present during the remineralization process, fluoride ions will become incorporated into the crystalline structure of hydroxyapatite replacing its hydroxyl groups resulting in a stronger, less porous, and more acid-insoluble crystal form called fluorapatite (\(\text{Ca}_{10} (\text{PO}_4)_6 \text{F}_2\)).\textsuperscript{22}

Thus, caries development is directly dependent upon the presence of acid-producing bacteria on the surface of the tooth, fermentable carbohydrates that when metabolized by these bacteria produce acid, and the ability of salivary factors to remineralize the tooth structure after acidic demineralization.\textsuperscript{23} In the orthodontic population, fixed appliances increase further the difficulty experienced by patients when they try to clean their teeth to remove both the acidogenic bacteria and fermentable carbohydrates thereby significantly increasing caries risk. To re-emphasize, these factors favor demineralization of enamel resulting in white spot lesions and caries.
Figure 1. The caries process results from an imbalance favoring the demineralization of enamel.

1.3.2 PATHOPHYSIOLOGY & DISEASE STATES

Dental caries is a slowly progressing disease characterized by the progressive destruction of tooth structure by acidic metabolites produced by oral bacteria. Caries can affect the crown or the root of the primary or permanent teeth, and can occur on smooth surfaces (i.e., buccal, lingual, and/or mesio-distal) as well as pitted and fissured surfaces (occlusal). There is a continuum of caries disease states ranging from incipient subclinical demineralization of enamel to complete destruction of the dental hard tissues with bacterial ingress into the dentin and the pulp chamber of the teeth. There are two critical markers in the progression of caries. The first is when there is visible enamel demineralization resulting in the appearance of a clinical white spot lesion, and it is possible to arrest and potentially reverse the lesion at this
The second is when the demineralization progresses to the dentin with an irreversible cavitation of the enamel surface signaling the need for restorative intervention. The initial stage of subclinical caries occurs when sufficient demineralization has occurred on the enamel surface resulting in a visible, opaque, white lesion in the enamel. This appearance is the result of a loss of mineral content which increases the porosity between the enamel rods and decreases the optical translucency of the enamel. Porous enamel has a rough surface and more light is scattered by porous enamel than by sound enamel, resulting in alterations in the refractive index and internal reflection of the enamel and a loss of surface shine. Hence, the surface of a white spot lesion often appears opaque and chalky due to these changes. If the surface of the lesion is intact, it is possible to arrest or even reverse the lesion. If the lesion persists, it will result in an esthetically unacceptable outcome.

Clearly, if the biofilm cannot be or is not removed, the production of acids will continue leading to demineralization of deeper layers of the enamel and eventually the dentin. The spread of the demineralization into the underlying dentin marks a point at which sufficient mineral structure has been removed from the tooth structure such that remineralization is unlikely. It is very difficult to detect when the dentin is first involved in developing caries, but radiographic progression of caries past the dentinoenamel junction into dentin is an indication for restorative treatment. Concomitantly, the surface enamel continues to become less mineralized and more porous and becomes increasingly vulnerable to a break in its surface continuity ultimately resulting in a cavitation of the enamel that is clinically detectable. When this occurs, removing the biofilm from the irregular surface of the cavitated enamel becomes practically impossible and therefore stopping the progression of caries becomes extremely difficult. Hence at this point, an irreversible carious process will have ensued meaning that the tooth is so affected it will require restorative intervention.

If the carious lesion is not intercepted once cavitation has occurred, it will progress much more rapidly, and bacteria will progressively infiltrate the dentin. Dentin, being much more rich in protein than enamel, is broken down not only by acid-mediated demineralization but also by proteolytic enzymes produced by the invading bacteria, meaning that once caries has reached...
the dentin the process of breakdown begins to accelerate.\textsuperscript{24} If the breakdown approaches the pulp without necessarily invading the pulp chamber, a reversible pulpitis might then develop. The odontoblasts in the pulp chamber react to this pulpitis by forming reparative or sclerotic dentin in the pulp chamber to prevent the ingress of bacteria into the pulp.\textsuperscript{24} If no restorative treatment is undertaken and the lesion is allowed to persist and progress, it will eventually reach the pulp chamber and will cause an irreversible bacterial pulpal infection, or an irreversible pulpitis, requiring endodontic therapy.

1.3.3 DETAILED DESCRIPTION OF CONTRIBUTING FACTORS

Several factors have been reported to contribute to the development of caries including the formation of dental plaque with cariogenic bacteria, inadequate oral hygiene, diet, salivary factors such as flow rate, pH and buffer capacity, and the presence of fixed orthodontic appliances.\textsuperscript{5}

DENTAL PLAQUE & CARIOGENIC BACTERIA

Formation of a dental biofilm, generally known as dental plaque, is fundamental to the caries process. Immediately after the surface of enamel is cleaned, it is coated by saliva. Within minutes, proteins and glycoproteins in the saliva selectively bind to the enamel surface forming a layer called the dental pellicle.\textsuperscript{28} Microorganisms can adhere and bind to complementary salivary receptors in the dental pellicle (i.e., sialylated mucins, proline-rich proteins, $\alpha$-amylase, salivary agglutinins, and bacterial cell fragments) resulting in the formation of a biofilm on the surface of the tooth known as dental plaque.\textsuperscript{29} As microorganisms accumulate in the biofilm, so does the concentration of bacterial metabolites on the teeth and around the gingival tissues resulting in various dentoalveolar diseases.\textsuperscript{30} Oral biofilms that are located on exposed enamel surfaces (supragingival plaque) are associated with caries, whereas biofilms that form below the gum-line and within the periodontal pocket (subgingival plaque) are associated with gingivitis and periodontal disease.\textsuperscript{29} The remainder of this discussion will be focused on the oral bacterial colonization of supragingival plaque.
The first bacteria to bind to the dental pellicle and begin the formation of dental plaque are *S. gordonii*, *S. mitis*, *S. oralis* and *S. sanguinis*. Other bacteria, such as actinomyces can then bind to these streptococci in a process known as coaggregation, and these two genera form the predominant initial colonizers of plaque. The biofilm matures as interspecies co-aggregation allows other species to colonize and proliferate, and the pattern of establishment of different colonies of bacteria (bacterial succession) is dependent upon the natural microflora present in the oral cavity and saliva. Ultimately, the final microbial composition of the plaque will depend on the ability of each species to grow and compete with neighboring cells for their own ‘domains’. It is important to note that within the biofilm different nutrient and physical microenvironments or niches are created in which different types bacteria are able to colonize preferentially. It has been shown that the predominant bacterial species found in the oral cavity is specific to the site and depth of sampling (i.e., supragingival plaque, subgingival plaque, tongue, etc.). Changes in the local environment can also cause ecological transitions within these specialized microbiomes that may lead to disease. For example, extrinsic factors such a frequent intake of fermentable carbohydrates could lead to organic acid production by acidogenic bacteria, whereas intrinsic factors such as bacterial metabolic by-products within the biofilm may cause an increase in acidity or a decrease in oxygen levels and may lead to a biofilm that favors aciduric, anaerobic, or facultative bacteria.

Substantial epidemiologic evidence has shown that the presence of aciduric and acidogenic bacteria such as *S. mutans* and lactobacilli plays an important role in the pathobiological processes that regulate the formation of caries. However, modern molecular analysis of the microbial flora in the mouth has shown that the process of caries initiation and progression is actually more complex and involves more than the above-mentioned bacteria. In this regard, it has been shown that a variety of different strains of bacteria are associated with lesions at different stages of maturity. *S. mutans* was found to have an overall strong relationship to caries at all lesion depths, and most importantly *S. mutans* was associated significantly with *early* enamel demineralization and the initiation of caries resulting in white spot lesion formation. However, additional bacterial species were also found to be involved in the caries process, though to a lesser extent. In order of decreasing cell numbers, *Actinomyces*
gerencseriae, Bifidobacterium, Veillonella, S. salivarius, S. constellatus, S. parasanguinis, and Lactobacillus fermentum were found to be associated with caries, whereas S. sanguinis was associated with health.\textsuperscript{35} Notably, lactobacilli were only observed in carious dentin once a lesion had cavitated, and in very small numbers,\textsuperscript{35,39,40} whereas Bifidobacterium was present in cavitation lesions in significantly higher numbers and, thus, may be an important pathogen in deep caries.\textsuperscript{35}

Specific studies have compared the plaque biofilms associated with white spot lesions to those isolated from cavitated lesions as well as from adjacent sound tooth surfaces.\textsuperscript{37} Once again, S. mutans preferentially colonized the surfaces with the white spot lesions and the cavitated lesions, whereas lactobacilli were not detected in the plaque from the white spot lesions and were recovered from only a few samples of the surrounding non-diseased tooth surfaces. Another study confirmed these findings and reported that the proportion of S. mutans recovered from plaque covering white spot lesions was significantly higher and the plaque pH significantly lower compared to that from clinically healthy sites.\textsuperscript{41} The study also reported that the lactobacilli proportions were generally very low in both types of plaques. Most recently, it has been discovered that lactobacilli may actually have an inhibitory effect on S. mutans which could be beneficial to oral health.\textsuperscript{40}

Lastly, the bacteria in the dental plaque also produce extracellular components such as exopolysaccharides, glycoproteins, lipoteichoic acid, and extracellular DNA (eDNA).\textsuperscript{31,42} Essentially, these extracellular components provide a three-dimensional scaffold for biofilm development, allowing microbial cells to firmly attach and structure themselves into highly dynamic microcolonies – each with their own spatial, metabolic and microenvironmental heterogeneities.\textsuperscript{42} Thus, the extracellular matrix provides structure to the plaque allowing for microbial cohesion and adhesion, while also preventing the diffusion of substances from superficial to deeper regions of the plaque. As the biofilm matures, the presence of voids or water channels between the microcolonies allow for the transport of nutrients within the biofilm.\textsuperscript{43} Approximately, 15-20\% of the volume of plaque is composed of bacteria while the remaining 75-80\% are these extracellular components.\textsuperscript{44} Thus, as the volume of plaque
increases, saliva’s beneficial buffering and antimicrobial properties are unable to penetrate the extracellular matrix and protect the enamel. Similarly, bacteria inhabiting the deeper regions of the plaque are protected from the effects of antimicrobial agents, for example chlorhexidine, while the tooth surface is deprived of the calcium and phosphate ions it requires to remineralize and repair itself.

**ORAL HYGIENE**

Mechanical debridement disrupts biofilm formation, eliminates plaque from the enamel surface, and allows clearance from the oral cavity. Compared to other techniques, it is the most effective form of oral hygiene. Adequate mechanical debridement, however, can be a difficult task, especially in the presence of fixed orthodontic appliances which may restrict access of both the tongue and toothbrush alike to the various plaque accumulating surfaces of the tooth. In particular, patients often find brushing around orthodontic brackets and underneath orthodontic arch wires, coils, power chains, and other adjuncts to be very difficult, with large accumulations of visible plaque often easily seen specifically at these sites. Often times, auxiliaries like proxy brushes are required to augment traditional tooth brushing and debride these hard to reach areas.

In response to plaque accumulation around the teeth and orthodontic appliances, patients can also develop gingivitis and gingival overgrowth, which complicates problems even more making gingival access for oral hygiene around the bracket even more difficult. Flossing is hampered with the orthodontic arch wire in place, effectively blocking direct entry of the floss to the gingival crevice from the occlusal embrasure so flossing also requires ancillary ‘tools’ including floss-threaders or Superfloss® in order to be effective.

Unfortunately, all of these procedures add time and complexity to the already questionable oral hygiene routines of the typical teenage orthodontic patient.
Diet
Carbohydrates are fermentable substrates for bacteria in the oral cavity, and when metabolized by these bacteria, acids are produced which demineralize the enamel. The pH of the plaque decreases with carbohydrate consumption but then increases with salivary buffering resulting in periods of demineralization and remineralization, respectively. As the frequency of carbohydrate consumption increases, the frequency of acid attacks increases without a concomitant increase in the reparative capacity of saliva, resulting in a net demineralization over time (i.e., white spot lesions or even fully developed caries).  

Salivary Factors
Caries can be arrested and possibly reversed in the early stages if conditions are favorable. Saliva is one of the key determinants influencing the dynamics of enamel demineralization-remineralization. It can affect the degree of mineral loss at the enamel-plaque interface, the rate of lesion progression and the likelihood of repair. Saliva delivers the minerals required for remineralization directly to the surface of the enamel lesion – such as calcium, phosphate and fluoride. The pH of saliva relates to its buffering capacity and its ability to neutralize the acid produced by the plaque on the enamel surface. Further, while a higher salivary pH maintains a higher buffering capacity, a lower pH actually favors colonization by aciduric bacteria such as S. mutans. Lastly, saliva physically cleanses the teeth, dilutes and buffers the acids produced by cariogenic plaque, and facilitates clearance from the oral cavity through swallowing. Increases in salivary flow rate promote these protective effects of saliva, while a low salivary secretion rate has been found to accentuate the drop in pH and delay its recovery following oral exposure to carbohydrates.

Influence of Fixed Orthodontic Appliances
Intraoral appliances, as required for fixed orthodontics, create plaque retentive regions that are inaccessible and difficult to cleanse by mechanical debridement. Brackets, bands, and wires limit physiologic self-cleansing mechanisms; that is, they reduce salivary access and hamper the movement of oral musculature. A bacterial niche is created in these stagnant areas, accelerating the accumulation of plaque. In the presence of carbohydrates, plaque pH is
lowered and an ecological shift favors increased colonization by aciduric and acidogenic bacteria, particularly *S. mutans*.  

Several studies have shown that *S. mutans* levels increase significantly during fixed orthodontic treatment,\(^2\)\(^-\)\(^4\) and some have suggested that the increases can be as high as five-fold.\(^3\) When these same patients were sampled 1.5 to 4 months after active orthodontic treatment was completed, *S. mutans* levels had decreased significantly and were comparable to untreated, age-matched controls. However, the amounts or characteristics of plaque (particularly *S. mutans*) do not seem to be affected by the use of any particular type of bracket – be it conventional or self-ligating.\(^49\) Similarly, ligation material – be it steel ligatures or elastomeric rings – does not appear to have an effect on the amount of microbial colonization observed during active treatment in orthodontic patients.\(^50\)

### 1.4 WHITE SPOT LESIONS IN THE ORTHODONTIC POPULATION

Enamel demineralization is one of the most common, undesirable and potentially avoidable sequelae of orthodontic treatment and has become a substantial clinical problem ever since the introduction of fixed orthodontic appliances.\(^5\) Fixed orthodontic appliances complicate oral hygiene, and demineralization of the enamel surface adjacent to these appliances results in white spot lesions that are both an esthetic concern and vulnerable to further caries progression. Pre-existing demineralization and white spot lesions may be present in orthodontic patients; however, demineralization occurring during orthodontic treatment is an indicator of a current bacterial infectious disease and must be taken seriously, for it is considered a precursor to frank enamel caries if the demineralization cycle persists.
Figure 2. White spot lesions affecting the anterior maxillary teeth are a significant esthetic concern in the orthodontic population. White spot lesions (yellow arrows) commonly develop adjacent to where the orthodontic brackets used to be (black arrows) during treatment.

1.4.1 PREVALENCE

In general, orthodontic patients have significantly more white spot lesions than non-orthodontic patients. The prevalence of post-treatment white spot lesions among orthodontic patients varies tremendously in the literature from as low as 2% to as high as 97%. However, some studies did not measure and exclude the pre-treatment prevalence of white spot lesions and this may have artificially inflated the prevalence figures that were reported to be due to orthodontic treatment. For instance, cross-sectional study designs cannot measure the pre-treatment prevalence, and some studies tried to compare the post-treatment prevalence of white spot lesions to untreated controls in an attempt to control for this. Controlling for pre-treatment white spot lesions, prevalence still varies greatly from 26% to 89%, and it is important to note that the sample sizes between studies also varied significantly (n = 35 to 332 patients). Nevertheless, regardless of what actual value is used, it is clear that the prevalence of white spot lesions is higher in patients who have undergone fixed orthodontic treatment as opposed to patients who have not received treatment.
There are various reasons for this wide range in prevalence figures in white spot lesions. The principal reason relates to whether the prevalence was reported in terms of affected individuals or affected teeth. Small sample size is likely a potential factor also. For instance, one study reported that 49.6% of patients were affected by at least 1 or more white spot lesions after orthodontic treatment but only 10.8% of teeth were affected (n = 121 patient). Another study with a smaller sample size (n = 45 patients) reported much higher figures observing that 73% of orthodontic patients and 26% of teeth were affected.

These wide variations could be due to other factors as well. The use of different diagnostic techniques to assess white spot lesions could have played a significant role. For example, a recent study that utilized quantitative light-induced fluorescence reported the highest prevalence of any study with 97% of patients and 30% of buccal surfaces affected by white spot lesions, and thus is much more sensitive than direct visualization to detect demineralization. Similarly, different studies used different clinical endpoints when diagnosing white spot lesions. For instance, some studies diagnosed earlier-stage white spot lesions that were detectable on dried and dehydrated teeth, whereas other studies only counted late stage lesions that were visible on hydrated teeth. Variations may have also arisen because some studies assessed post-treatment white spot lesions on all the teeth whereas others only assessed the anterior six or eight teeth. Lastly, the fluoride experience of the study populations differed between studies. In some studies, patients were given fluoride rinses or gels whereas in others the fluoride experience was not measured at all. For example, the proportion of orthodontic patients that developed white spot lesions with and without fluoride gel application varied 31% within the same study, but even with fluoride gel application 27% of patients still developed white spot lesions during orthodontic treatment. Similarly, the use of different bonding cements could have also affected the reported white spot prevalence between studies. Fluoride-releasing bonding cements reduced the prevalence of surfaces affected by white spot lesions by as much as 16.5%, but even then 24% of tooth surfaces were still affected.
Despite the lack of consistency across studies, there are some important trends that can also be observed in patients undergoing orthodontic treatment. The reported prevalence of white spot lesions does not vary by sex.\textsuperscript{8,52,53,60,65} Interestingly, measurable demineralization was found to occur around orthodontic appliances as early as after 1 month after placement of brackets, indicating that white spot lesions can begin to develop around orthodontic brackets within merely one appointment interval.\textsuperscript{62,67} As well, there is a sharp increase in the prevalence of white spot lesions during the first 6 months of fixed orthodontic treatment, but a slower rise thereafter.\textsuperscript{61,65} After debonding, white spot lesions decrease over the time,\textsuperscript{53,60,68} but more commonly they persist and the prevalence was found to be statistically significant even 5 years after treatment, indicating that this is a long-term esthetic problem.\textsuperscript{53} Alarmingly, cavitation did occur in a small, although statistically irrelevant, number of white spot lesions after 5 years.\textsuperscript{53}

In terms of location, there are no differences in the prevalence of white spot lesions between the left and right sides of the maxilla or mandible.\textsuperscript{52,53} Lesions are most commonly observed gingival to the brackets where oral hygiene is often inadequate and plaque accumulation severe,\textsuperscript{7,52,53,57} with the distogingival region being the most commonly affected.\textsuperscript{7} The maxillary laterals, mandibular premolars and mandibular canines are the most commonly affected teeth in the mouth.\textsuperscript{52,53,57,59} In the anterior region in specific, visible white spot lesions are a significant esthetic concern affecting 23.4\% of the anterior six maxillary and mandibular teeth (n = 885 patients).\textsuperscript{8} The maxillary arch is two and a half times more frequently affected than the mandibular arch,\textsuperscript{8} with white spot lesions occurring most frequently on the maxillary laterals, maxillary canines and mandibular canines.\textsuperscript{7,8,63} Pre-existing white spot lesions, declining oral hygiene during treatment, poor pre-treatment oral hygiene, treatment time in excess of 36 months, and young pre-treatment age (preadolescent) were all found to be significant risks for developing anterior white spot lesions,\textsuperscript{8,63} but the best predictors for white spot lesion formation were visible plaque and \textit{S. mutans} levels.\textsuperscript{59}
1.4.2 PREVENTION

A prevention-centered approach should be taken in the overall management of white spot lesions. This is especially important considering that in the majority of cases, orthodontic treatment will span multiple years, with almost every tooth in the mouth being bracketed and thus susceptible. It therefore poses an immense challenge to treat significant, multi-site lesions in 26\% to 89\% of all patients.\(^8\) Since white spot lesions are the direct result of an uncoupled demineralization-remineralization process favoring increased demineralization, it follows that prevention of white spot lesions can be achieved by decreasing demineralization and/or increasing remineralization. Decreased demineralization can be achieved through means that disrupt or decrease the bacterial biofilm and thereby decrease acid production such as traditional oral hygiene, diet modification, antimicrobial administration and/or fluoride supplementation. Remineralization can be promoted through traditional oral hygiene, fluoride supplementation, and/or casein phosphopeptide amorphous calcium phosphate supplementation. Some unconventional approaches such as the use of a non-fermentable sugar alcohol, xylitol, have also been suggested.

Traditional oral hygiene treatments are typically either mechanical or chemical in nature. The oral microflora produces extracellular glycans and organizes itself in an adherent and protective biofilm. Mechanical debridement of oral biofilm and plaque is the most effective way to reduce the total bacterial load, and thus the source of acid production, in the oral cavity. Mechanical debridement also allows saliva containing calcium and phosphate to reach the tooth surface also promoting remineralization. Mechanical debridement measures such as brushing, flossing, scaling and root planning are therefore essential to oral hygiene. However, these procedures need be done both regularly and correctly to be effective. There is a significant association between poor oral hygiene compliance and the development of white spot lesions.\(^8,45\) Thus, ongoing oral hygiene instruction and patient education are of paramount importance in the prevention of white spot lesions.

Oral rinses with antimicrobial active ingredients, such as alcohols, trislocan, chlorhexidine gluconate, etc., are bactericidal agents that chemically inhibit or disrupt oral bacteria.\(^{45,69,70}\)
These measures are an effective adjunct to mechanical debridement in reducing the overall microbial load in the oral cavity. Chlorhexidine has been shown to have immediate bactericidal effects by destabilizing the bacterial cell wall and cytoplasmic membrane leading to cell death.\(^7\) Chlorhexidine also has prolonged bacteriostatic effects due to its substantive properties and its ability to adsorb to the pellicle coated tooth surface.\(^7\) Therefore, chlorhexidine may be a beneficial adjunct to the oral hygiene routine of orthodontic patients. Once a day administration of 0.2% chlorhexidine was shown to significantly decrease biofilm formation, but upon discontinuation plaque formation returned to normal rates.\(^7\) Similarly, \(S.\) \textit{mutans} has been shown to be particularly susceptible to chlorhexidine, but these organisms were seen to recolonize the dentition in the long-term.\(^7\) Therefore, there is little literature on the topic and the strongest evidence in support of chlorhexidine utilizes a 40% chlorhexidine varnish as chlorhexidine is harmful in high concentrations.\(^7\) Long-term use can cause staining of the teeth, tongue, and gingiva and can alter taste sensation.\(^7\)

Fluoride administration has been targeted by the greater dental community as an effective method of preventing caries. When fluoride ions are incorporated into the hydroxyapatite structure, the lesion remineralizes in a less soluble, fluoridated form known as fluorapatite that is more resistant to demineralization.\(^2\) Thus, fluoride is dually effective as a pro-remineralization and anti-demineralization agent. In the orthodontic context, a significant association was found between teeth without fluorosis and the development of white spot lesions.\(^8\) Further, topical fluoride application has been proven to have substantive effects, where the calcium fluoride may persist in the dental pellicle and plaque for multiple weeks after application.\(^2\) Standard fluoridated toothpastes, irrespective of community-based water fluoridation, account for the bulk of an individual’s fluoride exposure and provide a considerable positive benefit, especially with twice daily use.\(^7\) The added benefit of fluoride mouth rinses and gels, however, has shown inconsistent results in various clinical trials.\(^2\)

Fluoride-releasing orthodontic bonding agents, such as resin modified glass ionomer cements, have been developed to provide a compliance-free, long-term delivery system for fluoride.\(^5,4\) These cements have been shown to significantly reduce white spot lesions.\(^6,7\) Fluoride
Varnishes have also been developed that provide a protective coating over the entire tooth surface. Fluoride varnishes were seen to reduce the incidence of white spot lesions by 18%, but required reapplication at each appointment every 6 weeks resulting in increased costs and chair time for both the patient and practitioner. When resin modified glass ionomer cements were used rather than composite resin cements, fluoride varnishes did not seem to provide any added benefit, and they could be readily abraded away by tooth brushing and oral function within just a few days requiring three monthly re-applications of the varnish.  

Casein phosphopeptide-amorphous calcium phosphate (CPP-ACP) is a novel technique that has been suggested in the literature to prevent and treat white spot lesions. Casein phosphopeptide stabilizes calcium phosphate in solution thereby forming the molecule CPP-ACP, and this provides the tooth surface with calcium and phosphate ions that are freely available for remineralization. CPP-ACP may promote remineralization thus decreasing the formation of white spot lesions during treatment and the severity of the lesion after treatment. There is limited literature as to the preventive effects of CPP-ACP in the orthodontic context; rather, its use has been suggested for the post-orthodontic management of white spot lesions. The effect of topical 10% CPP-ACP application to post-orthodontic white spot lesions has been compared to standard fluoride toothpaste both in vitro and in vivo. Use of both agents resulted in a statistically significant regression in white spot lesions, but there were no significant differences when compared to fluoride. Thus, the remineralizing effects of CPP-ACP was not superior to daily fluoride use, and combination of the two agents did not provide any additive remineralization potential. More research on the effectiveness of this modality is needed.

Xylitol is non-fermentable sugar alcohol of the polyol family that occurs naturally and is as sweet as sucrose. Studies done in vitro have shown that xylitol is not only noncariogenic but may in fact be anticariogenic; oral bacteria such as S. mutans can only partially metabolize xylitol which instead accumulates intracellularly and disrupts lactic acid production and bacterial growth. In theory, this antimicrobial activity should hypothetically reduce demineralization. The in vivo effectiveness of xylitol, however, has been controversial,
primarily due to differences in opinion regarding the quality of the published trials.\textsuperscript{82} Quite recently, the results of the first large-scale, long-term, multi-center, randomized placebo-controlled trial with xylitol was published consisting of 691 adult participants who consumed 5g of xylitol or placebo lozenges daily for 33 months.\textsuperscript{85} Through clinical examination, it was reported that although xylitol reduced the caries increment by 10%, or one-third of a surface per year, the results were not statistically nor clinically significant, suggesting that xylitol as a supplement does not have a significant anticariogenic effect in adults.

Despite these numerous preventive strategies and considering the widespread use of fluoride products in toothpaste and during orthodontic treatment, white spot lesions are still quite prevalent. There remains a need to find others therapies for prevention. Unfortunately, both mechanical and chemical means of treatment are non-specific in that they eliminate both the pathogenic and innocuous oral microorganisms leaving the oral cavity available for recolonization by both of these types of bacteria. Alternatively, probiotics may provide a more specific adjunct to oral hygiene than those described previously, and the addition of innocuous and potentially commensal strains of microbes into the oral cavity may shift the balance of the oral microflora from cariogenic to non-carious bacteria providing a long-term preventive solution unlike many of the current modalities.

1.5 ORAL PROBIOTIC THERAPY

1.5.1 INTRODUCTION TO PROBIOTICS

Probiotic therapy is a natural and alternative method that has been used to combat infectious disease by displacing pathogenic microorganisms with non-pathogenic endogenous or commensal bacteria.\textsuperscript{40} According to the World Health Organization, probiotics are live microorganisms that when administered in sufficient amount produce a health benefit for the host with minimal risk of side effects.\textsuperscript{86} Consumption of foods that contain a live bacterial component such as milk, yogurt and cheese has been advocated for centuries for their benefit on gastrointestinal health.\textsuperscript{40} However, it was not until 1907 that the concept of probiotics was given shape when Nobel Laureate Élie Metchnikoff proposed that the administration of certain
strains of bacteria could alter the flora of the gastrointestinal tract and replace pathogenic bacteria with commensal organisms, some of which might be even beneficial, thereby preventing bacterially mediated gastrointestinal diseases such as diarrhea, irritable bowel syndrome, colitis, etc. Probiotics have gained popularity in recent years due to their natural origin and benefits to general health, and the use of probiotic bacterial strains for preventing dental disease is a novel application of an age-old concept that has only begun to be clinically investigated in the last decade.

1.5.2 PROPOSED MECHANISM OF ACTION

Since the mouth forms the beginning of the alimentary canal, many of the bacteria currently used as probiotics for the oral cavity are the same strains found in probiotic dairy products for gastrointestinal health, and their safety has been well established. While these strains have been well studied in the context of various gastrointestinal diseases, their applicability and effectiveness in the oral cavity for dental diseases like caries has not yet been clarified completely. Thus, suggested mechanisms of probiotic action have been derived largely from gastrointestinal studies, but the exact mechanism by which probiotics influence the oral cavity is multifactorial, complex, and at best understood only partially.

The use of probiotics to create a favorable microbial ecological change may occur in various ways. In the gastrointestinal system, probiotics have been shown to exert a wide array of actions from direct antagonism of pathogenic bacteria to inducing an increase in colonization resistance (for putative pathogenic bacteria ostensibly) to alterations in the intestinal epithelium and immunomodulation of the host. For instance, probiotic bacteria have been found to produce different antimicrobial compounds that may be able to directly inhibit or kill pathogens. These compounds include organic acids, hydrogen peroxide, carbon peroxide, diacetyl, low molecular weight antimicrobial substances, bacteriocins, and inhibitors of bacterial adhesion to the host or biofilm substrate. The latter effect is important since it has been suggested that when probiotic bacteria compete with pathogenic microorganisms for binding sites on intestinal mucosa, they also compete for available nutrients within the gut and thereby reinforce the intestinal mucosal barrier, restore normal microecological balance and
improve resistance to colonization of the gut by potentially pathogenic bacteria after, for example, diarrhea.\textsuperscript{87} Furthermore, certain strains of probiotics have been found to stimulate the intestinal mucosa directly causing the mucosa to produce both specific antibodies as well as non-specific secretory immunoglobulins.\textsuperscript{40}

Since there is a distinct difference in the epithelial structure and mucosal secretions within the gastrointestinal tract versus those of the oral cavity, it is unknown if probiotic therapy has the same mechanisms of action in the oral cavity and in saliva that have been implicated in the gut.\textsuperscript{86} In the oral cavity, it has been suggested that if probiotic bacteria can colonize the dental plaque, then a biofilm might be formed with bacteria that promote health as opposed to disease. Biofilm formation might prevent future colonization by pathogenic bacteria by reducing the number of available adhesion binding sites on the surface of the tooth or salivary pellicle.\textsuperscript{89} In relation to this, colonization could occur directly on saliva-coated hydroxyapatite surfaces of teeth as well as by inducing co-aggregation and colonization of other bacteria.\textsuperscript{86,89} If a probiotic can interfere with bacteria-to-bacteria attachment and compete with other oral microorganisms for available nutrients, then interspecies interactions such as these could cause ecological shifts in the biofilm microflora that would be more permissive of health.\textsuperscript{87} It is also noteworthy that probiotic bacteria might be able to produce antimicrobial substances (i.e., hydrogen peroxide, bacteriocins, adhesion inhibitors, etc.) that directly inhibit oral bacteria, and hopefully even more so for pathogenic bacteria.\textsuperscript{90} In theory, consumption of probiotics should lead to an increased concentration of favorable bacterial species in the host organism and this could mean that the putative beneficial effects of probiotic therapy might persist in the long-term if it results in a selection pressure on the developing microflora towards colonization by non-pathogenic or possibly even anti-pathogenic species.\textsuperscript{87} Lastly, it has been suggested that oral probiotic therapy could lead to activation of a host immune response that might improve the host’s ability to prevent the establishment of pathogenic microorganisms. However, while this has demonstrated predominantly in gastrointestinal tissues, it is unclear as to whether or not probiotics can activate immune inductive sites within the oral cavity, for instance lymphoid tissues in the lingual tonsils, pharyngeal tonsils, adenoids, and Waldeyer’s ring.\textsuperscript{86}
1.5.3 PROBIOTIC STRAINS FOR THE ORAL CAVITY

Unlike other therapeutic modalities, probiotics are unique in that they are administered in a live form. Oral persistence of the probiotic in the oral cavity is crucial for there to be any long-term effect of probiotic therapy. Therefore, one essential requirement for a microorganism to be considered for oral probiotic therapy is its ability to attach, adhere to and colonize surfaces in the oral cavity.\textsuperscript{86} Otherwise, if there is no permanent colonization, the effect of the probiotic will only be temporary at best. Equally as important, in order to confer any health benefits, the probiotic strain must be viable in its intended environment – whether it be the oral cavity or the gastrointestinal tract.\textsuperscript{86} Therefore, most of the strains that are used in probiotic therapy are common commensal flora isolated from healthy individuals. These strains should be viable, able to adhere and colonize, may have antimicrobial properties, and must be proven to be harmless.\textsuperscript{40}

LACTOBACILLUS AS A PROBIOTIC

\textit{Lactobacillus} is a diverse genus of rod-shaped, Gram-positive facultative anaerobic or microaerophilic bacteria.\textsuperscript{91} In humans, lactobacilli are endogenous to both the oral cavity and the gastrointestinal tract, and they are the principal strains found in the common dairy products used for gastrointestinal probiotic therapy.\textsuperscript{91} Thus, their safety in human populations has been well established over the past century of use within the gastrointestinal field.\textsuperscript{86}

Lactobacilli derive their antimicrobial activity from the metabolism of lactose and other sugars into lactic acid creating an acidic environment that inhibits the growth of harmful bacteria. In the gastrointestinal tract, this antimicrobial activity has made them a popular choice for probiotic therapy, but this same production of acid in the oral cavity has been associated with dental caries. It has been discussed previously that \textit{S. mutans} is involved in the initiation of dental caries at the enamel on the surface of the tooth and is considered to be a primary pathogen for dental caries at all stages, whereas lactobacilli is only associated with the progression of mature lesions in dentine and is considered to be a secondary pathogen.\textsuperscript{35,39,40} This concept of shifting from a more cariogenic to a significantly less cariogenic bacteria that is only harmful at later disease stages has supported the use of lactobacilli for dental probiotic
therapy in patients with no active carious lesions. In vivo probiotic studies have further supported this notion of a favorable ecological shift. Studies using lactobacilli strains resulting in increased plaque and salivary counts of lactobacilli observed no changes in the amount of lactic acid produced in plaque, and on the contrary increases in plaque pH and overall salivary buffering capacity were reported.\textsuperscript{92-94}

In the oral cavity, specific species of lactobacilli have been investigated as probiotic agents capable of inhibiting the growth of oral pathogens.\textsuperscript{95-99} In vitro studies have shown that the majority of lactobacilli strains suppressed growth of numerous oral pathogens including \textit{S. mutans}, \textit{Aggregatibacter actinomycetemcomitans}, \textit{Porphyromonas gingivalis}, and \textit{Prevotella intermedia}, but none inhibited \textit{C. albicans}.\textsuperscript{95} Further, out of 42 strains, \textit{L. plantarum}, \textit{L. paracasei}, \textit{L. salivarius}, and \textit{L. rhamnosus} expressed the highest antimicrobial activity and also displayed a high tolerance for environmental stress, therefore making them good candidates to be used as potential probiotics for the oral cavity.\textsuperscript{95}

Another in vitro study demonstrated that a different strain of \textit{Lactobacillus}, \textit{L. reuteri}, exhibited significant inhibitory effects on the growth of cariogenic \textit{S. mutans} biofilms and on the proliferation of periodontopathogenic bacteria including \textit{A. actinomycetemcomitans}, \textit{P. gingivalis}, \textit{Fusobacterium nucleatum} and \textit{Tannerella forsythia}.\textsuperscript{90} An additional study showed that \textit{L. reuteri} was not only able to inhibit the growth of gram-positive and gram-negative bacteria, but it also inhibited the growth of yeast, mold and protozoa.\textsuperscript{100} Specifically, \textit{L. reuteri}’s antibacterial activities were attributed to production of organic acids, hydrogen peroxide, a bacteriocin-like compound and a unique antimicrobial factor, reuterin.\textsuperscript{90,101} Reuterin, in particular, has been shown to inhibit specifically the growth of \textit{S. mutans}.\textsuperscript{102} Other studies have reported that lactobacilli’s co-aggregation abilities can form a barrier that may be able to prevent colonization of pathogenic bacteria such as \textit{S. mutans}, and this co-aggregation may even create a microenvironment concentrated with the antimicrobial substances they produce.\textsuperscript{86,103,104}

A recent in vivo experiment investigated lactobacillus-mediated inhibition of \textit{S. mutans} in caries-free and caries-active subjects.\textsuperscript{105} In the study population, 91 strains of naturally
occurring lactobacilli were isolated and 23 of these strains completely inhibited the growth of all strains of *S. mutans* tested. Interestingly, this effect was most pronounced in caries-free subjects whose endogenous lactobacilli more effectively inhibited growth of *S. mutans* compared to patients with arrested or active-caries lesions who were already colonized. It was also reported that *L. paracasei*, *L. plantarum*, and *L. rhamnosus* were the species with the maximum capacity to interfere with the growth of *S. mutans*.

Thus, specific strains of lactobacilli may be useful as probiotic agents given the evidence for promising therapeutic action against common oral pathogens such as *S. mutans*.

**S. SALIVARIUS AS A PROBIOTIC**

*Streptococcus salivarius* is a species of spherical, Gram-positive bacterial species which colonizes the oral cavity and upper respiratory tract of humans within a few hours after birth and remains the predominant inhabitant throughout an individual’s life. Thus, *S. salivarius* is a commensal bacteria and a key member of the healthy oral microflora in humans.

Investigations have been carried out that have used *S. salivarius* as a probiotic agent in the prevention of oral infections. Some strains of *S. salivarius* secrete antimicrobial peptides such as bacteriocins and bacteriocin-like inhibitory substances (BLIS) that can inhibit the growth of oral pathogens such as *Streptococcus pyogenes* (the bacteria notorious for streptococcal pharyngitis) and *Candida albicans* (the fungus responsible for oral candidiasis). In addition, *S. salivarius* has also been shown to reduce the levels of pro-inflammatory cytokines interleukin-6 (IL-6) and interleukin-8 (IL-8) which are secreted primarily by human gingival fibroblasts when facing a microbiological pathogenic challenge. Thus, *S. salivarius* may play an integral role in interspecies inhibition of pathogens in the oral cavity as well by inducing a reduction in host-mediated tissue-damaging inflammation in response to oral pathogens.

A recent randomized controlled trial (RCT) evaluated the safety and human tolerance of the oral probiotic *S. salivarius K12*. It was reported that daily, oral ingestion of *S. salivarius* K12 over a 28-day period did not affect human health adversely – as measured by blood clinical
chemistry, hematology, and the frequency and intensity of adverse events – leading the investigators to conclude that *S. salivarius* K12 was safe to use in the human population via oral delivery.\(^{110}\)

### 1.5.4 CLINICAL ORAL PROBIOTIC STUDIES

Over the past decade, various studies have been conducted within the dental context to investigate if probiotic therapy has a beneficial anticariogenic effect. A review of the literature revealed that from 2001-2015 there have been 38 studies in total that have investigated the effects of various oral probiotic regimes within the context of plaque and caries (Table 1).\(^{92-94,111-145}\) Of these 38 studies, 29 were RCTs while 9 were crossover studies. All 38 studies investigated the effect of oral probiotic therapy on the levels of *S. mutans* and lactobacilli. However, 9 of the 38 studies also utilized clinical measures such as caries experience or plaque levels as principal outcomes (Table 2),\(^{111,121,127,136,137,139,142,144,145}\) whereas microbial evaluation was the sole outcome for 29 of these studies (Table 1). Only 5 of the 38 studies investigated the effect of oral probiotic therapy within the orthodontic context (Table 3).\(^{120,138,140,141,144}\)

Hence, the designs of the studies referred to above were extremely heterogeneous. Almost all of the studies utilized different bacterial strains, concentrations/dosages, vehicles of administration, administration protocols, intervention durations, and patient populations. Altogether then, the above-noted heterogeneity makes interpretation and comparison of the studies difficult at best. For example, over two dozen strains of commensal bacteria were used as probiotics, the most common of which were *L. rhamnosus*, *L. reuteri*, *Bifidobacterium lactis* subsp. BB-12, and *L. paracasei*. Various vehicles were used to deliver the probiotics including milk, cheese, liquid, yogurt, lozenges, tablets, ice cream, gum and mouth rinse. The majority of studies compared probiotic therapy alone to a placebo, but some studies compared or supplemented probiotic therapy with chlorhexidine, fluoride or xylitol. Some studies administered the probiotic intervention for as little as 2 weeks whereas others intervened for as long as 23 months. Participants ranged in age from being newborns to participants as old as 84 years of age.
Given the wide array of approaches used in the previously referenced investigations, it is no wonder that the results of the studies are mixed. Nevertheless, over 60% of the investigations demonstrated that there was a beneficial effect of the oral probiotic therapy in relation to whatever outcome(s) were being measured, whereas in 40% of the studies no effects were shown. In particular, no studies have been done to assess the effectiveness of a novel probiotic complex, Lorodent, particularly within the dental and more specifically within the framework of orthodontic treatment with fixed appliances.

**PROBIOTIC STUDIES ASSESSING CARIES EXPERIENCE & PLAQUE LEVELS**

Of the 38 studies in Table 1, 9 studies evaluated the anticariogenic effectiveness of oral probiotic therapy using clinical measures for disease such as caries experience or clinical plaque scores (Table 2).

When investigating the effectiveness of any intervention at preventing a disease, ideally the primary outcome of the study should be the incidence of the disease itself. To date, there have been only 7 studies on oral probiotic effectiveness that utilized caries experience as the primary outcome measure. In these studies, caries experience was measured in various ways including measuring the dental caries rate, the number of decayed-missing-filled surfaces (DMFS), caries free status, root caries reversals, and the incidence of white spot lesions (precursors to smooth surface caries). Overall, 4 of the 7 studies reported decreases in caries experience following oral probiotic therapy, whereas 3 reported no change. These studies marginally suggest that oral probiotics have a protective effect against caries; however, it is unclear whether the effect of oral probiotics are short-lived or long-lasting, and if they have the greatest effect when administered early in life.

There were 2 remaining articles that evaluated clinical plaque levels over time instead of caries experience as an indirect measure of disease, and both studies reported significant decreases in plaque scores using probiotic lozenges for short durations.

Cumulatively, the results from these 9 studies are mixed as to whether oral probiotics do or do not have a protective effect against caries. Given the low number of studies and the
heterogeneity of their methodology and results, further studies are needed to investigate the possible dental benefits of oral probiotics, especially within the orthodontic context.

**PROBIOTIC STUDIES ASSESSING *S. MUTANS* & *LACTOBACCILI* LEVELS**

Although the intended outcome of oral probiotic administration is a decrease in the overall caries experience of the treated population, as shown, very few studies utilized this as a clinical endpoint. It is understandable that since caries initiation and progression is a slow multifactorial process which happens over the course of years, it would be difficult for investigators to use this ideal endpoint as the time needed for the development of lesions is very long, the possibility of confounding variables high, and thus difficulty in conducting a long-term trial with sufficient sample size is significant. That is, surrogate endpoints allow studies to be simpler, shorter, and less expensive. Hence, much of the literature investigating the potential effect of oral probiotics on caries reduction instead used a surrogate endpoint like changes in the levels of *S. mutans* and total lactobacilli as their primary outcome. This, unfortunately, limits the conclusions that can be drawn from these studies in the context of clinical implications.

Specifically, 20 of the 38 studies from Table 1 reported a decrease in *S. mutans* levels post-probiotic administration while 18 of the studies showed no significant changes in the *S. mutans* levels. Further, 27 studies investigated the short-term effect of oral probiotics on post-administration lactobacilli levels. Lactobacilli levels are often measured to assess if successful colonization of the probiotic strains has occurred. 19 of the 27 studies showed no change, 7 of the studies showed statistically significant increases in lactobacilli levels and 1 study showed a decrease in lactobacilli levels. These findings further suggest that administration of various oral probiotics has the potential to allow for a reduction of the pathogenic *S. mutans*; however, it remains unclear if the mechanism for decrease in *S. mutans* is a concomitant increase in the lactobacilli levels.
To date, there have only been 5 studies that have investigated the effects of probiotic therapy in adolescent patients undergoing orthodontic treatment with the use of bonded brackets (Table 3),\textsuperscript{120,138,140,141,144} and 1 of these studies was carried out in orthodontic patients with cleft lip and palate.\textsuperscript{141} Of the 5 orthodontic studies, only 1 used the development of white spot lesions or caries as the primary outcome,\textsuperscript{144} whereas the remainder all used microbial levels as their primary outcome.\textsuperscript{120,138,140,141} To reemphasize, microbial analysis alone is only a surrogate marker and cannot be unequivocally extrapolated to be related to the development or absence of white spot lesions/caries. Further, 3 of the 5 studies were RCTs,\textsuperscript{138,141,144} whereas 2 were crossover studies.\textsuperscript{120,140} Overall, 3 studies reported a beneficial effect of the oral probiotic therapy in relation to whatever outcome(s) were being measured,\textsuperscript{120,138,141} whereas the other 2 studies showed no significant change.\textsuperscript{140,144} It is difficult to compare the studies directly given the heterogeneity in terms of their designs, outcomes measured and probiotic treatment modalities used (i.e., probiotic strain(s), dosage, duration), and given the low total number of studies, there do not seem to be any discernable trends.

Given the diversity of experimental protocols and findings regarding probiotics in the at-risk orthodontic population, further studies are needed to elucidate the potential benefits of oral probiotics.
Table 1. Studies assessing oral probiotic effectiveness at reducing caries experience, plaque levels and/or microbial levels in all dental populations.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Design</th>
<th>Age (years)</th>
<th>Groups</th>
<th>Sample Size</th>
<th>Probiotic Strain</th>
<th>Probiotic Vehicle, Duration</th>
<th>Clinical Outcome</th>
<th>Microbial Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Näse et al., 2001</td>
<td>RCT</td>
<td>1-6</td>
<td>1. Probiotic</td>
<td>1. 231</td>
<td><em>L. rhamnosus</em> GG</td>
<td>Milk, 7 months</td>
<td>↓ in dental caries rate</td>
<td>↓ MS (saliva + plaque)</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>2. Placebo</td>
<td>2. 220</td>
<td></td>
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</tr>
<tr>
<td>Ahola et al., 2002</td>
<td>RCT</td>
<td>18-35</td>
<td>1. Probiotic</td>
<td>1. 38</td>
<td><em>L. rhamnosus</em> GG + <em>L. rhamnosus</em> LC 705</td>
<td>Cheese, 3 weeks</td>
<td></td>
<td>↓ MS (saliva)</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>2. Placebo</td>
<td>2. 36</td>
<td></td>
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</tr>
<tr>
<td>Montalto et al., 2004</td>
<td>RCT</td>
<td>23-37</td>
<td>1. Probiotic (capsule)</td>
<td>1. 14</td>
<td><em>L. sporogenes</em> + <em>L. bifidum</em> + <em>L. bulgaricus</em> + <em>L. termophilus</em> + <em>L. acidophilus</em> + <em>L. casei</em> + <em>L. rhamnosus</em> Liquid or tablet, 45 days</td>
<td></td>
<td></td>
<td>→ MS + ↑ LB (saliva)</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>2. Probiotic (liquid)</td>
<td>2. 16</td>
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<td></td>
<td></td>
<td></td>
<td>3. Placebo</td>
<td>3. 5</td>
<td></td>
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<tr>
<td>Nikawa et al., 2004</td>
<td>Crossover</td>
<td>20</td>
<td>Probiotic + placebo</td>
<td>40</td>
<td><em>L. reuteri</em></td>
<td>Yogurt, 2 weeks per treatment</td>
<td></td>
<td>↓ MS (saliva)</td>
</tr>
<tr>
<td>Caglar et al., 2005</td>
<td>Crossover</td>
<td>21-24</td>
<td>Probiotic + placebo</td>
<td>21</td>
<td><em>Bifidobacterium</em> DN-173</td>
<td>Yogurt, 2 weeks per treatment</td>
<td></td>
<td>↓ MS + → LB (saliva)</td>
</tr>
<tr>
<td>Caglar et al., 2006</td>
<td>RCT</td>
<td>21-24</td>
<td>1. Probiotic (liquid)</td>
<td>1. 30</td>
<td><em>L. reuteri</em> ATCC 55730</td>
<td>Liquid or tablet, 3 weeks</td>
<td></td>
<td>↓ MS + → LB (saliva)</td>
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<td></td>
<td></td>
<td></td>
<td>2. Probiotic (tablet)</td>
<td>2. 30</td>
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<td></td>
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<td></td>
<td>3. Placebo (liquid)</td>
<td>3. 30</td>
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<td>4. Placeo (tablet)</td>
<td>4. 30</td>
<td></td>
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<tr>
<td>Caglar et al., 2007</td>
<td>RCT</td>
<td>21-24</td>
<td>1. Probiotic</td>
<td>1. 20</td>
<td><em>L. reuteri</em> ATCC 55730 + <em>L. reuteri</em> ATCC PTA 5289</td>
<td>Xylitol (1.0g)</td>
<td>↓ MS + → LB (saliva)</td>
<td>Combination with xylitol did not enhance effect</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>2. Xylitol</td>
<td>2. 20</td>
<td></td>
<td>Gum, 3 weeks</td>
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<td></td>
<td></td>
<td></td>
<td>3. Probiotic + xylitol</td>
<td>3. 20</td>
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<td></td>
<td></td>
<td></td>
<td>4. Placebo</td>
<td>4. 20</td>
<td></td>
<td></td>
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<tr>
<td>Caglar et al., 2008</td>
<td>Crossover</td>
<td>20</td>
<td>Probiotic + placebo</td>
<td>24</td>
<td><em>Bifidobacterium</em> lactis subsp. BB-12</td>
<td>Ice-cream, 10 days per treatment</td>
<td></td>
<td>↓ MS + → LB (saliva)</td>
</tr>
<tr>
<td>Caglar et al., 2008</td>
<td>RCT</td>
<td>20</td>
<td>1. Probiotic</td>
<td>1. 10</td>
<td><em>L. reuteri</em> ATCC 55730 + <em>L. reuteri</em> ATCC PTA 5289</td>
<td>Lozenge, 10 days</td>
<td></td>
<td>↓ MS + → LB (saliva)</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>2. Placebo</td>
<td>2. 10</td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Cildir et al., 2009</td>
<td>Crossover</td>
<td>12-16</td>
<td>1. Probiotic</td>
<td>1. 12</td>
<td><em>Bifidobacterium</em> animalis subsp. lactis</td>
<td>Yogurt, 2 weeks per treatment</td>
<td></td>
<td>↓ MS + → LB (saliva)</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>2. Placeo</td>
<td>2. 12</td>
<td></td>
<td></td>
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<tr>
<td>Stecksén-Blicks et al.,</td>
<td>RCT</td>
<td>1-5</td>
<td>1. Probiotic + fluoride</td>
<td>1. 110</td>
<td><em>L. rhamnosus</em> LB21 + Fluoride (2.5 mg/L)</td>
<td>Milk, 21 months</td>
<td></td>
<td>↓ caries experience (DMFS)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2. Placebo</td>
<td>2. 76</td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Study</td>
<td>Design</td>
<td>Duration</td>
<td>Intervention 1</td>
<td>Intervention 2</td>
<td>Intervention 3</td>
<td>Intervention 4</td>
<td>Dosage</td>
<td>Treatment Details</td>
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<tr>
<td>Cogulu et al., 2010</td>
<td>RCT</td>
<td>20-27</td>
<td>1. Probiotic 1x/day</td>
<td>2. Probiotic 2x/day</td>
<td>3. Placebo</td>
<td></td>
<td>1.35</td>
<td>Lactococcus lactis ssp. lactis + Lactococcus lactis ssp. cremoris + Lactococcus lactis ssp. diactylactis + Leuconostoc mesenteroides ssp. cremoris + Kluyveromyces marxianus + Saccaromyces unisporus + L. kefyr Kefir, 3 weeks</td>
</tr>
<tr>
<td>Lexner et al., 2010</td>
<td>RCT</td>
<td>Adolescents</td>
<td>1. Probiotic</td>
<td>2. Placebo</td>
<td>18</td>
<td>L. rhamnosus LB21 Milk, 2 weeks</td>
<td>1.35</td>
<td>MS + LB (saliva) in group 2</td>
</tr>
<tr>
<td>Aminabadi et al., 2011</td>
<td>RCT</td>
<td>6-12</td>
<td>1. Probiotic</td>
<td>2. Chlorhexidine</td>
<td>3. Chlorhexidine then probiotic</td>
<td></td>
<td>1.35</td>
<td>MS + LB (saliva + plaque)</td>
</tr>
<tr>
<td>Chuang et al., 2011</td>
<td>RCT</td>
<td>20-26</td>
<td>1. Probiotic + xylitol</td>
<td>2. Xylitol</td>
<td></td>
<td>1.42</td>
<td>L. paracasei GMNL-33 + xylitol (11%) Tablet, 2 weeks</td>
<td>2.36</td>
</tr>
<tr>
<td>Jindal et al., 2011</td>
<td>RCT</td>
<td>7-14</td>
<td>1. Probiotic formula 1</td>
<td>2. Probiotic formula 2</td>
<td>3. Placebo</td>
<td></td>
<td>1.50</td>
<td>L. rhamnosus + Bifidobacterium longum + Saccharomyces cerevisiae 2. Bacillus coagulans Mouth rinse, 2 weeks</td>
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<tr>
<td>Singh et al., 2011</td>
<td>Crossover</td>
<td>12-14</td>
<td>Probiotic + placebo</td>
<td></td>
<td>39</td>
<td>Bifidobacterium lactis subsp. BB-12 + L. acidophilus LA-5 Ice cream, 10 days per treatment</td>
<td></td>
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<tr>
<td>Cildir et al., 2012</td>
<td>Crossover</td>
<td>4-12</td>
<td>Probiotic + placebo</td>
<td></td>
<td>19</td>
<td>L. reuteri DSM 17939 + L. reuteri ATCC PTA 5289 Liquid drops, 25 days per treatment</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Juneja &amp; Kakade, 2012</td>
<td>RCT</td>
<td>12-15</td>
<td>1. Probiotic</td>
<td>2. Placebo</td>
<td>1.18</td>
<td>2.18</td>
<td>L. rhamnosus HCT 70 Milk, 3 weeks</td>
<td></td>
</tr>
<tr>
<td>Keller &amp; Twetman, 2012</td>
<td>Crossover</td>
<td>26 (mean)</td>
<td>Probiotic + placebo</td>
<td></td>
<td>18</td>
<td>L. reuteri DSM 17938 + L. reuteri ATCC PTA 5289 Tablet, 2 weeks</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Study</td>
<td>Design</td>
<td>Duration</td>
<td>Group A</td>
<td>Group B</td>
<td>Treatment</td>
<td>Outcome</td>
<td></td>
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<tr>
<td>Keller et al., 2012&lt;sup&gt;32&lt;/sup&gt;</td>
<td>RCT</td>
<td>23 (mean)</td>
<td>1. Probiotic after chlorhexidine course</td>
<td>1. 32</td>
<td>L. reuteri DSM 17938 + L. reuteri ATCC PTA 5289 Lozenge, 6 weeks</td>
<td>$\uparrow$ MS (saliva) $\downarrow$ MS due to CHX</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Marttinen et al., 2012&lt;sup&gt;92&lt;/sup&gt;</td>
<td>Crossover</td>
<td>25 (mean)</td>
<td>Probiotic formula 1 + Probiotic formula 2</td>
<td>13</td>
<td>1. L. rhamnosus GG 2. L. reuteri SD2112 &amp; PTA 5289 Tablet, 2 weeks</td>
<td>$\uparrow$ MS + $\uparrow$ LB (plaque)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mortazavi &amp; Akhlaghi, 2012&lt;sup&gt;133&lt;/sup&gt;</td>
<td>RCT</td>
<td>18-37</td>
<td>1. Probiotic 2. Placebo</td>
<td>1. 29</td>
<td>L. casei Cheese, 2 weeks</td>
<td>$\downarrow$ MS (saliva)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sudhir et al., 2012&lt;sup&gt;134&lt;/sup&gt;</td>
<td>RCT</td>
<td>10-12</td>
<td>1. Probiotic 2. Placebo</td>
<td>1. 20</td>
<td>L. acidophilus Curd, 30 days</td>
<td>$\downarrow$ MS (saliva)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Taipale et al., 2012&lt;sup&gt;135&lt;/sup&gt;</td>
<td>RCT</td>
<td>1 month</td>
<td>1. Probiotic 2. Xylitol 3. Sorbitol</td>
<td>1. 32</td>
<td>Bifidobacterium lactis subsp. BB-12 Tablets in pacifier or by spoon, 22-23 months</td>
<td>$\downarrow$ plaque scores Overall: $\uparrow$ MS (saliva) Where M18 colonized: $\downarrow$ MS $\downarrow$ MS + $\downarrow$ LB (saliva) No evidence of oral colonization of F19</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Burton et al., 2013&lt;sup&gt;136&lt;/sup&gt;</td>
<td>RCT</td>
<td>5-10</td>
<td>1. Probiotic 2. Placebo</td>
<td>1. 40</td>
<td>S. salivarius M18 Lozenge, 3 months</td>
<td>$\downarrow$ plaque scores</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hasslöf et al, 2013&lt;sup&gt;137&lt;/sup&gt;</td>
<td>RCT</td>
<td>4 months</td>
<td>1. Probiotic 2. Placebo</td>
<td>1. 56</td>
<td>L. paracasei F19 Cereal, 9 months</td>
<td>No change in DMFS $\downarrow$ MS + $\downarrow$ LB (saliva) No evidence of oral colonization of F19</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Jose et al., 2013&lt;sup&gt;138&lt;/sup&gt;</td>
<td>RCT</td>
<td>14-19</td>
<td>1. Probiotic curd 2. Probiotic toothpaste 3. Placebo</td>
<td>1. 20</td>
<td>Strains not specified Curd and toothpaste, 30 days</td>
<td>$\downarrow$ MS (plaque) No difference between either probiotic vehicle</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Taipale et al., 2013&lt;sup&gt;139&lt;/sup&gt;</td>
<td>RCT</td>
<td>1 month</td>
<td>1. Probiotic 2. Xylitol 3. Sorbitol</td>
<td>1. 32</td>
<td>Bifidobacterium lactis subsp. BB-12 Tablet in pacifier or by spoon, 22-23 months</td>
<td>No $\uparrow$ or $\downarrow$ in caries rate at 4 year follow up $\uparrow$ MS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Campus et al., 2014&lt;sup&gt;93&lt;/sup&gt;</td>
<td>RCT</td>
<td>6-8</td>
<td>1. Probiotic 2. Placebo</td>
<td>1. 91</td>
<td>L. brevis CD2 Lozenge, 6 weeks</td>
<td>$\downarrow$ MS (plaque) $\uparrow$ plaque pH</td>
<td></td>
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</tr>
</tbody>
</table>
Nishihara et al., 2014<sup>44</sup> | RCT | 25 (mean) | 1. Probiotic 1 + xylitol 2. Probiotic 2 + xylitol 3. S. mutans Ab + xylitol 4. Xylitol | 1. L. salivarius WB21 + xylitol (280 mg) 2. L. salivarius TI 2711 + xylitol (450 mg) 3. Antibody against glucosyltransferase from S. mutans + xylitol (100 mg) 4. Xylitol (280 mg) Tablet, 2 weeks | 1. ↓ MS + ↑ LB (saliva) 2. ↓ MS + ↑ LB (saliva) 3. ↓ MS + → LB (saliva) | 1. ↓ MS + ↑ LB (saliva) 2. ↓ MS + ↑ LB (saliva) 3. ↓ MS + → LB (saliva) |

Pinto et al., 2014<sup>40</sup> | Crossover | 10-30 | 1. Probiotic 2. Placebo | Bifidobacterium animalis subsp. lactis DN-173010 Yogurt, 2 weeks per treatment | → MS + → LB (saliva + plaque) |

Ritthagol et al., 2014<sup>41</sup> | RCT | 19 (mean) | 1. Probiotic 2. Placebo | L. paracasei SD1 Milk, 4 weeks | ↓ MS + ↑ LB (saliva) SD1 successfully colonized at 4 weeks |

Stensson et al., 2014<sup>42</sup> | RCT | From birth | 1. Probiotic 2. Placebo | L. reuteri ATC 55730 Oral drops, 1 year | ↑ in # of caries free subjects | → MS, → LB, → SIgA (saliva) |

Teanpaisan & Piwat, 2014<sup>43</sup> | RCT | 18-25 | 1. Probiotic 2. Placebo | L. paracasei SD1 Milk; 8 weeks | ↓ MS + ↑ LB (saliva) SD1 detected at 4 weeks |

Gizani et al., 2015<sup>44</sup> | RCT | 16 (mean) | 1. Probiotic 2. Placebo | L. reuteri DSM 17938 + L. reuteri ATCC PTA 5289 Lozenge, 17 months | No difference in WSL incidence | → MS + → LB (saliva) |

Toiviainen et al., 2015<sup>45</sup> | RCT | 24 (mean) | 1. Probiotic 2. Placebo | L. rhamnosus GG + Bifidobacterium animalis subsp. lactis BB-12 Lozenge, 4 weeks | ↓ plaque scores | → MS, → LB (saliva) |

*↓ = Decrease, → = No change, ↑ = Increase  
† MS = S. mutans, LB = lactobacilli  
‡ Green cells indicate a beneficial effect from probiotic therapy while red cells indicate no effect.  
‡‡ Blue cells indicate studies that used caries experience or plaque levels as the primary outcome.
<table>
<thead>
<tr>
<th>Reference</th>
<th>Design</th>
<th>Age (years)</th>
<th>Groups</th>
<th>Sample Size</th>
<th>Probiotic Strain</th>
<th>Probiotic Vehicle, Duration</th>
<th>Clinical Outcome</th>
<th>Microbial Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Näse et al., 2001&lt;sup&gt;111&lt;/sup&gt;</td>
<td>RCT</td>
<td>1-6</td>
<td>1. Probiotic</td>
<td>1. 231</td>
<td>L. rhamnosus GG</td>
<td>Milk, 7 months</td>
<td>↓ in dental caries rate</td>
<td>↓ MS (saliva + plaque)</td>
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<td></td>
<td></td>
<td></td>
<td>2. Placebo</td>
<td>2. 220</td>
<td></td>
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<tr>
<td>Stecksén-Blicks et al., 2009&lt;sup&gt;121&lt;/sup&gt;</td>
<td>RCT</td>
<td>1-5</td>
<td>1. Probiotic + fluoride</td>
<td>1. 110</td>
<td>L. rhamnosus LB21 + Fluoride (2.5 mg/L)</td>
<td>Milk, 21 months</td>
<td>↓ caries experience (DMFS)</td>
<td>← MS + ↑ LB (saliva + plaque)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2. Placebo</td>
<td>2. 76</td>
<td></td>
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<tr>
<td>Petersson et al., 2011&lt;sup&gt;127&lt;/sup&gt;</td>
<td>RCT</td>
<td>58-84</td>
<td>1. Probiotic</td>
<td>1. 27</td>
<td>L. rhamnosus LB21</td>
<td>Fluoride (5 ppm)</td>
<td>↑ root caries reversals</td>
<td>↓ MS + ↑ LB (saliva + plaque)</td>
</tr>
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<td></td>
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<td>2. Fluoride</td>
<td>2. 22</td>
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<td></td>
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<td></td>
<td>3. Probiotic + fluoride</td>
<td>3. 26</td>
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<td>4. Placebo</td>
<td>4. 25</td>
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<tr>
<td>Burton et al., 2013&lt;sup&gt;136&lt;/sup&gt;</td>
<td>RCT</td>
<td>5-10</td>
<td>1. Probiotic</td>
<td>1. 40</td>
<td>S. salivarius M18</td>
<td>Lozenge, 3 months</td>
<td>↓ plaque scores</td>
<td>Overall: ← MS (saliva) Where M18 colonized: ↓ MS</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2. Placebo</td>
<td>2. 43</td>
<td></td>
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<tr>
<td>Hasslöf et al, 2013&lt;sup&gt;137&lt;/sup&gt;</td>
<td>RCT</td>
<td>4 months</td>
<td>1. Probiotic</td>
<td>1. 56</td>
<td>L. paracasei F19</td>
<td>Cereal, 9 months</td>
<td>No change in DMFS</td>
<td>← MS + ↑ LB (saliva) No evidence of oral colonization of F19 ← MS</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2. Placebo</td>
<td>2. 62</td>
<td></td>
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</tr>
<tr>
<td>Taipale et al., 2013&lt;sup&gt;138&lt;/sup&gt;</td>
<td>RCT</td>
<td>1 month</td>
<td>1. Probiotic</td>
<td>1. 32</td>
<td>Bifidobacterium lactis subsp. BB-12</td>
<td>Tablet in pacifier or by spoon, 22-23 months</td>
<td>No ↑ or ↓ in caries rate at 4 year follow up</td>
<td>← MS</td>
</tr>
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<td></td>
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<td>2. Xylitol</td>
<td>2. 33</td>
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<td></td>
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<td>3. Sorbitol</td>
<td>3. 29</td>
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<tr>
<td>Stensson et al., 2014&lt;sup&gt;140&lt;/sup&gt;</td>
<td>RCT</td>
<td>From birth</td>
<td>1. Probiotic</td>
<td>1. 60</td>
<td>L. reuteri ATC 55730</td>
<td>Oral drops, 1 year</td>
<td>↑ in # of caries free subjects</td>
<td>← MS, ← LB, ← S IgA (saliva)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2. Placebo</td>
<td>2. 53</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gizani et al., 2015&lt;sup&gt;144&lt;/sup&gt;</td>
<td>RCT</td>
<td>16 (mean)</td>
<td>1. Probiotic</td>
<td>1. 42</td>
<td>L. reuteri DSM 17938 + L. reuteri ATCC PTA 5289</td>
<td>Lozenge, 17 months</td>
<td>No difference in WSL incidence</td>
<td>← MS + ↑ LB (saliva)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2. Placebo</td>
<td>2. 43</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Toiviainen et al., 2015&lt;sup&gt;146&lt;/sup&gt;</td>
<td>RCT</td>
<td>24 (mean)</td>
<td>1. Probiotic</td>
<td>1. 29</td>
<td>L. rhamnosus GG + Bifidobacterium animalis subsp. lactis BB-12</td>
<td>Lozenge, 4 weeks</td>
<td>↑ plaque scores</td>
<td>← MS, ← LB (saliva)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>2. Placebo</td>
<td>2. 31</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*↑↓ = Decrease, ← = No change, ↑ = Increase
† MS = S. mutans, LB = lactobacilli
‡ Green cells indicate a beneficial effect from probiotic therapy while red cells indicate no effect.
‡‡ Orange cells indicate studies in orthodontic populations.
Table 3. Studies assessing oral probiotic effectiveness in only orthodontic populations.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Design</th>
<th>Age (years)</th>
<th>Groups</th>
<th>Sample Size</th>
<th>Probiotic Strain</th>
<th>Probiotic Vehicle, Duration</th>
<th>Probiotic Effect (†, ‡, ‡‡)*</th>
<th>Clinical Outcome</th>
<th>Microbial Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cildir et al., 2009*</td>
<td>Crossover 12-16</td>
<td>1. Probiotic 2. Placebo</td>
<td>1. 12 2. 12</td>
<td>Bifidobacterium animalis subsp. lactis DN-173010 Yogurt, 2 weeks per treatment</td>
<td>↓ MS + ↑ LB (saliva)</td>
<td>↓ MS (plaque)</td>
<td>No difference between either probiotic vehicle</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Jose et al., 2013‡</td>
<td>RCT</td>
<td>14-19</td>
<td>1. Probiotic curd 2. Probiotic toothpaste 3. Placebo</td>
<td>1. 20 2. 20 3. 20</td>
<td>Strains not specified Curd and toothpaste, 30 days</td>
<td>↑ MS (saliva)</td>
<td>No difference</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pinto et al., 2014‡‡</td>
<td>Crossover 10-30</td>
<td>1. Probiotic 2. Placebo</td>
<td>26</td>
<td>Bifidobacterium animalis subsp. lactis DN-173010 Yogurt, 2 weeks per treatment</td>
<td>↑ MS + ↑ LB (saliva + plaque)</td>
<td>↓ MS + ↑ LB (saliva + plaque)</td>
<td>SD1 successfully colonized at 4 weeks</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ritthagol et al., 2014†‡</td>
<td>RCT 19 (mean)</td>
<td>1. Probiotic 2. Placebo</td>
<td>1. 15 2. 15</td>
<td>L. paracasei SD1 Milk, 4 weeks</td>
<td>↓ MS + ↑ LB (saliva)</td>
<td>↓ MS + ↑ LB (saliva)</td>
<td>SD1 successfully colonized at 4 weeks</td>
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<td></td>
</tr>
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<td>Gizani et al., 2015‡‡</td>
<td>RCT 16 (mean)</td>
<td>1. Probiotic 2. Placebo</td>
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<td>L. reuteri DSM 17938 + L. reuteri ATCC PTA 5289 Lozenge, 17 months</td>
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<td></td>
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</tbody>
</table>

*↓↓ = Decrease, ↑ = No change, † = Increase
† MS = S. mutans, LB = lactobacilli
‡ Green cells indicate a beneficial effect from probiotic therapy while red cells indicate no effect.
‡‡ Blue cells indicate studies that used caries experience, WSLs, or plaque levels as the primary outcome.
1.6 STUDY CONSIDERATIONS

1.6.1 LORODENT PROBIOTIC COMPLEX

The Lorodent probiotic complex, developed by Integra Medical Inc., is a blend of six probiotic bacteria including *S. salivarius* K12 and five lactobacilli strains *L. paracasei, L. plantarum, L. acidophilus, L. salivarius* and *L. reuteri*. The Lorodent probiotic complex has never been investigated previously within the dental context, nor has any other multistrain probiotic with a similar combination of strains. In fact, studies on multistrain probiotic therapy are lacking in general. Only 5 of the 38 probiotic studies from Table 1 used multistrain probiotics, whereas the other 33 studies used monostrain probiotics. Of these, 4 of the 5 studies reported decreases in either plaque scores or *S. mutans* levels following multistrain probiotic therapy, suggesting that multistrain probiotic preparations such as the Lorodent probiotic complex may be effective in the oral cavity. As well, none of the studies from Table 3 have investigated multistrain probiotics within the orthodontic context. While monostrain probiotic studies allow investigation into the effectiveness of specific strains, gastrointestinal research has suggested that monostrain probiotic preparations may have a lower chance of successful colonization whereas multiple strains with divergent properties may increase the odds of having at least one strain survive. Further, successfully combatting multifactorial diseases may also require a combination of probiotic properties, such properties may be strain-specific, and there may be synergistic effects by combining different strains together.

1.6.2 DURATION OF PROBIOTIC THERAPY

The current dental and orthodontic literature has indicated that short-term probiotic therapy can be effective at decreasing surrogate endpoints for caries, such as plaque levels or *S. mutans* levels. From the 38 studies outlined in Table 1, 25 studies used probiotic therapy durations of 4 weeks or less, and within this group of investigations decreases in either plaque or *S. mutans* levels were shown in 16 whereas no changes in these parameters were demonstrated in the other 9. Specifically within the orthodontic context, 4 of the 5 studies (Table 3) also used durations for probiotic therapy of 4 weeks or less, and of these studies, 3 reported significant
decreases in S. mutans levels whereas 2 did not. Since there is really no clear consensus as to what is the optimal duration of therapy, it was decided to carry out a study that employed a shorter duration of probiotic therapy in order to increase the feasibility of completing the study with adequate power and sample size.

1.6.3 SURROGATE VS. CLINICAL ENDPOINTS

Ideally, for this type of study the true clinical endpoint in an orthodontic population should be the incidence of white spot lesion formation in association with orthodontic therapy. It is difficult to assess white spot lesions when the orthodontic brackets are bonded to the buccal surfaces of the tooth, and thus white spot lesions are best assessed before the brackets are placed to establish the baseline, pre-treatment levels and after the brackets are removed for the post-treatment levels. Unfortunately, this would require monitoring participants over the entire length of their orthodontic treatment – on average around two to three years, and would make conducting such a clinical trial and achieving adequate sample size and power unfeasible within the Graduate Orthodontic setting. Thus, short duration studies require surrogate endpoints to be considered as being truly indicative of the disease of import (i.e., S. mutans levels as will be done in this study) when the ‘true’ outcomes, in this case white spot lesions/caries, take so long to develop making the study impracticable within a 3-year graduate clinical specialty training program, particularly with regard to collecting enough data to make a reliable series of conclusions. Hence, for the purposes of this investigation and as outlined below in more detail, it was decided to assess the impact of probiotic therapy on the levels of S. mutans. Indeed, as already discussed, 29 of the 38 dental studies on probiotic therapy and 4 of the 5 actual orthodontic studies have used surrogate endpoints such as plaque levels or S. mutans levels as their primary outcomes, and so it seems justifiable to use this microbe as the outcome parameter in this investigation, which will also improve the ability to compare the outcomes obtained in this investigations with other in a more reliable fashion (i.e., the same outcomes will be used).
1.6.4 VALIDITY OF ASSESSING PLAQUE & S. MUTANS LEVELS AS SURROGATE ENDPOINTS FOR CARIES & WHITE SPOT LESIONS

Numerous studies have attempted to develop predictive models for caries activity. Many risk factors for caries have been identified including past and current caries experience, plaque levels, oral hygiene habits, diet, microbiological factors (i.e., S. mutans counts), salivary buffering capacity, general medical history, socioeconomic factors (i.e., income and education), and psychosocial factors (i.e., health attitudes). However, the contribution of each of these risk factors towards being reliable indicators of disease, in this case white spot lesions or caries, has been debated heavily. Thus, caries risk assessment is a complex subject where no one model has proven to be applicable to all dental populations. Instead, the importance of risk factors varies based on the age group at which they are targeted. Past caries experience has been cited numerous as the most accurate single predictor for future caries activity in adults. On the other hand, visible plaque was found to be the best indicator for caries risk in young children with over 90% of children correctly classified as being at risk for the development of caries by use of this risk factor. It was also found that children with high S. mutans levels were likely to have high caries activity in the future, but that this may be partly compensated for by other factors such as good/improved oral hygiene, low plaque levels and a non-cariogenic diet. Generally, the best predictors for future caries activity were found by combining factors that are important contributors to the pathogenesis of dental caries, and the most accurate prediction models were seen to use plaque levels as well as salivary S. mutans levels together.

Within the orthodontic context, patients with high past caries experience, high numbers of S. mutans, and high plaque scores (measured by the Plaque Index) were reported to have a significantly higher overall caries experience during orthodontic treatment. The incidence of smooth surface carious lesions in orthodontic patients was most strongly related to increases in Plaque Index scores, but initial caries experience was not found to be significantly correlated. Visible plaque and S. mutans levels were reported to be the best predictors for orthodontically related white spot lesions. Therefore, plaque and S. mutans levels may be the most valid surrogates for white spot lesions and smooth surface caries within this specific patient subset,
which is sensible given that they are the most significant factors to change when oral hygiene becomes impaired due to the placement of fixed orthodontic appliances.

1.6.5 PLAQUE ASSESSMENT

Plaque indices to grade plaque levels provide a straightforward, inexpensive, systematic technique that can be easily performed on a high number of patients. Several plaque indices have been used in dental research to quantify levels of plaque for research purposes including the Simplified Oral Hygiene Index by Greene and Vermillion,\textsuperscript{154} Plaque Index by Silness and Löe,\textsuperscript{153} Quigley-Hein Plaque Index by Quigley and Hein,\textsuperscript{155} and the Modified Navy Plaque Index by Rustogi et al.\textsuperscript{156} Of the 2 probiotic studies that reported significant decreases in plaque levels following probiotic therapy, one study used the Simplified Oral Hygiene Index whereas the other used the Plaque Index.\textsuperscript{136,145} As discussed previously, the Plaque Index has specifically been found to be highly related to smooth surface caries risk in orthodontic patients, and thus was chosen to be used in our study.\textsuperscript{56}

Furthermore, previous studies have demonstrated the epidemiologic validity of the Ramfjord teeth in representing whole mouth gingival inflammation and plaque levels, and this was the basis for the selection of teeth that were to be assessed using the Plaque Index.\textsuperscript{157-159} However, a modification was made to the Ramfjord teeth to replace the first premolars with the canines, since the canines have one of the highest incidences of white spot lesion formation during orthodontic treatment.\textsuperscript{8,160} Also, by excluding first premolars from the teeth to be examined, this allowed orthodontic patients undergoing premolar extraction as part of their orthodontic treatment to be included in the study. Premolar extraction patients represent a significant subset of the orthodontic population – over a third of the patients at the Department of Orthodontics at the Faculty of Dentistry, University of Toronto. If they were excluded, this would have severely limited the patient population from which participants could have been recruited and would have tremendously increased the difficulty in achieving the desired sample size.
1.6.6 MICROBIAL EVALUATION

Several studies demonstrate a strong correlation between the proportion of *S. mutans* in plaque and saliva and current or future caries experience. Therefore, the cariogenic bacterial load, measured as the proportion of *S. mutans* present in plaque or saliva, may serve as a surrogate endpoint for caries susceptibility or, more specifically in this context, susceptibility to demineralization and the formation of white spot lesions.

There are a variety of methods available to accurately detect and quantify *S. mutans* in dental plaque and saliva samples. Classic microbiological methods include cultivation on selective media and rely on cell multiplication under *in vitro* conditions. Detection of a species is dependent upon selectively culturing the species, accuracy depends on the culture medium used, quantification is done manually through plating and counting, and culture-dependent methods offer limited information on the total diversity present in the plaque.

Contemporary techniques using culture-independent methods, such as polymerase chain reaction (PCR) or DNA hybridization techniques, have been developed that offer excellent opportunities for detection of bacteria by amplification of defined DNA sequences. Several authors have described the successful application of quantitative real-time PCR (qPCR) to detect and quantify both absolute and relative amounts of bacteria such as *S. mutans*, lactobacilli, *S. salivarius*, *F. nucleatum*, *A. actinomycetemcomitans* and total bacterial load using genus specific oligonucleotide primers. This can be advantageous as, for instance, *F. nucleatum*, and *A. actinomycetemcomitans* cannot be cultured on selective media due to their strict anaerobic requirements; that is, these bacteria do not survive upon oxygen exposure, often making them unculturable. The use of qPCR to detect the levels of bacteria has a high degree of specificity as the sequences of 16S rRNA genes are known for numerous bacterial species. As well, because there are several gene copies of 16S rRNA per bacterial cell, the technique also has a high degree of sensitivity. Additionally, qPCR allows for simultaneous analysis of numerous bacterial species with the addition of additional genus specific primers, and quantification is automated and much less laborious than conventional techniques.
1.7 OBJECTIVES

The aim of the study is to investigate the efficacy of the Lorodent probiotic lozenge in reducing the accumulation of plaque and cariogenic bacterial load in adolescent patients with fixed orthodontic appliances.

RESEARCH QUESTIONS

Does the probiotic significantly reduce plaque accumulation compared to the placebo in adolescents with fixed orthodontic appliances?

Does the probiotic significantly reduce the amount of *S. mutans* in supragingival plaque in adolescents with fixed orthodontic appliances?

Do any potential effects of the probiotic persist after one month of discontinued use?

Are there any serious adverse effects from oral probiotic use?

Can this intervention be practically implemented in a clinical orthodontic setting?

1.8 HYPOTHESIS

Our hypothesis was that oral exposure of the Lorodent probiotic lozenge for 28 days in an at-risk adolescent orthodontic population will lead to significant decreases in plaque and *S. mutans* levels in both supragingival plaque and saliva in the short-term compared to the placebo group.
2. METHODS & MATERIALS

2.1 OVERVIEW

This study was a randomized, double-blind, parallel-group, placebo-controlled trial which evaluated the effectiveness of the Lorodent probiotic complex in reducing clinical plaque scores and S. mutans bacterial levels in patients undergoing full fixed appliance orthodontic treatment over an 8-week time period.

2.2 FACILITIES

This clinical trial was conducted in the Graduate Orthodontic Clinic at the Faculty of Dentistry at the University of Toronto. Microbiological analysis was conducted at the Department of Microbiology and Immunology at the University of Western Ontario (by Kyle MacDonald, Dr. Burton, and Dr. Cadieux).

2.3 ETHICAL APPROVAL, TRIAL REGISTRATION & FUNDING

Ethical approval was obtained from the University of Toronto Health Sciences Research Ethics Board (#30148) (Appendix 1) and the University of Western Ontario (#101955) (Appendix 2). The clinical trial was registered and conducted in compliance with Health Canada (#185428) (Appendix 3) and funded by the Ontario Centres for Excellence (#20964) (Appendix 4). Probiotic and placebo lozenges were provided at no cost by Integra Medical Inc. as part of the collaboration with the Ontario Centres for Excellence.

2.4 STUDY DESIGN

Eligible participants were assigned randomly into 2 parallel groups using a 1:1 allocation ratio to receive either the Lorodent probiotic lozenge or the placebo lozenge for a 28-day period. Participants were monitored throughout and then recalled for a 28-day follow-up, for a total clinical trial length of 56 days. Once the trial commenced, there were no changes to the methodology and design of the clinical trial (Figure 3).
2.4.1 DATA COLLECTION

Participants were examined at 4 time points: 0 days (T1, baseline), 14 days (T2, mid-intervention), 28 days (T3, lozenges stopped), and at 56 days (T4, follow up). Monthly sampling intervals were chosen for practicality as patients are usually seen on a monthly basis during regular orthodontic care.

At each appointment, the following clinical and microbiological data was collected:

1. Plaque Index (PI) scores
2. Supragingival plaque samples
3. Saliva samples

Once a participant was enrolled in the study, the same examiner completed all data collection for that particular subject in order to maintain consistency between assessments. As described in Section 2.9.2, the examiners were blinded as to which treatment group the participant was receiving.

Figure 3. Study design.
2.4.2  ORAL HYGIENE INSTRUCTIONS

Participants were instructed to maintain the current standard of care regarding oral hygiene; that is, to brush two times per day and floss once per day at a minimum. As an exception, participants were instructed not to brush their teeth before the appointments or upon arrival to the clinic so that adequate plaque samples could be taken.

As mouth rinse is not considered the standard of care for oral hygiene and is optional, participants enrolled in this study were instructed to abstain from using mouth rinses as part of their oral health regimen or they would be excluded from the study.

2.4.3  COMPLIANCE MONITORING

In order to both assist and monitor compliance, a compliance calendar was provided to each participant to indicate the days and amounts when lozenges were to be taken (Figure 4). Participants were instructed to mark one box for every one lozenge taken but if a lozenge was not taken, to leave the corresponding box empty.

Overall compliance of each participant was assessed as a percentage of the number of lozenges actually taken out of the total number of lozenges that were supposed to be taken (70 total). ‘Compliant’ was defined as a percentage of 70% or higher, and those with a percentage lower than 70% were identified as ‘non-compliant’.

Figure 4. Compliance calendar.
2.4.4 SAFETY MONITORING

A thorough medical history was taken on each participant prior to enrollment. Participants were then monitored throughout the 56 days and asked at each appointment about changes in their medical health or adverse events that could be related to probiotic therapy such as nausea, fever, vomiting, bloody diarrhea, and severe abdominal pain.

2.5 PARTICIPANTS

2.5.1 ELIGIBILITY

Subjects who were receiving orthodontic treatment as patients of the Graduate Orthodontic Clinic at the Faculty of Dentistry, University of Toronto were selected to participate in the clinical trial based on the following criteria.

The inclusion criteria included individuals who:

- Were male or female between the ages of 11 to 18 years
- Were undergoing fixed orthodontic therapy on both arches with attachments on at least 20 teeth including bonded 1st molars for a minimum of 6 months
- Had fully erupted teeth #16, #21, #23, #36, #41, and #43
- Had mild to moderate plaque accumulation (PI score of at least 1) (Table 4)
- Were caries inactive prior to study initiation
- Were in a healthy systemic condition
- Were non-smokers and do not consume alcohol
- Who were able or whose legal guardian had given informed consent to participate in the study and were able to communicate in English

The exclusion criteria excluded individuals who:

- Had participated in a clinical trial within 30 days prior to randomization
• Had major dental conditions such as periodontal disease, dental caries and/or xerostomia or systemic diseases which could directly or indirectly affect plaque formation

• Were using any antimicrobial mouth rinses, probiotics (unrelated to the study), antibiotics or anti-inflammatories medications within one month prior to and during the study that could have influenced the outcome

• Had allergies to milk or milk products, gluten or soy or any other ingredients present in the Lorodent probiotic complex

• Had experienced any nausea, fever, vomiting, bloody diarrhea or severe abdominal pain within the 30 days prior to commencing the study

• Were immunocompromised and/or had a major underlying medical condition or ENT problem

• Had recent (within the past 45 days) or planned (within the past 90 days) surgery of any kind

• Had a history of smoking or alcohol consumption

• Were pregnant females

• Were unable to make informed consent

Participants enrolled in the study had to have undergone the following standard orthodontic bonding procedure:

• Maxillary and mandibular fixed orthodontic treatment using standard edgewise brackets with attachments on at least 20 teeth including bonded 1st molars

• Standard bonding procedure undertaken using 37% phosphoric acid, Transbond™ Plus Self Etching primer (3M Unitek), and Transbond™ light cure adhesive (3M Unitek) (light cured Bis-GMA composite resin)
2.5.2 SCREENING & RECRUITMENT

The principal investigators requested their co-residents in the Graduate Orthodontic Clinic to recommend compliant orthodontic patients that were 18 years or younger. One of the two principal investigators then screened potential subjects at the subject’s next routine orthodontic appointment for eligibility for the clinical trial. Screening involved a review of their medical and clinical histories and performance of a dental examination to ensure that potential subjects met the inclusion criteria or contrarily to disqualify potential subjects that met any of the criteria for exclusion.

Once a subject was deemed eligible, recruitment was made by verbal request. One of the two principal investigators verbally informed potential participants and their parents and/or guardians of the study rationale, design and potential benefits and risks. Additionally, they were provided with written documentation in the form of a “Patient Information & Consent Form” (Appendix 5) which further detailed the purpose of the study, study procedures, participant eligibility, time requirement, potential benefits and risks including any potential adverse events associated with probiotic therapy, right to refuse, right to withdraw, compensation, how the data collected was to be used, and the privacy and confidentiality policies for use of their personal information. Potential participants, parents and guardians were then given the opportunity to review the documents at their leisure and had all questions answered in order to make a voluntary decision regarding study participation.

2.5.3 CONSENT PROCESS

Informed consent to participate in the clinical trial was obtained verbally and in writing from all included participants or, if the participant was below the age of 18, from their parents and/or guardians (Appendix 5). Further, since compliance was an important factor in this study, it was in the study’s best interest that the participants themselves – and not only their parents/guardians – understood the study and were motivated to comply with the study procedures. Therefore if the participant was under the age of 18, informed consent was obtained from their parent and/or guardian and verbal assent was obtained from the participant in addition.
Each participant had also previously provided consent to be treated as patients of the Graduate Orthodontic Clinic at the Faculty of Dentistry, University of Toronto. This consent included both consent for care and consent for collection of personal information to be used in their health management and research purposes (Appendix 6).

2.5.4 BURDEN OF PARTICIPATION

In order to minimize participant burden, the study was designed such that subjects were to participate in the clinical trial within the context of their routine orthodontic care at the Graduate Orthodontic Clinic at the Faculty of Dentistry, University of Toronto. That is, clinical data was collected from the participants during their regularly scheduled orthodontic appointments which were typically at 4-week intervals; however, one additional appointment was required at 2 weeks. The participants were not inconvenienced in any other way, and there was no disruption or modification to their orthodontic treatment during the clinical trial.

2.5.5 COMPENSATION

Participants received a total compensation of $75 for their completion of the clinical trial, which required them to take the study lozenges daily for 28 days as an adjunct to their orthodontic treatment and attend 4 appointments in total including 1 extra appointment at 2 weeks. Compensation was given in installments as participants proceeded through each phase of the study. Specifically, a $25 gift card to a shopping mall was given at the additional appointment at 2 weeks (visit 2), and a $50 gift card to the mall was given upon study completion at the routine orthodontic appointment at 8 weeks (visit 4).

2.5.6 WITHDRAWAL & EXCLUSION

Participants were removed from the study based on the following criteria:

- Personal reasons: Participants could withdraw from the study for any reason at any time without any repercussion. Any partial data was used up to the last follow up point. For example, if a patient decided to withdraw from the study halfway through, the data from the first half of the study was still included in data analysis.
• Exclusion criteria including adverse events: A participant was withdrawn from the study if they satisfied any of the exclusion criteria, including but not limited to use of antimicrobial mouth rinses or experiencing adverse events related to probiotic therapy such as nausea, fever, vomiting, bloody diarrhea or severe abdominal pain. Participants that were removed from the study were informed at the time of exclusion, and data from participants who met the exclusion criteria were excluded from data analysis.

Participants had the right to refuse any data that had been collected to be kept up to the time of data analysis, after which point it was unrealistic to omit data. A 10% drop out rate was incorporated into the study sample size in order account for dropouts or non-compliance.

2.5.7 CONFIDENTIALITY
Each participant’s identity was kept confidential at all times. After issuing informed consent, each participant was given a unique numerical identifier, ‘Subject ID’, in order to maintain patient confidentiality. All data and samples collected were labeled using the participant’s Subject ID and no identifiable data was used throughout the course of the study (Figure 5E). All information collected from the participant’s electronic chart and any data collected during the clinical trial was done in accordance with the University of Toronto, Faculty of Dentistry Consent for Care and Privacy Policies (Appendix 6).

2.6 INTERVENTION
Two blueberry-flavored lozenges provided by Integra Medical Inc. were used in this study:

1. Lorodent probiotic lozenge – contained 1.6 billion CFUs of active probiotics

2. Placebo lozenge – did not contain any live microorganisms

The lozenges were manufactured in August 2011 by Nutraceutix Inc. (Redmond, Washington, USA). Product information about both lozenges can be found in Appendix 7.
Once distributed to the investigators, all lozenges were stored in a -80°C freezer in secured laboratory until they were given to participants at the first appointment (T1). Participants were asked to store the lozenges in their fridge during the trial.

2.6.1 LORODENT PROBIOTIC LOZENGES
The probiotic lozenges contained the active Lorodent probiotic complex (S. salivarius K12, L. paracasei, L. plantarum, L. acidophilus, L. salivarius and L. reuteri), lactitol, inulin, dicalcium phosphate, blueberry flavor (natural), dextrose, fructose, stearic acid, citric acid, vanilla flavor (natural), and stevia rebaudioside A (97%) as excipients. As per manufacturer specifications, the Lorodent lozenges could be stored at room temperature for 18 months.

The probiotic strain stability of the Lorodent probiotic lozenge was confirmed throughout the lozenge’s shelf life by Integra Medical Inc. using their Lorodent Enumeration Protocol (Appendix 7). In July 2014, Integra Medical Inc. confirmed there were a total of 1.6 x 10⁹ active CFUs per probiotic lozenge.

2.6.2 PLACEBO LOZENGES
The placebo lozenges contained lactitol, inulin, dicalcium phosphate, blueberry flavor (natural), dextrose, fructose, stearic acid, citric acid, vanilla flavor (natural), and stevia rebaudioside A (97%).

2.6.3 DOSAGE & ADMINISTRATION
As per recommendation from Integra Medical Inc., participants were instructed to take two lozenges two times a day (after breakfast and dinner) for the first 7 days (loading dose) and then two lozenges once a day (after breakfast) for the next 21 days (maintenance dose). Therefore, participants were instructed to take a sum of 70 lozenges over a total administration period of 28 days. Participants were instructed to slowly dissolve the lozenges on their tongue for five minutes; that is, not to swallow or chew the lozenges.
2.6.4 RISKS OF THE LORODENT PROBIOTIC COMPLEX

No significant side effects were expected from oral probiotic therapy using the Lorodent probiotic complex based on preclinical studies of Lorodent and previous studies using similar bacterial formulation. Theoretically, while the administration of live bacterial strains could result in a bacterial infection, this risk was minimal because the Lorodent bacterial strains were endogenous to the human oral cavity and gastrointestinal tract and were highly susceptible to the commonly used antibiotics amoxicillin and erythromycin. However, participants were cautioned that nausea, fever, vomiting, bloody diarrhea and severe abdominal pain might occur when taking live microorganisms, but that the incidence of these side effects was rare. Specifically, if digestive upset or diarrhea were to occur and persist for 3 or more days, the use of probiotics was to be immediately discontinued. If pregnancy or severe medical conditions were to arise during probiotic therapy, the participant was to be withdrawn and the probiotic was to be immediately discontinued.

Although the Lorodent lozenges contained a small amount of sugar, this sugar was not expected to affect the outcomes of this study significantly – namely plaque accumulation and *S. mutans* levels – as the amount of sugar in each lozenge was miniscule compared to the amount of sugar in the average diet. Each Lorodent lozenge (500 mg) was approximately 6% fermentable sugar by weight and contained 15 mg of dextrose and 15 mg of fructose. Given a dosage regimen of 2 to 4 lozenges a day, the overall increase in fermentable sugar in the participants diet was 60-120 mg/day. In comparison, there is approximately 80-160 fold more sugar in an average sized apple. Furthermore, in comparison to the average sugar intake by Canadian children aged 9-18 years of 145 g, consumption of the Lorodent lozenges would increase the overall sugar consumption of children by 0.01% per lozenge up to a maximum of 0.04%. Therefore, despite the fact that the lozenge was designed to be slow releasing, the effects of the fermentable sugars were deemed to be negligible.
2.7 EXAMINERS

Data was collected by the two principal investigators – Dr. Fatima Ebrahim & Dr. Sarah Habib – who are licensed dentists and current graduate students in the Department of Orthodontics at the Faculty of Dentistry, University of Toronto. Both examiners were providing ongoing orthodontic care to patients in the Graduate Orthodontic Clinic at the Faculty of Dentistry, University of Toronto.

2.7.1 ALIGNMENT & REPRODUCIBILITY

Prior to commencement of the clinical trial, the two examiners underwent alignment to establish good or higher inter-rater reliability for Plaque Index scores following a methodology similar to the one recommended by Hefti and Preshaw.171

First, the two examiners were aligned to develop mutual agreement between them. The purpose of alignment was not to necessarily produce perfect agreement with a theoretical standard, but rather to establish consistency in how they distinguished various scores from each other.171 Initially, the examiners had a meeting to critically discuss the Plaque Index scoring system (Table 4). Then the two examiners examined the intra-oral photos from a sample of 5 carefully selected subjects (Sample A) who represented the full range of the scoring system. Together the examiners took turns examining the buccal, mesial and distal surfaces of teeth #21, #23, #41 & #43. The lingual surfaces and teeth #16 and #36 were excluded as they are not easily visible in intra-oral photographs. Scores were called audibly, site after site, and any discrepancies between examiners were discussed in order to improve agreement. The scores were not recorded during this alignment period.

Next the reproducibility of the examiners’ scores was assessed. This time, examiners scored another sample of subjects independently, who were once more selected to represent the full range of the scoring system. Intra-oral photos of another 5 subjects (Sample B) were scored. Then, the examiners also scored 3 orthodontic patients (Sample C) using all 4 surfaces (buccal, mesial, distal and lingual) for all 6 teeth (#16, #21, #23, #36, #41 & #43) as described in the protocol of the study. This time, all scores from Samples B and C were recorded to measure
the examiners’ inter-rater reliability. Then after study completion, the examiners re-scored the intra-oral photos from Sample B to measure their intra-rater reliability.

Similarly, examiners also assimilated their techniques for plaque and saliva sample collection.

**Note:** The intraoral photos and orthodontic patients used for calibration and reproducibility were sampled from the Graduate Orthodontic Clinic at the Faculty of Dentistry, University of Toronto. None of the patients used during this process were participants in the study.

## 2.8 OUTCOMES OF INTEREST

### 2.8.1 PLAQUE LEVELS – PRIMARY OUTCOME (CLINICAL)

The Plaque Index (PI), as outlined by Silness and Löe in 1967, was used to clinically grade the extent and severity of plaque accumulation (Table 4). Using this system, PI scores from 0 to 3 were assigned to each of the 4 surfaces (buccal, lingual, mesial and distal) of each of the 6 selected teeth (#16, #21, #23, #36, #41 and #43). Taking the mean of ordinal, non-parametric data may confound clinical outcomes so individual scores were recorded for each tooth at each surface and were not averaged. Therefore, a total of 24 PI scores were recorded per participant at any given time point.

A composite PI (cPI) score, comprised of the sum of all 24 PI scores, was also calculated for each participant to represent the participant’s overall plaque status at a particular time point (maximum score of 72). Further, cPI subscores were generated for each participant at each time point by summing 8 PI scores for tooth type (4 surfaces per tooth x 2 teeth per tooth type, maximum score of 24) and 6 PI scores for surface (6 teeth x 1 surface per tooth, maximum score of 18).
Both plaque and salivary samples were collected at each time point for microbial analyses. Supragingival plaque was collected with a sterile stainless steel periodontal Gracey curette (Figure 5A) by taking two strokes from the facial surface around the orthodontic brackets of each of #23 and #43 (Figure 5B). The supragingival plaque from #23 and #43 was then combined and placed in 1.5mL Eppendorf tubes (Figure 5C). Approximately 2 mL of unstimulated saliva was collected by having the participants expectorate in an upright position into a 15 mL Falcon tube (Figure 5D).

Each Eppendorf and Falcon tube was alphanumerically coded with the patient’s Subject ID and lozenge group A or B so that the microbiologists performing the laboratory analysis were unaware of the identity of the subject or the type of lozenge administered (Figure 5E). The samples were immediately stored in a -20°C freezer in secured laboratory and shipped on dry ice to the University of Western Ontario for analysis.

Quantitative real-time PCR was used to determine the levels of *S. mutans*, total lactobacilli and *S. salivarius* K12 from both the supragingival plaque and saliva samples collected at baseline (T1) and 28 days (T3) for each participant.
**DNA EXTRACTION**

DNA was extracted from the plaque and saliva samples using the PowerSoil®-htp 96 Well Soil DNA Isolation Kit (MoBio). Saliva samples were thawed prior to extraction, while the plaque samples were suspended and mixed thoroughly in 400 µL of 1x TE buffer (10 mM Tris [pH 8.0], 1 mM EDTA). The extraction was carried out according to the manufacturer’s protocol (*Appendix 8*) with two changes: the addition of a 10-minute incubation step at 65°C in a bead bath before the bead-beating step, and a doubling of all centrifugation times. 200 µL of saliva and suspended plaque were used for the extractions. Extracted DNA was stored at -20°C until used for qPCR.

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**Figure 5.** Stainless steel Gracey curette (A). Supragingival plaque collection around the orthodontic bracket on #23 (B). Placement of supragingival plaque into a 1.5 mL Eppendorf tube (C). Participant expectorating unstimulated saliva into a 15 mL Falcon tube (D). Labeling of Eppendorf and Falcon tubes with participant’s subject ID, lozenge group, appointment number, and sample type (E).
DNA QUANTIFICATION

DNA extracted from plaque and saliva samples was then quantified using a NanoDrop 1000 Spectrophotometer (Thermo Scientific) with NanoDrop v3.8.1 Measurement Software. Elution buffer from the DNA extraction kit was used as a blank, with 1 µL of each sample placed on the spectrophotometer to determine the DNA concentration of each sample.

QUANTITATIVE REAL-TIME PCR (qPCR)

Validated primers specific for *S. mutans*, *S. salivarius* K12, total lactobacilli, and total bacteria were identified from previous studies (Table 5). Selected primers were tested against isolated DNA from pure cultures of *S. salivarius* K12, *S. mutans*, and *L. reuteri* as a positive control to confirm their efficiency and specificity.

<table>
<thead>
<tr>
<th>Bacterial Species</th>
<th>Primer Sequence (5'-3')</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>S. mutans</em></td>
<td>F: GCCTACAGCTCAGAGATGCTATTCT</td>
</tr>
<tr>
<td></td>
<td>R: GCCATAACCCACTCATGAATTGA</td>
</tr>
<tr>
<td><em>S. salivarius</em> K12</td>
<td>F: CGGCAAAAACCAAAGCTAGAG</td>
</tr>
<tr>
<td></td>
<td>R: ACGTGGTTTTTGGGGTTAG</td>
</tr>
<tr>
<td>Total lactobacilli</td>
<td>F: TGGAAACAGRTGCTAATACCG</td>
</tr>
<tr>
<td></td>
<td>R: GTCCATTGTGGAAGATTCCC</td>
</tr>
<tr>
<td>Total bacteria</td>
<td>F: TCCTACGGGAGGCAGCAGT</td>
</tr>
<tr>
<td></td>
<td>R: GGACTACCAGGGTGATCTAATGCTGTT</td>
</tr>
</tbody>
</table>

Table 5. Forward (F) and reverse (R) qPCR primer sequences for various bacterial species.

All qPCR reactions were carried out in 384-well reaction plates using the 7900 HT Sequence Detection System with SDS 2.3 Sequencing Software (Applied Biosystems, Foster City, California, USA) under the following program: Stage 1, 50°C for 2 minutes; Stage 2, 95°C for 10 minutes; then 95°C for 15 sec, and 60°C for 1 min, with the program run for 40 cycles. Reactions were carried out in 20 µL volumes with 5 µL extracted template DNA, 10 uL 1× Power SYBR Green PCR Master Mix (Applied Biosystems), 4.5 uL PCR-grade water, and 0.25 µL of each forward and reverse primer (100 µM stock).
Standard curves were generated for each primer set using serial 10-fold dilutions of known concentrations of isolated DNA from pure cultures of their respective species. Bacterial species were quantified in triplicate (technical replicates with the exact same reaction mixture) for each biological sample to control for errors from inaccurate pipetting. The mean cycle threshold (Ct) values of the triplicates were used to determine raw DNA concentrations based on the standard curves. However, since the amount of plaque or saliva per sample was not standardized, the amount of extracted DNA loaded in each qPCR reaction was different for each sample. To control for this and to allow for comparison across samples, the total amount of bacterial DNA was set as an internal reference for each sample, and relative proportions of *S. mutans* DNA, total lactobacilli DNA, and *S. salivarius* K12 DNA were calculated as a percentage of that.

### 2.8.3 LOZENGE SATISFACTION

At the conclusion of the study, each participant was asked to complete a 10-item “End of Study Questionnaire” that evaluated his or her satisfaction with the lozenge intervention and the clinical trial (*Appendix 9*). The questionnaire has been adapted from a similar questionnaire used by the Xylitol for Adult Caries Trial.175

### 2.9 RANDOMIZATION, BLINDING & DATA SECURITY

#### 2.9.1 RANDOMIZATION

Once informed consent was obtained, eligible participants were randomized into 2 parallel groups to receive either the intervention (Lorodent probiotic) or control (placebo).

Blocked randomization was carried out to ensure that the comparison groups were generated according to a predetermined 1:1 allocation ratio with each group being of the same size. The principal investigators generated a random allocation sequence using an online randomization service offered by random.org. Based on the sequence of enrollment, participants were given a unique Subject ID, which was used to identify them throughout the course of the study. The random allocation sequence from random.org was then sequentially matched to the Subject
IDs. Random.org was then used to assign the first block of integers (1-30) from the random allocation sequence to one lozenge group while the second block of integers (31-60) was assigned to the other. The ultimate randomization schedule can be seen in Appendix 10.

2.9.2 BLINDING

Integra Medical Inc. concealed the identities of the probiotic and placebo lozenges and provided the principal investigators with bottles marked either Lozenge A or Lozenge B. Thus, the participants, principal investigators, microbiologists, orthodontic residents providing routine treatment, and the statistician were all blinded from the true identity of the lozenges, which was only known by Integra Medical Inc. Lozenges A and Lozenges B looked identical in color, shape and size (Figure 6A), and packaging for each bottle of lozenges was identical except for a marking on each bottle of either “A” or “B” to indicate the lozenge group (Figure 6B). As per the Ontario Centres of Excellence guidelines, Integra Medical Inc. provided the blinded, pre-coded lozenges directly to the primary investigators but was not involved in any collection or analysis of data. The principal investigators did, however, maintain a sealed master list from Integra Medical Inc. of the lozenges’ identities in case of emergency. This sealed master list was never opened.

![Figure 6](image)

**Figure 6.** Probiotic and placebo lozenges were identical in color, shape and size (A). The packaging for each bottle of lozenges was identical except for a marking on each bottle of “A” or “B to indicate the lozenge group (B).

After data analysis was completed, Integra Medical Inc. informed the investigators that Lozenge A was the placebo and Lozenge B was the Lorodent probiotic. These identities were
then confirmed by Integra Medical Inc. using bacterial enumeration following their Lorodent Enumeration Protocol (Appendix 7).

2.9.3 DATA SECURITY & RETENTION
Any data collected during the clinical trial including any personally identifiable information was stored in a locked filing cabinet in a secured office at the Faculty of Dentistry, University of Toronto. Any samples collected were stored in Dr. Siew-Ging Gong’s secured laboratory at the Faculty of Dentistry, University of Toronto and then subsequently at Dr. Burton’s secured laboratory at the Department of Microbiology and Immunology, University of Western Ontario. Access to the data collected was limited to the investigators, microbiologists, primary supervisor, and department head. After dissemination of the results, the data will be retained in the Department of Orthodontics, Faculty of Dentistry, University of Toronto for 7 years as per Health Canada’s guidelines for clinical trials.

2.10 STATISTICAL METHODS
All data analyses was completed per-protocol including only those participants who completed the treatment they were originally allocated to and excluding any participants that were non-compliant, met the exclusion criteria and/or were lost to follow up. Since no participants had less than 70% compliance and only 2 participants in total were lost to follow-up (1 from the probiotic group, 1 from the placebo group), the per-protocol approach was deemed sufficient and no additional intent to treat analyses was carried out.

Unless otherwise stated, all statistical analysis was performed using IBM SPSS Statistics 23.0 with a 5% significance level (p = 0.05).

2.10.1 SAMPLE SIZE DETERMINATION
Sample size was determined to provide sufficient power to test the hypothesis that an oral probiotic lozenge would significantly reduce clinical plaque levels compared to the placebo group. Sample size determination was conducted a priori to ensure the study had sufficient
statistical power to detect a difference between treatments if one truly existed and to reduce the risk of an underpowered (false negative) result.\textsuperscript{176}

In most cases, an \textit{a priori} power analysis often involves simplifying a number of assumptions in order to make the problem solvable; determining an appropriate sample size before the study commenced would otherwise have been too complex. Therefore, a power analysis was conducted using a test of two independent proportions, which had the benefit of testing relative differences between groups rather than absolute changes and thus did not require the standard deviation of the groups to be known (i.e., from a pilot study).

Sample size calculations were based on:

- One tailed – this study was directional testing the hypothesis that the probiotic reduces clinical plaque levels, not that it increases it
- Type I error: $\alpha = 0.05$ (standard)
- Type II error: $\beta = 0.20$ (standard)
- Statistical power: $1 - \beta = 0.80$ (standard)
- Minimum detectable difference between groups (i.e., difference in proportions): 25%
- Primary outcome measure: Reduction in PI score (clinical)

It was decided that for the probiotic to be a worthwhile adjunct to orthodontic treatment, the probiotic should reduce clinical plaque scores by 25% compared to the placebo group. This was broken down by systematically calculating that if there were 4 surfaces per tooth (buccal, mesial, distal, and lingual) and 6 teeth examined per patient, then there were 24 total sites assessed and it seemed reasonable that if the probiotic was effective, a minimum of 6 out of 24 total sites (25%) would show a reduction in clinical plaque levels. Reduction of clinical plaque was defined as a lowering in the Plaque Index score by at least 1 score out of 4. Thus, simply stated, patients would experience a reduction of plaque in 25% of sites if the probiotic was effective.
To account for a possible placebo effect, it was estimated that the placebo group would see plaque scores reduce by 5%.

The statistical program G*Power 3.1 was used to determine that 28 subjects per group would be needed to test a difference in proportion of 0.25 with 80% power (1-sided, $\alpha = 0.05$) (Figure 7). It was then assumed that a potential loss to follow-up of 10% could occur due to either dropout or non-compliance, thus requiring a total recruitment of 30-31 subjects per group (total n = 60-62) to ensure adequate sample size.

**Figure 7.** A priori sample size determination with G*Power 3.1 for a difference between proportions of 0.25, a power of 0.8 and an alpha of 0.05.
2.10.2 BASELINE COMPARISONS

Demographic, clinical and microbial baseline data was tested for balance across the probiotic and placebo treatment groups. This included baseline comparisons for sex, age at enrollment, PI and cPI scores at T1, and microbial proportions of S. mutans and total lactobacilli at T1.

A chi-square test was used to compare the proportions of each sex between the probiotic and placebo groups. The age at enrollment between the lozenge groups was compared using an independent samples t-test (parametric) and a Mann-Whitney U-test (non-parametric). Baseline plaque and microbial comparisons are described in further detail in Sections 2.10.3 and 2.10.4 respectively.

2.10.3 PLAQUE ANALYSIS

Plaque analyses were carried out using the PI scores (24 per participant per time point) or the cPI scores (sum of 24 PI scores per participant per time point). Datasets for the mesial and distal surfaces were combined (not averaged) for all PI analyses.

Two approaches were used to compare the baseline PI scores recorded at T1 between the probiotic and placebo groups. First, frequency distributions of the PI scores at baseline were generated for both the probiotic and placebo groups, and the proportions in each group were compared using chi-square tests. Second, the baseline cPI scores for the placebo and probiotic groups were compared using a Mann-Whitney U-test. This test was also repeated to compare baseline cPI scores between male and female participants. Exploratory analyses were then done for both approaches by subgrouping the baseline PI and cPI data by tooth type (incisor, canine, molar) and by surface (buccal, lingual, combined mesio-distal) and comparing them using chi-square or Mann-Whitney U-tests, respectively.

The differences in the PI and cPI scores between T1 and T2, T1 and T3, and T1 and T4 for each participant were calculated to represent the improvement in PI and cPI scores from baseline to each time point. In this context, a difference yielding a positive number represented an improvement in the PI score while a negative number represented a worsening of the PI score. Wilcoxon Signed Rank tests were carried out to assess if the improvements within each
lozenge group were significant. Then, Mann-Whitney U-tests were used to compare if the improvement in one lozenge group was more significant than the improvement in the other. PI and cPI scores were then subgrouped by tooth type and surface, and were again compared between groups using Mann-Whitney U-tests. Frequency distributions, negative ranks, positive ranks, and mean ranks were used to evaluate what direction any potential changes were occurring.

2.10.4 MICROBIAL ANALYSIS

The mathematical derivations of the relative proportions of *S. mutans* DNA, total lactobacilli DNA, and *S. salivarius* K12 DNA were described in detail in the methods for qPCR (Section 2.8.2). The samples where DNA did not enumerate were eliminated from the analysis. Thus, 8 qPCR datasets were created for the proportions of *S. mutans* DNA and total lactobacilli DNA in both supragingival plaque and saliva at 0 days (T1, baseline) and 28 days (T3, lozenges stopped). From this data, the differences between T3 and T1 for each sample were calculated resulting in another 4 data sets that evaluated the within group changes in the proportions of DNA for *S. mutans* and total lactobacilli in supragingival plaque and saliva. In this context, a difference yielding a positive number represented an increase in microbial counts whereas a negative number represented a decrease in microbial counts.

All 12 qPCR datasets failed both the Kolmogorov-Smirnov (with Lilliefors Significance Correction) and the Shapiro-Wilk tests of normality (p < 0.05) (Table 6). Thus, the data was treated non-parametrically and the microbial DNA proportions of the probiotic and placebo lozenge groups were compared. Mann-Whitney U-tests were used to compare the microbial DNA proportions between the lozenge groups at baseline. Wilcoxon Signed Rank tests were carried out to assess if the changes from T1 to T3 in each lozenge group were significant. Then, Mann-Whitney U-tests were used to compare if the change in one lozenge group was more significant than the change in the other. Medians of the various datasets were assessed to evaluate what direction any potential changes were occurring.
2.10.5  INTER-RATER & INTRA-RATER RELIABILITY

Inter-rater and intra-rater reliability was assessed using weighted kappa statistics performed by the statistical program STATA 13. Weights were assigned such that an exact match was given a full match rating (1.0), a difference in the PI score of one unit was given a half-match rating (0.5), and a difference bigger than 1 unit was given a zero rating (0). Interpretation of the kappa statistics to determine the level of agreement for categorical data between examiners and between themselves was based off of Landis and Koch’s recommendations (Table 7).\textsuperscript{178}

Table 6. Tests of normality for various qPCR datasets at T1 and T3.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Kolmogorov-Smirnov\textsuperscript{†} (p)</th>
<th>Shapiro-Wilk (p)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plaque</td>
<td>% of S. mutans DNA at T1</td>
<td>0.000*</td>
</tr>
<tr>
<td></td>
<td>% of S. mutans DNA at T3</td>
<td>0.000*</td>
</tr>
<tr>
<td></td>
<td>Change in % of S. mutans DNA (T3-T1)</td>
<td>0.000*</td>
</tr>
<tr>
<td></td>
<td>% of lactobacilli DNA at T1</td>
<td>0.000*</td>
</tr>
<tr>
<td></td>
<td>% of lactobacilli DNA at T1</td>
<td>0.002*</td>
</tr>
<tr>
<td></td>
<td>Change in % of lactobacillus DNA (T3-T1)</td>
<td>0.000*</td>
</tr>
<tr>
<td>Saliva</td>
<td>% of S. mutans DNA at T1</td>
<td>0.000*</td>
</tr>
<tr>
<td></td>
<td>% of S. mutans DNA at T3</td>
<td>0.000*</td>
</tr>
<tr>
<td></td>
<td>Change in % of S. mutans DNA (T3-T1)</td>
<td>0.000*</td>
</tr>
<tr>
<td></td>
<td>% of lactobacilli DNA at T1</td>
<td>0.000*</td>
</tr>
<tr>
<td></td>
<td>% of lactobacilli DNA at T1</td>
<td>0.000*</td>
</tr>
<tr>
<td></td>
<td>Change in % of lactobacillus DNA (T3-T1)</td>
<td>0.000*</td>
</tr>
</tbody>
</table>

\textsuperscript{†} Lilliefors Significance Correction.

*p < 0.05.
Compliance was assessed at two time points: 14 days (T2) and 28 days (T3, lozenges stopped).

Mann-Whitney U-tests were used to compare the difference in the frequencies of lozenges consumed between the probiotic and placebo groups and between men and women at both time points T2 and T3.

Originally, the proportion of ‘compliant’ (≥70% lozenge consumption) and ‘non-compliant’ (<70% lozenge consumption) participants between probiotic and placebo groups was to be compared. However, since no patients reported a compliance of less than 70% at either time points, it was decided to instead compare the proportion of participants with ‘perfect compliance’ (100% lozenge consumption) to those with ‘less than perfect compliance’ (<100% lozenge consumption) between the two groups. This comparison was carried out between the probiotic and placebo groups at T2 and T3, between males and females at T2 and T3, and between participants examined by examiner 1 and 2 at T2 and T3. If there was a count of 5 or more in each subgroup, a chi-square test (2-sided) was carried out for the comparison. If there were less than 5 in each subgroup (violation of an assumption of the chi-square test), Fisher’s Exact test (2-sided) was used instead.

<table>
<thead>
<tr>
<th>Table 7. Landis and Koch interpretation of the kappa statistic for rater reliability.178</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.00</td>
</tr>
<tr>
<td>0.00-0.20</td>
</tr>
<tr>
<td>0.21-0.40</td>
</tr>
<tr>
<td>0.41-0.60</td>
</tr>
<tr>
<td>0.61-0.80</td>
</tr>
<tr>
<td>0.81-1.00</td>
</tr>
<tr>
<td>1.00</td>
</tr>
</tbody>
</table>

---

2.10.6 **COMPLIANCE ASSESSMENT**

Compliance was assessed at two time points: 14 days (T2) and 28 days (T3, lozenges stopped).

Mann-Whitney U-tests were used to compare the difference in the frequencies of lozenges consumed between the probiotic and placebo groups and between men and women at both time points T2 and T3.

Originally, the proportion of ‘compliant’ (≥70% lozenge consumption) and ‘non-compliant’ (<70% lozenge consumption) participants between probiotic and placebo groups was to be compared. However, since no patients reported a compliance of less than 70% at either time points, it was decided to instead compare the proportion of participants with ‘perfect compliance’ (100% lozenge consumption) to those with ‘less than perfect compliance’ (<100% lozenge consumption) between the two groups. This comparison was carried out between the probiotic and placebo groups at T2 and T3, between males and females at T2 and T3, and between participants examined by examiner 1 and 2 at T2 and T3. If there was a count of 5 or more in each subgroup, a chi-square test (2-sided) was carried out for the comparison. If there were less than 5 in each subgroup (violation of an assumption of the chi-square test), Fisher’s Exact test (2-sided) was used instead.

---
Between August to October 2014, 87 patients from the Graduate Orthodontic Clinic at the Faculty of Dentistry, University of Toronto were screened for eligibility for the clinical trial. Sixty patients met the eligibility criteria and provided informed consent. The remainder of the subjects were excluded for various reasons: 19 subjects did not meet the inclusion criteria (i.e., participants had banded first molars, unerupted canines, multiple missing teeth, only one arch bonded, outside of the age range, caries, etc.) and 8 subjects declined to participate. Screening stopped when the desired sample size was achieved.

**Figure 8.** Participant flow.
Once enrolled, the 60 participants were then randomized into two equal groups of 30 that received either the probiotic or placebo lozenges. Of these, 2 participants (3.3%) did not complete the trial and were lost to follow up. Specifically, 1 participant from the probiotic group was withdrawn from the study because the subject’s elbow had been broken requiring surgery and the administration of systemic and oral antibiotics; the latter possibly affecting the levels of *S. mutans*. As well, 1 participant from the placebo group reported adverse events and withdrew from the study.

Thus, of the 60 enrolled participants, 58 completed the clinical trial and were available for the analyses (probiotic group, n = 29; placebo group n = 29). Data was collected from August until December 2014 at which point the clinical trial was considered to be complete. Figure 8 depicts the participants’ progression through the study.

### 3.1 BASELINE CHARACTERISTICS

Baseline demographic and clinical characteristics did not significantly differ between the two groups. There were no statistically significant differences for both sex and age of enrollment (Table 8).

Overall, more females were enrolled in the study compared to men (56.9% female, 43.1% male). In the probiotic group, 16 of the 29 participants were male (55.2%) and 13 were female (44.8%). In the placebo group, 9 of the 29 participants were male (31.0%) and 20 were female (69.0%). Although there were no significant differences in the ratio of males to females between the two groups (p = 0.063), there was a trend for more males (16 out of 25) in the probiotic group and more females (20 out of 33) in the placebo group (Figure 9).

Participants averaged 15.69 ± 1.70 years in age and the mean age in the probiotic group was 15.75 ± 1.67 years compared to 15.64 ± 1.75. Differences between ages in both groups were not significant whether the group was compared parametrically (p = 0.812) or non-parametrically (p = 0.828).
Table 8. Demographic data.

<table>
<thead>
<tr>
<th></th>
<th>Probiotic group n = 29 (50%)</th>
<th>Placebo group n = 29 (50%)</th>
<th>Total cohort n = 58 (100%)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sex</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>16 (55.2%)</td>
<td>9 (31.0%)</td>
<td>25 (43.1%)</td>
<td>0.063†</td>
</tr>
<tr>
<td>Female</td>
<td>13 (44.8%)</td>
<td>20 (69.0%)</td>
<td>33 (56.9%)</td>
<td></td>
</tr>
<tr>
<td><strong>Age (years)</strong></td>
<td>15.75 ± 1.67*</td>
<td>15.64 ± 1.75*</td>
<td>15.69 ± 1.70*</td>
<td>0.812‡</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.828§</td>
</tr>
</tbody>
</table>

*Mean ± s.d.
† Chi-square test.
‡ Independent samples t-test.
§ Mann-Whitney U-test.

Figure 9. Male and female distributions in the probiotic and placebo groups.
There were no significant differences in the frequency distributions of the baseline PI scores recorded at T1 between the probiotic and placebo groups (p = 0.249) (Table 9 and Figure 10). Similarly, there were no significant differences when baseline cPI scores were compared between the lozenge groups (p = 0.882) (Table 12 and Figure 13).

However, there were significant sex differences in the frequency distributions of the baseline PI scores, with males having a greater proportion of higher plaque scores compared to females (p = 0.05) (Table 9 and Figure 10). On the contrary, there were no significant sex differences when baseline cPI scores were compared (p = 0.271) (Table 12 and Figure 13), but cPI scores have a much lower sample size than PI scores and may not have been powerful enough to detect the differences.

When baseline PI scores were subdivided based on tooth type, there were no significant differences between the frequency distributions of the probiotic and placebo groups for the incisors (p = 0.108), canines (p = 0.622), or molars (p = 0.576) (Table 10 and Figure 11). When subdivided by surface, there were no significant differences between the frequency distributions of the probiotic and placebo groups for the buccal (p = 0.186), lingual (p = 0.271) and combined mesio-distal surfaces (p = 0.484) (Table 11 and Figure 12).

Similarly, when the two groups were compared using the baseline cPI subscores, there were no significant differences when subdivided by tooth type for the incisors (p = 0.778), canines (p = 0.863), or molars (p = 0.478) (Table 13 and Figure 14) and no significant differences when subdivided by surface type for the buccal (p = 0.736), lingual (p = 0.833), or combined mesio-distal surfaces (p = 0.731) (Table 14 and Figure 15).

There were no significant differences in the baseline proportions of \textit{S. mutans} DNA or total lactobacilli DNA between the probiotic and placebo groups in either the plaque or saliva samples (p > 0.05) (Table 15).
Table 9. Comparison of the frequency distributions of the baseline PI scores in each lozenge group.

<table>
<thead>
<tr>
<th>Group</th>
<th>PI Scores</th>
<th></th>
<th>Total</th>
<th>P-value†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>Overall</td>
<td>646 (46.4%)</td>
<td>653 (46.9%)</td>
<td>93 (6.7%)</td>
<td>1392 (100%)</td>
</tr>
<tr>
<td>Lozenge Group</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Probiotic</td>
<td>328 (47.1%)</td>
<td>315 (45.3%)</td>
<td>53 (7.6%)</td>
<td>696 (100%)</td>
</tr>
<tr>
<td>Placebo</td>
<td>318 (45.7%)</td>
<td>338 (48.6%)</td>
<td>40 (5.7%)</td>
<td>696 (100%)</td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>266 (44.3%)</td>
<td>279 (46.5%)</td>
<td>55 (9.2%)</td>
<td>600 (100%)</td>
</tr>
<tr>
<td>Female</td>
<td>380 (48.0%)</td>
<td>374 (47.2%)</td>
<td>38 (4.8%)</td>
<td>792 (100%)</td>
</tr>
</tbody>
</table>

† Chi-square test.

Figure 10. Comparison of the frequency distributions of the baseline PI scores between lozenge groups and between sexes (*p < 0.05).
Table 10. Comparison of the frequency distributions of the baseline PI scores in each lozenge group subgrouped by tooth type.

<table>
<thead>
<tr>
<th>Group</th>
<th>PI Scores</th>
<th>Total</th>
<th>P-value†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Incisor</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Probiotic</td>
<td>127 (54.7%)</td>
<td>87 (37.5%)</td>
<td>18 (7.8%)</td>
</tr>
<tr>
<td>Placebo</td>
<td>114 (49.1%)</td>
<td>107 (46.1%)</td>
<td>11 (4.7%)</td>
</tr>
<tr>
<td>Canine</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Probiotic</td>
<td>116 (50.0%)</td>
<td>95 (40.9%)</td>
<td>21 (9.1%)</td>
</tr>
<tr>
<td>Placebo</td>
<td>109 (49.0%)</td>
<td>105 (45.3%)</td>
<td>18 (7.8%)</td>
</tr>
<tr>
<td>Molar</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Probiotic</td>
<td>85 (36.6%)</td>
<td>133 (57.3%)</td>
<td>14 (6.0%)</td>
</tr>
<tr>
<td>Placebo</td>
<td>95 (40.9%)</td>
<td>126 (54.3%)</td>
<td>11 (4.7%)</td>
</tr>
</tbody>
</table>

† Chi-square test.

Table 11. Comparison of the frequency distributions of the baseline PI scores in each lozenge group subgrouped by surface.

<table>
<thead>
<tr>
<th>Group</th>
<th>PI Scores</th>
<th>Total</th>
<th>P-value†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Buccal</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Probiotic</td>
<td>69 (39.7%)</td>
<td>83 (47.7%)</td>
<td>22 (12.6%)</td>
</tr>
<tr>
<td>Placebo</td>
<td>57 (32.8%)</td>
<td>100 (57.5%)</td>
<td>17 (9.8%)</td>
</tr>
<tr>
<td>Lingual</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Probiotic</td>
<td>140 (80.5%)</td>
<td>33 (19.0%)</td>
<td>1 (0.6%)</td>
</tr>
<tr>
<td>Placebo</td>
<td>131 (75.3%)</td>
<td>43 (24.7%)</td>
<td>0 (0.0%)</td>
</tr>
<tr>
<td>Mesio-Distal**</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Probiotic</td>
<td>119 (34.2%)</td>
<td>199 (57.2%)</td>
<td>30 (8.6%)</td>
</tr>
<tr>
<td>Placebo</td>
<td>130 (37.4%)</td>
<td>195 (56.0%)</td>
<td>23 (6.6%)</td>
</tr>
</tbody>
</table>

† Chi-square test.
Figure 11. Comparison of the frequency distributions of the baseline PI scores in each lozenge group subgrouped by tooth type.

Figure 12. Comparison of the frequency distributions of the baseline PI scores in each lozenge group subgrouped by surface.
Table 12. Comparison of the baseline cPI scores between lozenge groups and between sexes.

<table>
<thead>
<tr>
<th>Group</th>
<th>Median</th>
<th>Range</th>
<th>IQR</th>
<th>Mean Rank</th>
<th>P-value†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Overall (n = 58)</td>
<td>39</td>
<td>25-54</td>
<td>11</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Lozenge Group</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Probiotic (n = 29)</td>
<td>39</td>
<td>26-54</td>
<td>11.5</td>
<td>29.83</td>
<td>0.882</td>
</tr>
<tr>
<td>Placebo (n = 29)</td>
<td>39</td>
<td>25-51</td>
<td>12</td>
<td>29.17</td>
<td></td>
</tr>
<tr>
<td><strong>Sex</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male (n = 25)</td>
<td>40</td>
<td>25-54</td>
<td>12.5</td>
<td>32.30</td>
<td>0.271</td>
</tr>
<tr>
<td>Female (n = 33)</td>
<td>38</td>
<td>29-51</td>
<td>10</td>
<td>27.38</td>
<td></td>
</tr>
</tbody>
</table>

* cPI score = sum of 24 sites (4 surfaces x 6 teeth)
† Mann-Whitney U-test.

**Figure 13.** Comparison of the baseline cPI scores between lozenge groups and between sexes. Box plots show median values (solid vertical line), interquartile range (box outline), highest and lowest values within the upper and lower limits (1.5 IQR) (whiskers), and any outliers (circles).
Table 13. Comparison of the baseline cPI subscores between lozenge groups subgrouped by tooth type.

<table>
<thead>
<tr>
<th>Group</th>
<th>Median</th>
<th>Range</th>
<th>IQR</th>
<th>Mean Rank</th>
<th>P-value†</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Incisor</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Probiotic</td>
<td>12</td>
<td>8-19</td>
<td>2.5</td>
<td>28.88</td>
<td>0.778</td>
</tr>
<tr>
<td>Placebo</td>
<td>12</td>
<td>8-17</td>
<td>5</td>
<td>30.12</td>
<td></td>
</tr>
<tr>
<td><strong>Canine</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Probiotic</td>
<td>12</td>
<td>8-19</td>
<td>3</td>
<td>29.12</td>
<td>0.863</td>
</tr>
<tr>
<td>Placebo</td>
<td>13</td>
<td>9-18</td>
<td>4.5</td>
<td>29.88</td>
<td></td>
</tr>
<tr>
<td><strong>Molar</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Probiotic</td>
<td>14</td>
<td>8-17</td>
<td>4</td>
<td>31.05</td>
<td>0.478</td>
</tr>
<tr>
<td>Placebo</td>
<td>13</td>
<td>8-16</td>
<td>5</td>
<td>27.95</td>
<td></td>
</tr>
</tbody>
</table>

* cPI subscore by tooth type = sum of 8 sites (4 surfaces per tooth x 2 teeth per tooth type)
† Mann-Whitney U-test.

Figure 14. Comparison of the baseline cPI subscores between lozenge groups subgrouped by tooth type. Box plots show median values (solid vertical line), interquartile range (box outline), highest and lowest values within the upper and lower limits (1.5 IQR) (whiskers), and any outliers (circles).
Table 14. Comparison of the baseline cPI subscores between lozenge groups subgrouped by surface.

<table>
<thead>
<tr>
<th>Group</th>
<th>Median</th>
<th>Range</th>
<th>IQR</th>
<th>Mean Rank</th>
<th>P-value†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buccal</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Probiotic (n = 29)</td>
<td>10</td>
<td>6-17</td>
<td>3</td>
<td>28.76</td>
<td>0.736</td>
</tr>
<tr>
<td>Placebo (n = 29)</td>
<td>11</td>
<td>6-17</td>
<td>3</td>
<td>30.24</td>
<td></td>
</tr>
<tr>
<td>Lingual</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Probiotic (n = 29)</td>
<td>7</td>
<td>6-10</td>
<td>2</td>
<td>29.97</td>
<td>0.833</td>
</tr>
<tr>
<td>Placebo (n = 29)</td>
<td>6</td>
<td>6-11</td>
<td>3</td>
<td>29.03</td>
<td></td>
</tr>
<tr>
<td>Mesio-Distal**</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Probiotic (n = 29)</td>
<td>21</td>
<td>14-31</td>
<td>5.5</td>
<td>28.79</td>
<td>0.731</td>
</tr>
<tr>
<td>Placebo (n = 29)</td>
<td>21</td>
<td>13-27</td>
<td>7</td>
<td>30.21</td>
<td></td>
</tr>
</tbody>
</table>

*cPI subscore by surface = sum of 6 sites (6 teeth x 1 surface per tooth)
**Mesial and distal cPI subscores were combined (sum of 12 sites)
† Mann-Whitney U-test.

Figure 15. Box-and-whisker plots comparing the baseline cPI subscores between lozenge groups subgrouped by surface. Box plots show median values (solid vertical line), interquartile range (box outline), highest and lowest values within the upper and lower limits (1.5 IQR) (whiskers), and any outliers (circles).
Table 15. Comparison of the proportions of *S. mutans* DNA and lactobacilli DNA between lozenge groups at baseline.

<table>
<thead>
<tr>
<th>Sample</th>
<th>% of <em>S. mutans</em> DNA at T1</th>
<th>% of lactobacilli DNA at T1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Median</td>
<td>Mean Rank</td>
</tr>
<tr>
<td>Plaque</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Probiotic</td>
<td>0.050123</td>
<td>16.67</td>
</tr>
<tr>
<td>Placebo</td>
<td>0.207975</td>
<td>22.05</td>
</tr>
<tr>
<td>Saliva</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Probiotic</td>
<td>0.116199</td>
<td>12.57</td>
</tr>
<tr>
<td>Placebo</td>
<td>0.773649</td>
<td>14.58</td>
</tr>
</tbody>
</table>

† Mann-Whitney *U*-test.
3.2 PLAQUE LEVELS (PI & cPI SCORES)

Plaque accumulation was assessed clinically by recording 24 PI scores at each time point from each of the 58 participants. In total, there were 1392 PI scores recorded per time point, or specifically 696 PI scores per lozenge group per time point. The frequency distributions of the PI scores at each time point for the probiotic and placebo groups are shown in Table 16 and Figure 16. For both lozenge groups, over 90% of the PI scores were assigned to PI scores of 1 or 2. There were almost no PI scores of 0 and very few PI scores of 3 (<10%).

Within each group, improvements from baseline to each time point were assessed using both PI and cPI scores (Table 17 and Figure 17). For PI scores, both lozenge groups experienced more sites of improvement than worsening, although the bulk of the sites (>70%) did not change in score. Using cPI scores, most participants either improved or worsened, whereas very few participants experienced no overall change in their cumulative plaque scores. No significant improvements in both PI and cPI scores from baseline were seen throughout the intervention period for the probiotic group at any time frame (p > 0.05), whereas significant improvements were seen in the PI and cPI scores of the placebo group at each time frame (p < 0.01). When the improvements observed in the probiotic and placebo groups were compared between groups, the degree of improvement in the placebo group was found to be significantly more than that observed in the probiotic group at each time frame for both PI (p < 0.01) and cPI scores (p < 0.05) (Table 18 and Figure 18).

However, the overall frequency and magnitude of the improvements observed in each group were very small, as is indicated by the median amount of improvement in PI and cPI scores in Table 17. For instance, over 70% of the sites showed no change in PI scores; thus, the improvements in each group were only observed in a very small subset of the sites assessed. When the magnitude of the improvement was considered, the median improvement over the 28-day administration period was 1 cPI unit in the probiotic group and 3 cPI units in the placebo group. Considering that cPI is scored out of 72, 1 and 3 units of improvement out of 72 is marginal and clinically insignificant.
When the improvements in PI and cPI scores from each time frame were subdivided based on tooth type, there was significantly more of an improvement in the PI and cPI scores of the placebo group for the canines at each time frame (p < 0.05) and for the molars from T1 to T2 and T1 to T3 (p < 0.05) (Table 19 and Figure 19). There were no significant differences between the improvements of the two lozenge groups for the incisors at any time frame (p > 0.05).

When subdivided by surface, there was significantly more of an improvement in the PI and cPI scores in the placebo group at the buccal surface at each time frame (p < 0.05) and at the lingual surface from T1 to T3 and T1 to T4 (p < 0.05) (Table 20 and Figure 20). There were no significant differences between the improvements of the two lozenge groups for the mesio-distal surfaces at any time frame (p > 0.05).

When subdivided by both tooth type and surface, there were significantly more improvements in the PI scores of the placebo group for the buccal and lingual surfaces of the incisors from T1 to T3 and T1 to T4 (p < 0.05), at the buccal surfaces of the canine from T1 to T2 and T1 to T3 (p < 0.05), and at the mesio-distal surfaces of the molars at each time frame (p < 0.05) (Table 21).

When the improvements in PI and cPI scores from each time frame were compared between sexes, there were no significant differences in the amount of improvement experienced by either sex (p > 0.05) (Table 22).
**Table 16. Frequency distribution of the PI scores from T1-T4 for the probiotic and placebo groups.**

<table>
<thead>
<tr>
<th>Group</th>
<th>PI Scores</th>
<th>Median</th>
<th>Pi</th>
<th>cPi</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>Probiotic</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1 (n = 696)</td>
<td>0 (0.0%)</td>
<td>328 (47.1%)</td>
<td>315 (45.3%)</td>
<td>53 (7.6%)</td>
</tr>
<tr>
<td>T2 (n = 696)</td>
<td>1 (0.1%)</td>
<td>341 (49.0%)</td>
<td>298 (42.8%)</td>
<td>56 (8.0%)</td>
</tr>
<tr>
<td>T3 (n = 696)</td>
<td>3 (0.4%)</td>
<td>336 (48.3%)</td>
<td>315 (45.3%)</td>
<td>42 (6.0%)</td>
</tr>
<tr>
<td>T4 (n = 696)</td>
<td>5 (0.7%)</td>
<td>334 (48.0%)</td>
<td>307 (44.1%)</td>
<td>50 (7.2%)</td>
</tr>
<tr>
<td>Placebo</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1 (n = 696)</td>
<td>0 (0.0%)</td>
<td>318 (45.7%)</td>
<td>338 (48.6%)</td>
<td>40 (5.7%)</td>
</tr>
<tr>
<td>T2 (n = 696)</td>
<td>0 (0.0%)</td>
<td>368 (52.9%)</td>
<td>304 (43.7%)</td>
<td>24 (3.4%)</td>
</tr>
<tr>
<td>T3 (n = 696)</td>
<td>0 (0.0%)</td>
<td>400 (57.5%)</td>
<td>275 (39.5%)</td>
<td>21 (3.0%)</td>
</tr>
<tr>
<td>T4 (n = 696)</td>
<td>1 (0.1%)</td>
<td>389 (55.9%)</td>
<td>286 (41.1%)</td>
<td>20 (2.9%)</td>
</tr>
</tbody>
</table>

**Figure 16.** Frequency distributions of the PI scores at each time point for the probiotic and placebo groups.
**Table 17.** Comparison of the improvement, worsening or ties in PI scores and cPI scores from baseline to each time point within each lozenge group.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Negative Ranks</th>
<th>Positive Ranks</th>
<th>Ties</th>
<th>P-value†</th>
<th>Median Improvement</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PI Scores</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Probiotic from T1 to T2 (n = 696)</td>
<td>n = 76</td>
<td>n = 63</td>
<td>n = 557</td>
<td>0.325</td>
<td>0</td>
</tr>
<tr>
<td>Probiotic from T1 to T3 (n = 696)</td>
<td>n = 97</td>
<td>n = 73</td>
<td>n = 526</td>
<td>0.070</td>
<td>0</td>
</tr>
<tr>
<td>Probiotic from T1 to T4 (n = 696)</td>
<td>n = 101</td>
<td>n = 84</td>
<td>n = 511</td>
<td>0.188</td>
<td>0</td>
</tr>
<tr>
<td>Placebo from T1 to T2 (n = 696)</td>
<td>n = 103</td>
<td>n = 40</td>
<td>n = 553</td>
<td>0.000*</td>
<td>0</td>
</tr>
<tr>
<td>Placebo from T1 to T3 (n = 696)</td>
<td>n = 133</td>
<td>n = 39</td>
<td>n = 524</td>
<td>0.000*</td>
<td>0</td>
</tr>
<tr>
<td>Placebo from T1 to T4 (n = 696)</td>
<td>n = 126</td>
<td>n = 45</td>
<td>n = 525</td>
<td>0.000*</td>
<td>0</td>
</tr>
<tr>
<td><strong>cPI scores</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Probiotic from T1 to T2 (n = 29)</td>
<td>n = 12</td>
<td>n = 11</td>
<td>n = 6</td>
<td>0.462</td>
<td>0</td>
</tr>
<tr>
<td>Probiotic from T1 to T3 (n = 29)</td>
<td>n = 15</td>
<td>n = 9</td>
<td>n = 5</td>
<td>0.181</td>
<td>1</td>
</tr>
<tr>
<td>Probiotic from T1 to T4 (n = 29)</td>
<td>n = 16</td>
<td>n = 11</td>
<td>n = 2</td>
<td>0.399</td>
<td>1</td>
</tr>
<tr>
<td>Placebo from T1 to T2 (n = 29)</td>
<td>n = 21</td>
<td>n = 6</td>
<td>n = 2</td>
<td>0.003*</td>
<td>3</td>
</tr>
<tr>
<td>Placebo from T1 to T3 (n = 29)</td>
<td>n = 25</td>
<td>n = 2</td>
<td>n = 2</td>
<td>0.000*</td>
<td>3</td>
</tr>
<tr>
<td>Placebo from T1 to T4 (n = 29)</td>
<td>n = 20</td>
<td>n = 5</td>
<td>n = 4</td>
<td>0.000*</td>
<td>4</td>
</tr>
</tbody>
</table>

**Note:** Negative rank = the number of cases with a decrease (improvement) in PI or cPI scores from T1. Positive rank = the number of cases with an increase (worsening) in PI or cPI scores from T1.

† Wilcoxon Signed Rank test.
* p < 0.05.

**Table 18.** Comparison of the improvements in PI and cPI scores from baseline to each time point between the probiotic and placebo groups.

<table>
<thead>
<tr>
<th>Outcome</th>
<th>Probiotic Mean Rank</th>
<th>Placebo Mean Rank</th>
<th>P-value†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PI</td>
<td>cPI</td>
<td>PI</td>
</tr>
<tr>
<td>T1 to T2 Improvement</td>
<td>673.82</td>
<td>24.36</td>
<td>719.18</td>
</tr>
<tr>
<td>T1 to T3 Improvement</td>
<td>665.34</td>
<td>23.07</td>
<td>727.66</td>
</tr>
<tr>
<td>T1 to T4 Improvement</td>
<td>668.31</td>
<td>25.03</td>
<td>724.69</td>
</tr>
</tbody>
</table>

† Mann-Whitney U-test.
* p < 0.05.
**Figure 17.** Comparison of the improvement, worsening or ties in PI scores and cPI scores from baseline to each time point within each lozenge group (*p < 0.05).

**Figure 18.** Comparison of the PI scores from baseline to each time point between lozenge groups (*p < 0.05).
Table 19. Comparison of the improvements in PI and cPI scores subgrouped by tooth type from baseline to each time point between the lozenge groups.

<table>
<thead>
<tr>
<th>Outcome</th>
<th>Probiotic Mean Rank</th>
<th>Placebo Mean Rank</th>
<th>P-value†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PI</td>
<td>cPI</td>
<td>PI</td>
</tr>
<tr>
<td><strong>Incisor</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1 to T2 Improvement</td>
<td>229.07</td>
<td>28.09</td>
<td>235.93</td>
</tr>
<tr>
<td>T1 to T3 Improvement</td>
<td>225.35</td>
<td>26.72</td>
<td>239.65</td>
</tr>
<tr>
<td>T1 to T4 Improvement</td>
<td>223.56</td>
<td>26.43</td>
<td>241.44</td>
</tr>
<tr>
<td><strong>Canine</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1 to T2 Improvement</td>
<td>222.44</td>
<td>25.48</td>
<td>242.56</td>
</tr>
<tr>
<td>T1 to T3 Improvement</td>
<td>219.50</td>
<td>24.03</td>
<td>245.50</td>
</tr>
<tr>
<td>T1 to T4 Improvement</td>
<td>219.51</td>
<td>25.21</td>
<td>245.49</td>
</tr>
<tr>
<td><strong>Molar</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1 to T2 Improvement</td>
<td>223.23</td>
<td>26.09</td>
<td>241.77</td>
</tr>
<tr>
<td>T1 to T3 Improvement</td>
<td>221.55</td>
<td>24.69</td>
<td>243.45</td>
</tr>
<tr>
<td>T1 to T4 Improvement</td>
<td>226.48</td>
<td>27.38</td>
<td>238.52</td>
</tr>
</tbody>
</table>

† Mann-Whitney U-test.

*p < 0.05.
Table 20. Comparison of the improvements in PI and cPI scores subgroupped by surface at each time point to baseline between the lozenge groups.

<table>
<thead>
<tr>
<th>Outcome</th>
<th>Probiotic Mean Rank</th>
<th>Placebo Mean Rank</th>
<th>P-value†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PI</td>
<td>cPI</td>
<td>PI</td>
</tr>
<tr>
<td>Buccal</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1 to T2 Improvement</td>
<td>164.55</td>
<td>24.19</td>
<td>184.45</td>
</tr>
<tr>
<td>T1 to T3 Improvement</td>
<td>159.79</td>
<td>23.76</td>
<td>189.21</td>
</tr>
<tr>
<td>T1 to T4 Improvement</td>
<td>164.78</td>
<td>25.00</td>
<td>184.22</td>
</tr>
<tr>
<td>Lingual</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1 to T2 Improvement</td>
<td>172.11</td>
<td>27.52</td>
<td>176.89</td>
</tr>
<tr>
<td>T1 to T3 Improvement</td>
<td>167.57</td>
<td>24.91</td>
<td>181.43</td>
</tr>
<tr>
<td>T1 to T4 Improvement</td>
<td>166.25</td>
<td>25.71</td>
<td>182.75</td>
</tr>
<tr>
<td>Mesio-Distal</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1 to T2 Improvement</td>
<td>338.34</td>
<td>25.72</td>
<td>358.66</td>
</tr>
<tr>
<td>T1 to T3 Improvement</td>
<td>339.11</td>
<td>26.90</td>
<td>357.89</td>
</tr>
<tr>
<td>T1 to T4 Improvement</td>
<td>338.39</td>
<td>25.59</td>
<td>358.61</td>
</tr>
</tbody>
</table>

† Mann-Whitney U-test.

*p < 0.05.
Figure 19. Comparison of the PI scores from baseline to each time point between lozenge groups subdivided by tooth type (*p < 0.05).

Figure 20. Comparison of the PI scores from baseline to each time point between lozenge groups subdivided by surface (*p < 0.05).
Table 21. Comparison of the improvements in PI scores subgrouped by tooth type and by surface at each time point to baseline between the lozenge groups.

<table>
<thead>
<tr>
<th>Outcome</th>
<th>Buccal</th>
<th>Lingual</th>
<th>Mesio-Distal</th>
</tr>
</thead>
<tbody>
<tr>
<td>T1 to T2 Improvement</td>
<td>0.274</td>
<td>0.244</td>
<td>0.832</td>
</tr>
<tr>
<td>T1 to T3 Improvement</td>
<td>0.006*</td>
<td>0.017*</td>
<td>0.209</td>
</tr>
<tr>
<td>T1 to T4 Improvement</td>
<td>0.053*</td>
<td>0.017*</td>
<td>0.770</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Tooth Type</th>
<th>Buccal</th>
<th>Lingual</th>
<th>Mesio-Distal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Incisor</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1 to T2 Improvement</td>
<td>0.041*</td>
<td>0.996</td>
<td>0.163</td>
</tr>
<tr>
<td>T1 to T3 Improvement</td>
<td>0.048*</td>
<td>0.513</td>
<td>0.078</td>
</tr>
<tr>
<td>T1 to T4 Improvement</td>
<td>0.081</td>
<td>0.066</td>
<td>0.229</td>
</tr>
<tr>
<td>Canine</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>T1 to T2 Improvement</td>
<td>0.214</td>
<td>0.743</td>
<td>0.031*</td>
</tr>
<tr>
<td>T1 to T3 Improvement</td>
<td>0.215</td>
<td>0.769</td>
<td>0.019*</td>
</tr>
<tr>
<td>T1 to T4 Improvement</td>
<td>0.896</td>
<td>0.553</td>
<td>0.041*</td>
</tr>
<tr>
<td>Molar</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

† Mann-Whitney U-test.
*p < 0.05.

Table 22. Comparison of the improvements in PI and cPI scores from baseline to each time point between sexes.

<table>
<thead>
<tr>
<th>Outcome</th>
<th>Male Mean Rank</th>
<th>Female Mean Rank</th>
<th>P-value†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PI</td>
<td>cPI</td>
<td>PI</td>
</tr>
<tr>
<td>T1 to T2 Improvement</td>
<td>682.28</td>
<td>25.40</td>
<td>707.28</td>
</tr>
<tr>
<td>T1 to T3 Improvement</td>
<td>685.75</td>
<td>27.20</td>
<td>704.64</td>
</tr>
<tr>
<td>T1 to T4 Improvement</td>
<td>695.33</td>
<td>29.84</td>
<td>697.39</td>
</tr>
</tbody>
</table>

† Mann-Whitney U-test.
3.3 BACTERIAL LEVELS (qPCR)

Following DNA extraction, the average DNA concentrations from the supragingival plaque and saliva samples were 1.2 ng/µL and 5.5 ng/µL, respectively. Microbial evaluation using qPCR was then performed to quantify the relative proportions of *S. mutans* DNA and total lactobacilli DNA in the plaque samples from all 58 participants at baseline (T1) and 28 days (T3). The same microbial assays were undertaken on the first half of the salivary samples (n = 29). Based on the preliminary results and given the substantial financial expense of qPCR, it was not deemed meaningful to analyze the remaining 29 salivary samples.

The proportion of *S. mutans* DNA relative to the total bacterial DNA was extremely low in the supragingival plaque samples, and for 20 participants was completely undetectable at one of the two time points (Figure 21A). Similarly, the salivary proportions of *S. mutans* were detectable in only 26 of 29 samples, and the vast majority of these samples had very low expression values (Figure 21B). The expression of total lactobacilli was much better than *S. mutans* in both plaque and saliva with detectable levels in all 58 supragingival plaque samples (Figure 21C) and all 29 saliva samples (Figure 21D). *S. salivarius* K12 was assayed for in 29 plaque samples but none adequately expressed, so no further quantification of *S. salivarius* K12 was undertaken in the remaining plaque or salivary samples.

![Figure 21. Expression of supragingival S. mutans (A), salivary S. mutans (B), supragingival total lactobacilli (C), and salivary total lactobacilli (D) for each participant at T1 and T3. In the majority of samples, S. mutans DNA levels were not detectable, unlike lactobacilli.](image-url)
Figure 21, continued.
The frequency distributions for the proportions of *S. mutans* DNA and total lactobacilli DNA in both plaque and salivary samples at T1 and T3 exhibited marked non-normal distributions (p < 0.05) with a bulk of the frequencies in each histogram around 0% (Figure 22). This was especially true for the *S. mutans* populations.

Overall, the proportion of *S. mutans* DNA observed within the probiotic group as well as the placebo groups did not show any significant differences from T1 to T3 in both the plaque and saliva samples (p > 0.05) (Table 23). In the probiotic group, there was a trend for the proportion of *S. mutans* DNA in plaque to decrease and for the proportion of *S. mutans* DNA in saliva to increase from T1 to T3. The opposite trends were seen in the placebo group from T1 to T3 where the proportion of *S. mutans* DNA in plaque tended to increase while the proportion of *S. mutans* DNA in the saliva tended to decrease. Similarly, there were no significant differences in the proportions of total lactobacilli DNA from T1 to T3 in the plaque or saliva samples within the probiotic or placebo groups (p > 0.05) (Table 24). There was a general trend towards decreasing proportions of lactobacilli DNA as the study progressed, especially in the saliva samples of the placebo group.

There were no significant differences in the amount of change experienced by the probiotic and placebo groups in their proportions of *S. mutans* DNA from T1 to T3 for either plaque or saliva samples (p = 0.599 and p = 0.304, respectively) (Table 25). The amount of change of total lactobacilli proportions between the probiotic and placebo groups was also insignificant for both biological samples (p = 0.680 and p = 0.154, respectively).
Figure 22. Frequency distributions of the proportion of *S. mutans* DNA and total lactobacilli DNA at T1, T3 and the differences from T1 to T3 in both supragingival plaque and saliva. Note the non-normal distribution of frequencies in each histogram and the high number of frequencies around 0%.
Figure 22, continued.
Table 23. Changes in the proportions of S. mutans DNA within the probiotic and placebo groups.

<table>
<thead>
<tr>
<th>Sample</th>
<th>T1 Median</th>
<th>T3 Median</th>
<th>Trend from T1 to T3</th>
<th>P-value†</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Probiotic</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% of S. mutans DNA in plaque (n = 18)</td>
<td>0.050123</td>
<td>0.039007</td>
<td>decrease</td>
<td>0.372</td>
</tr>
<tr>
<td>% of S. mutans DNA in saliva (n = 14)</td>
<td>0.116199</td>
<td>0.258695</td>
<td>increase</td>
<td>0.875</td>
</tr>
<tr>
<td><strong>Placebo</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% of S. mutans DNA in plaque (n = 20)</td>
<td>0.207975</td>
<td>0.584166</td>
<td>increase</td>
<td>0.247</td>
</tr>
<tr>
<td>% of S. mutans DNA in saliva (n = 12)</td>
<td>0.773649</td>
<td>0.077346</td>
<td>decrease</td>
<td>0.117</td>
</tr>
</tbody>
</table>

† Wilcoxon Signed Rank test.

Table 24. Changes in the proportions of total lactobacilli DNA within the probiotic and placebo groups.

<table>
<thead>
<tr>
<th>Sample</th>
<th>T1 Median</th>
<th>T3 Median</th>
<th>Trend from T1 to T3</th>
<th>P-value†</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Probiotic</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% of lactobacilli DNA in plaque (n = 29)</td>
<td>1.322577</td>
<td>1.387280</td>
<td>same</td>
<td>0.974</td>
</tr>
<tr>
<td>% of lactobacilli DNA in saliva (n = 14)</td>
<td>4.984790</td>
<td>4.512778</td>
<td>decrease</td>
<td>0.826</td>
</tr>
<tr>
<td><strong>Placebo</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% of lactobacilli DNA in plaque (n = 29)</td>
<td>1.716559</td>
<td>1.435498</td>
<td>decrease</td>
<td>0.689</td>
</tr>
<tr>
<td>% of lactobacilli DNA in saliva (n = 14)</td>
<td>5.430305</td>
<td>3.779750</td>
<td>decrease</td>
<td>0.096</td>
</tr>
</tbody>
</table>

† Wilcoxon Signed Rank test.
3.4 RATER RELIABILITY

The inter-rater reliability, or the degree of agreement between the two examiners at assigning PI scores, was assessed before the study, after the study, and overall. Similarly, the intra-rater reliability, or the degree of agreement among repeated assignments of PI scores by each examiner, was also assessed.

Inter-rater reliability was measured using the PI scores assigned to Samples B and C from both examiners (Table 26 and Figure 23). Examiner alignment before the study commenced resulted in ‘substantial’ agreement between the examiners for both Sample B (intra-oral photos: kappa = 0.7240, std. error = 0.0973) and for Sample C (live clinical patients: kappa = 0.7394, std. error = 0.0779). After the study, the inter-rater agreement improved to ‘almost perfect’ (kappa = 0.8200, std. error = 0.0965) when the examiners re-scored Sample B. The overall inter-rater reliability of all PI scores for Sample B (pre- and post-study combined) showed ‘substantial’ agreement (kappa = 0.7707, std. error = 0.0685).

Intra-rater reliability was measured using the PI scores assigned to Sample B from before and after the study (Table 27 and Figure 24). Both examiners independently had ‘almost perfect’ agreement (examiner 1: kappa = 0.8287, std. error = 0.0971; examiner 2: kappa = 0.8439, std. error = 0.0970).

\[\text{Mann-Whitney U-test.}\]
error = 0.0968) with their pre- and post-study ratings of PI scores. The combined intra-rater reliability of both examiners maintained this ‘almost perfect’ agreement (kappa = 0.8362, std. error = 0.0685).

**Table 26. Inter-rater reliability for the Plaque Index.**

<table>
<thead>
<tr>
<th>Sample</th>
<th># of values</th>
<th>Agreement</th>
<th>Expected agreement</th>
<th>Weighted kappa</th>
<th>Std. error</th>
<th>Z</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample B</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(intra-oral photos)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre-Study</td>
<td>60 pairs</td>
<td>89.17%</td>
<td>60.75%</td>
<td>0.7240</td>
<td>0.0973</td>
<td>7.44</td>
<td>0.0000</td>
</tr>
<tr>
<td>Post-Study</td>
<td>60 pairs</td>
<td>93.33%</td>
<td>62.97%</td>
<td>0.8200</td>
<td>0.0965</td>
<td>8.49</td>
<td>0.0000</td>
</tr>
<tr>
<td>Pre- &amp; Post-Study Combined</td>
<td>120 pairs</td>
<td>91.25%</td>
<td>61.83%</td>
<td>0.7707</td>
<td>0.0685</td>
<td>11.25</td>
<td>0.0000</td>
</tr>
<tr>
<td>Sample C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(clinical patients)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre-Study</td>
<td>72 pairs</td>
<td>90.28%</td>
<td>63.33%</td>
<td>0.7394</td>
<td>0.0779</td>
<td>9.43</td>
<td>0.0000</td>
</tr>
</tbody>
</table>

**Table 27. Intra-rater reliability for the Plaque Index.**

<table>
<thead>
<tr>
<th>Examiner</th>
<th># of values</th>
<th>Agreement</th>
<th>Expected agreement</th>
<th>Weighted kappa</th>
<th>Std. error</th>
<th>Z</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample B</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(intra-oral photos)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Examiner 1</td>
<td>60 pairs</td>
<td>94.17%</td>
<td>62.64%</td>
<td>0.8439</td>
<td>0.0968</td>
<td>8.72</td>
<td>0.0000</td>
</tr>
<tr>
<td>Examiner 2</td>
<td>60 pairs</td>
<td>93.33%</td>
<td>61.08%</td>
<td>0.8287</td>
<td>0.0971</td>
<td>8.53</td>
<td>0.0000</td>
</tr>
<tr>
<td>2 Examiners Combined</td>
<td>72 pairs</td>
<td>93.75 %</td>
<td>61.83%</td>
<td>0.8362</td>
<td>0.0685</td>
<td>12.21</td>
<td>0.0000</td>
</tr>
</tbody>
</table>
Figure 23. Inter-rater reliability for the Plaque Index.

Figure 24. Intra-rater reliability for the Plaque Index.
3.5 COMPLIANCE

All participants self-reported a lozenge consumption compliance over 90%, and 80% of participants reported perfect compliance. Overall lozenge consumption at 14 days (T2) and at 28 days (T3) of all participants (n = 58) is shown in Figure 25 and Figure 26 respectively. Overall, the median lozenges consumption at 14 days was 42 (IQR = 0) and at 28 days was 70 (IQR = 2).

Between the probiotic and placebo groups, there were no significant differences in the number of lozenges consumed at T2 (p = 0.967) and T3 (p = 0.721) (Table 28). Similarly, there were no significant differences in the proportion of participants with perfect compliance to those with less than perfect compliance at either T2 (p = 1.000) or T3 (p = 0.557) between the probiotic and placebo groups (Table 29).

Significant differences were seen in the number of lozenges consumed between male and female participants at T2 (p = 0.026), but these differences did not exist by T3 (p = 0.617) (Table 28). Similarly, the differences in the proportion of male and female participants with perfect compliance to those with less than perfect compliance was significant at T2 (p = 0.032) but not significant by T3 (p = 0.595) (Table 30).

When assessing whether the examiner that enrolled the patient affected patient compliance, no differences were seen between the proportion of participants with perfect compliance to those with less than perfect compliance at T2 (p = 0.670) and T3 (p = 0.557) (Table 31).
Figure 25. Lozenge consumption of all participants at 14 days (T2).

Figure 26. Lozenge consumption of all participants at 28 days (T3).
Table 28. Comparison of number of lozenges taken between lozenge groups and between sexes.

<table>
<thead>
<tr>
<th>Time point</th>
<th>Mean rank of lozenges taken @ T2 (14 days)</th>
<th>P-value†</th>
<th>Mean rank of lozenges taken @ T3 (28 days)</th>
<th>P-value†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Probiotic</td>
<td>29.55</td>
<td>0.967</td>
<td>28.88</td>
<td>0.721</td>
</tr>
<tr>
<td>(n = 29)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Placebo</td>
<td>29.45</td>
<td></td>
<td>30.12</td>
<td></td>
</tr>
<tr>
<td>(n = 29)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male</td>
<td>32.50</td>
<td>0.026*</td>
<td>30.50</td>
<td>0.617</td>
</tr>
<tr>
<td>(n = 25)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>27.23</td>
<td></td>
<td>28.74</td>
<td></td>
</tr>
<tr>
<td>(n = 33)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

† Mann-Whitney U-test.
* p < 0.05.

Table 29. Compliance by lozenge group.

<table>
<thead>
<tr>
<th>Time point</th>
<th>Probiotic group</th>
<th>Placebo group</th>
<th>Total cohort</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Probiotic group</td>
<td>Placebo group</td>
<td>Total cohort</td>
<td>P-value</td>
</tr>
<tr>
<td></td>
<td>n = 29 (50%)</td>
<td>n = 29 (50%)</td>
<td>n = 58 (100%)</td>
<td></td>
</tr>
<tr>
<td><strong>T2: 14 days</strong> (42 lozenges)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Perfect = 42</td>
<td>26 (89.7%)</td>
<td>26 (89.7%)</td>
<td>52 (89.7%)</td>
<td>1.000†</td>
</tr>
<tr>
<td>Less than &lt;42</td>
<td>3 (10.3%)</td>
<td>3 (10.3%)</td>
<td>6 (10.3%)</td>
<td></td>
</tr>
<tr>
<td><strong>T3: 28 days</strong> (70 lozenges)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Perfect = 70</td>
<td>20 (69.0%)</td>
<td>22 (75.9%)</td>
<td>42 (72.4%)</td>
<td>0.557‡</td>
</tr>
<tr>
<td>Less than &lt;70</td>
<td>9 (31.0%)</td>
<td>7 (24.1%)</td>
<td>16 (27.6%)</td>
<td></td>
</tr>
</tbody>
</table>

† Fisher’s Exact test (since n < 5 in both probiotic and placebo ‘less than perfect compliance’ subgroups).
‡ Chi-square test.
<table>
<thead>
<tr>
<th>Table 30. Compliance by sex.</th>
<th>Time point</th>
<th>Male</th>
<th>Female</th>
<th>Total cohort</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>n = 25 (43.1%)</td>
<td>n = 33 (56.9%)</td>
<td>n = 58 (100%)</td>
<td></td>
</tr>
<tr>
<td><strong>T2: 14 days</strong> (42 lozenges)</td>
<td>Perfect = 42</td>
<td>25 (100.0%)</td>
<td>27 (81.8%)</td>
<td>52 (89.7%)</td>
<td>0.032†</td>
</tr>
<tr>
<td></td>
<td>Less than &lt;42</td>
<td>0 (0.0%)</td>
<td>6 (18.2%)</td>
<td>6 (10.3%)</td>
<td></td>
</tr>
<tr>
<td><strong>T3: 28 days</strong> (70 lozenges)</td>
<td>Perfect = 70</td>
<td>19 (76.0%)</td>
<td>22 (69.7%)</td>
<td>42 (72.4%)</td>
<td>0.595‡</td>
</tr>
<tr>
<td></td>
<td>Less than &lt;70</td>
<td>6 (24.0%)</td>
<td>10 (30.3%)</td>
<td>16 (27.6%)</td>
<td></td>
</tr>
</tbody>
</table>

† Fisher’s Exact test (since n < 5 in the male ‘less than perfect compliance’ subgroup).
‡ Chi-square test.

<table>
<thead>
<tr>
<th>Table 31. Compliance by examiner.</th>
<th>Time point</th>
<th>Examiner 1</th>
<th>Examiner 2</th>
<th>Total cohort</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>n = 29 (50%)</td>
<td>n = 29 (50%)</td>
<td>n = 58 (100%)</td>
<td></td>
</tr>
<tr>
<td><strong>T2: 14 days</strong> (42 lozenges)</td>
<td>Perfect = 42</td>
<td>25 (86.2%)</td>
<td>27 (93.1%)</td>
<td>52 (89.7%)</td>
<td>0.670†</td>
</tr>
<tr>
<td></td>
<td>Less than &lt;42</td>
<td>4 (13.8%)</td>
<td>2 (6.9%)</td>
<td>6 (10.3%)</td>
<td></td>
</tr>
<tr>
<td><strong>T3: 28 days</strong> (70 lozenges)</td>
<td>Perfect = 70</td>
<td>20 (69.0%)</td>
<td>22 (75.9%)</td>
<td>42 (72.4%)</td>
<td>0.557‡</td>
</tr>
<tr>
<td></td>
<td>Less than &lt;70</td>
<td>9 (31.0%)</td>
<td>7 (24.1%)</td>
<td>16 (27.6%)</td>
<td></td>
</tr>
</tbody>
</table>

† Fisher’s Exact test (since n < 5 in the both examiners’ ‘less than perfect compliance’ subgroups).
‡ Chi-square test.
**Figure 27.** Comparison of the proportion of participants with ‘perfect compliance’ to ‘less than perfect compliance’ in probiotic vs. placebo, male vs. female, and examined by examiner 1 vs. examiner 2 subgroups (*p < 0.05).

### 3.6 ADVERSE EVENTS

One participant had been taking the lozenges for over 2 weeks uneventfully and then suddenly developed stomach upset and diarrhea, at which time she stopped taking the lozenges. The participant was not sure if the stomach upset or diarrhea was caused by the lozenges or was incidental. Symptoms went away within 48 hours and were gone by the time the investigators were notified by the participant and parents. The investigators withdrew the participant from the study and instructed the participant not to take any further lozenges. The participant was monitored for another 6 weeks with no recurrence of the symptoms. When the lozenge identities were revealed at the study’s end, this particular participant was identified to be in the placebo group.

No adverse events were experienced in the probiotic group.
3.7 SATISFACTION QUESTIONNAIRE

The following is a summary of the responses to the “End of Study Questionnaire” (Appendix 9) from all participants that completed the clinical trial (n = 58):

1. **Satisfaction with taste of lozenges**: 89.6% responded very satisfied or satisfied with the lozenge taste, no responses for dissatisfaction.

2. **Success at taking 2 lozenges per day for 28 days**: 84.5% felt completely successful, 15.5% felt they were mostly successful, no responses for unsuccessful.

3. **Difficult to take 2 lozenges per day**: 93.2% disagreed or strongly disagreed, 5.2% were neutral, and 1.7% agreed.

4. **Difficult to remember to take the lozenges everyday**: 67.3% disagreed or strongly disagreed, 19.0% were neutral and 13.7% agreed.

5. **Study length was too long**: 74.1% disagreed or strongly disagreed, 20.7% were neutral, and 5.2% agreed.

6. **Participants did not feel the need to prevent white spots, decay or gum disease**: 77.6% disagreed or strongly disagreed, 17.2% neutral, 5.2% agreed.

7. **Participants lost interest in the study**: 86.2% disagreed or strongly disagreed, 13.8% were neutral.

8. **Effectiveness of the lozenges**: 62% believed the lozenges were effective or very effective, 36.2% were neutral, and 1.7% did not believe they were effective.

9. **Type of lozenges participants believed they were taking**: 86.2% believed there were taking the probiotic, 13.8% believed they were taking the placebo.

10. **Likelihood of participants using lozenges if they were shown to be effective at reducing white spots, decay or gum disease**: 81% responded they would be very likely or moderately likely to use them, 15.5% were somewhat likely, and 3.4% were somewhat not likely.
3.8 SUMMARY OF RESULTS

The results of this study can be summarized as follows:

- No significant improvements in plaque levels (PI and cPI scores) were observed in the probiotic group whereas significant improvements were observed in the placebo group. There were significantly more improvements in the placebo group than in the probiotic group. However, none of the observed improvements in either group were of any practical, clinical significance as the fluctuations observed between time points were exceedingly small in frequency and magnitude.

- There were no significant changes in the levels of \textit{S. mutans} or lactobacilli after probiotic or placebo intervention in either supragingival plaque or saliva samples. There were no significant differences in the amount of change experienced in one group relative to the other.

- \textit{S. salivarius} K12 expression levels were below the detectable limits in all supragingival plaque samples screened.

- There was substantial inter-rater agreement between examiners and almost perfect intra-rater agreement for each examiner from the beginning to the end of the study.

- There were no significant differences in compliance between the probiotic and placebo groups at any time point. All participants self-reported a lozenge consumption compliance over 90%, and 80% of participants reported perfect compliance.

- No adverse events were experienced in the probiotic group.

- 89.6% of participants were very satisfied or satisfied with the lozenge taste and 81% responded they would be fairly likely to use them if they were shown to be effective at reducing white spots, decay or gum disease.
4. DISCUSSION

The present study was undertaken to investigate whether daily consumption of the Lorodent probiotic complex – consisting of *S. salivarius* K12 and five lactobacilli strains *L. paracasei*, *L. plantarum, L. acidophilus, L. salivarius* and *L. reuteri* – was effective in reducing plaque accumulation and *S. mutans* levels during orthodontic treatment with fixed appliances. To our knowledge, this is the seventh clinical oral probiotic study in the orthodontic population and only the second with a primary outcome that was clinical. The current study is the first clinical study to investigate the effectiveness of multistrain probiotic therapy in orthodontic patients and is the first study to specifically evaluate the Lorodent probiotic complex in any dental or orthodontic population.

The results from this study rejected our hypothesis that oral exposure of the Lorodent probiotic lozenge for 28 days would cause a significant decrease in levels of plaque and *S. mutans* over time in adolescent orthodontic patients compared to placebo-treated control group.

4.1 COMPARISON OF THE RESULTS OF THE STUDY TO THE EXISTING PROBIOTIC LITERATURE

In the following section, the clinical and microbial results of this study will be separately compared to the existing probiotic literature with primary emphasis on the other orthodontic probiotic studies and secondary emphasis on other dental probiotic studies. As shown previously, orthodontic patients have a unique caries challenge that is unlike other dental populations due to the extreme build-up of plaque and significant rises in *S. mutans* levels. Because the probiotic literature is very heterogeneous in terms of the study designs, the strongest conclusions can be drawn about probiotic efficacy when comparing within similar populations.

4.1.1 EFFECT OF THE LORODENT PROBIOTIC ON PLAQUE LEVELS

To date, no orthodontic study has assessed the effect of probiotic therapy on plaque levels. However, one orthodontic study did evaluate a different clinical outcome – white spot lesions
– and, like our study, this study found that oral probiotic therapy had no clinical benefit.\textsuperscript{144} Specifically, this study reported that oral probiotic therapy for 17 months did not decrease the prevalence of white spot lesions, assessed using intraoral photographs, in adolescent patients undergoing fixed orthodontic therapy. This study also had the strongest design, highest sample size, and longest treatment duration out of all the orthodontic probiotic studies. Further, the methodology of this study was also most similar to ours as they investigated \textit{L. reuteri}, which is one of the strains in the Lorodent probiotic complex, and used lozenges as the delivery vehicle. Since plaque accumulation is a precursor to white spot lesion development, these findings support the conclusions from our study that oral probiotic therapy had no effect on plaque levels in orthodontic patients.

On the contrary, when considering studies in non-orthodontic populations, the results from our study are in disagreement with most of the other probiotic studies that evaluated clinical outcomes such as caries experience and plaque levels.\textsuperscript{111,121,127,136,142,145} However, there are number of critical differences between our study and the others that may have affected the outcomes including the length of the probiotic administration, bacterial strains used in the probiotics, vehicles in which the probiotics were delivered, the presence or absence of fluoride in the probiotics, and the sample population in which the probiotics were tested. Most of the studies with decreased caries experience and plaque levels had long probiotic administration periods ranging from 3 to 21 months.\textsuperscript{111,121,127,136,142} Secondly, the bacterial strains that were used in those studies differed from the strains used in our study. For example, significant decreases in caries experience and plaque levels were found in studies that used \textit{L. rhamnosus},\textsuperscript{111,121,127} \textit{L. rhamnosus} with \textit{Bifidobacterium animalis subsp. lactis},\textsuperscript{145} and \textit{S. salivarius} M18.\textsuperscript{136} Thirdly, in most of the studies showing decreased caries experience the probiotic, \textit{L. rhamnosus}, was delivered in milk,\textsuperscript{111,121,127} and one of these studies also supplemented the \textit{L. rhamnosus} milk with fluoride, both of which facilitate remineralization.\textsuperscript{121} Lastly, most of the studies with decreased caries experience used probiotic therapy in infants soon after birth in an attempt to establish the probiotic strains in the mouth before a pathogenic oral microbiome could be established.\textsuperscript{111,121,142}
Possible explanations for why the probiotic group did not show any changes in plaque levels are discussed in Section 4.2 whereas explanations for why the probiotic group showed beneficial changes in plaque levels are discussed in Section 4.3.

4.1.2 EFFECT OF THE LORODENT PROBIOTIC ON S. MUTANS LEVELS

The literature appears to be split regarding the effect of oral probiotic therapy on S. mutans levels whether in the orthodontic or general dental populations. Our results reporting no changes in S. mutans levels in orthodontic patients are corroborated by 2 orthodontic studies, but contradicted by 3 others. The most robust orthodontic study, however, with the highest sample size, longest probiotic duration and similar methodology to ours was in agreement with our study and did not report any change in S. mutans levels after 17 months of probiotic therapy. A similar split in the literature is seen in all dental populations in which 18 of the 38 studies reported no changes in S. mutans levels post-probiotic administration, whereas 20 of the studies showed significant decreases (Table 1). However, a common element in studies that showed significant decreases in S. mutans levels was the use of a dairy delivery vehicle for probiotic therapy, and this will be discussed further in Section 4.2.

One important consideration when interpreting the literature is the sensitivity and specificity of the laboratory techniques used to assess S. mutans levels. Notably, 37 of the 38 dental probiotic studies measured S. mutans levels using selective media culturing and most used commercial chair-side kits (i.e., Dentocult® SM, CRT® Bacteria). These techniques are less sensitive and specific than the qPCR technique used in our study. Further, most of these studies assessed S. mutans levels in saliva which is a less accurate biomarker for caries experience than S. mutans levels in plaque. On the other hand, only 1 study assessed S. mutans levels using qPCR like our study. The methodology of this study was similar to ours in that S. mutans levels were assessed from plaque samples in 60 orthodontic patients after 30 days of probiotic therapy, but their results disagreed reporting significant decreases in S. mutans levels. This study differed from ours in terms of the vehicle of delivery (a dairy product), the qPCR primers that were used to detect S. mutans, and unfortunately the authors
did not report the probiotic strains used in their study. Further, it is important to note that in our study many participants did not have detectable levels of \textit{S. mutans} in either their plaque or saliva samples, effectively decreasing the sample size for analysis, and this may have resulted in insufficient power to detect significant differences between the groups. Possible explanations for this are discussed in Section 4.4.

### 4.1.3 EFFECT OF THE LORODENT PROBIOTIC ON LACTOBACILLI LEVELS

The levels of lactobacilli have been assayed in only 4 probiotic studies in the orthodontic population.\cite{120,140,141,144} In theory, a rise in total lactobacilli levels would indicate persistence and successful colonization of the lactobacilli probiotic strains in the oral cavity.\cite{113,141} Unfortunately, half of the orthodontic studies that assayed for lactobacilli did not use probiotics containing lactobacilli, and unsurprisingly noted no changes in lactobacilli levels.\cite{120,140} Of the remaining orthodontic studies that actually used lactobacilli probiotic strains, the results are split. One study reported an increase in total lactobacilli levels using \textit{L. paracasei} in milk,\cite{141} whereas the most robust orthodontic study using \textit{L. reuteri} in a lozenge similar to ours saw no changes in total lactobacilli levels.\cite{144} Thus, it is difficult to draw conclusions from the orthodontic literature given the low number of studies and the heterogeneity of their designs.

When assessing the effect of probiotic therapy on lactobacilli levels in all dental populations, our results agreed with the majority of the studies – 19 of the 27 studies showed no change, 7 of the studies showed statistically significant increases, and 1 study showed decreases in lactobacilli levels (Table 1). Most of the studies that reported either no changes or increases in lactobacilli levels used probiotics composed mainly of lactobacilli strains, but no further trends could be identified. Interestingly, the 1 study that showed decreased lactobacilli levels used a probiotic composed mainly of lactococci strains.\cite{122} All 27 oral probiotic studies assessed total lactobacilli levels using selective media culturing, most using commercial chair-side kits (i.e., Dentocult® LB, CRT® Bacteria). No studies used qPCR to evaluate total lactobacilli, and our study was the first to do so.
4.1.4 **S. SALIVARIUS K12 PERSISTENCE IN SUPRAGINGIVAL PLAQUE**

The majority of previous probiotic literature on *S. salivarius* K12 has investigated this strain within the context of oral halitosis or upper respiratory tract infections like pharyngitis.\(^{180-184}\) Only one study extended their investigation of *S. salivarius* K12 to various sites in the oral cavity that could be applicable to caries.\(^{184}\) This study demonstrated that *S. salivarius* K12 preferentially colonizes the dorsum of the tongue (relevant to halitosis), but is not found on any tooth or gingival surfaces; however, this study only had a sample size of 1. Since this strain was part of the Lorodent probiotic complex and given the much larger sample size of our study and the unique orthodontic patient population, we felt it would be an interesting and novel opportunity to screen for *S. salivarius* K12 in plaque – directly at the site of enamel demineralization. In our study, *S. salivarius* K12 was not detected in any of the plaque samples from 29 participants, regardless of whether they had received the probiotic or placebo and these results confirm that *S. salivarius* K12 does not colonize supragingival plaque.

4.1.5 **COMPLIANCE & SAFETY WITHIN THE ORTHODONTIC SETTING**

Compliance was commendably high in both groups with greater than 90% compliance from all participants, and there were no significant differences in compliance between the probiotic and placebo groups at any time point. Thus, compliance was not a possible factor for the negative findings in the probiotic group. Further, blueberry flavored lozenges were very well accepted by the adolescent orthodontic participants in this study, with almost 90% of the participants fairly satisfied with the taste and 81% of participants motivated to use probiotic therapy if it was shown to be effective. This study also established the safety of the Lorodent probiotic complex in the human population as no adverse events were experienced in the probiotic group. Thus, our study illustrated that probiotic therapy can be practically implemented in the clinical orthodontic setting.
4.2 POSSIBLE EXPLANATIONS FOR NEGATIVE FINDINGS IN THE PROBIOTIC GROUP

There are numerous possible explanations for the negative findings in the probiotic group, such as ineffective probiotic strains, ineffective delivery vehicle for the probiotic, suboptimal daily dosage, short duration of the intervention, and the paramount cariogenic bacterial challenge of orthodontic patients.

As discussed previously, the strains in the Lorodent probiotic complex – *S. salivarius* K12, *L. paracasei*, *L. plantarum*, *L. acidophilus*, *L. salivarius* and *L. reuteri* – may not have been the most effective strains at decreasing plaque levels and oral *S. mutans* levels where other strains like *L. rhamnosus* have been shown to be quite effective.\textsuperscript{111,121,127,145} From the literature, there were multiple studies with mixed results for the effectiveness of *L. reuteri* and *L. paracasei*, but only 3 out of 38 investigated *L. acidophilus*,\textsuperscript{113,128,134} only 1 study investigated *L. salivarius*,\textsuperscript{94} and no dental probiotic studies investigated *L. plantarum* or *S. salivarius* K12. Thus, the individual effectiveness of these strains are unknown, and so is the effectiveness of these strains in combination with each other.

Multistrain probiotics are often desired for their synergistic and symbiotic activities towards each other, but there is very limited research that suggests that probiotic strains showing mutually inhibitory properties – for instance, the production of hydrogen peroxide and bacteriocin-like substances – may not only have the desired effect of inhibiting endogenous strains like *S. mutans*, but in multistrain combinations may also undesirably inhibit each other.\textsuperscript{185} It is difficult to ascertain but this may have been a possibility in the Lorodent probiotic as *L. reuteri* produces hydrogen peroxide,\textsuperscript{90,101} and *S. salivarius* K12 produces a very potent bacteriocin-like substance.\textsuperscript{107-109} Ironically, the very bacterial products that make probiotic therapy effective against pathogenic bacteria like *S. mutans* may be the same products that in combination prevented the multistrain Lorodent probiotic from being effective.

Similarly, a lozenge may have been an ineffective delivery vehicle to establish the Lorodent probiotic strains in the oral cavity. Firstly, even though participants were instructed to slowly
dissolve the lozenges, they may have chewed and/or swallowed them instead, thereby clearing the probiotics quickly from the oral cavity. Secondly, probiotics may thrive better in nutrient-rich mediums like dairy products compared to relatively inert delivery vehicles like lozenges that are designed to have long shelf lives, and dairy products contain calcium phosphate and casein phosphopeptides which enhance enamel remineralization.\textsuperscript{186-190} From the literature (Table 1), 14 of the 20 studies that reported a significant decrease in \textit{S. mutans} levels used dairy products as the delivery vehicle for the probiotic. On the other hand, 13 of the 18 studies that did not show changes in \textit{S. mutans} levels used non-dairy delivery vehicles. This dichotomy was statistically significant (\( p = 0.022 \)) (Table 32). Notably, half the studies that saw an improvement in caries experience used milk with \textit{L. rhamnosus} – this may be an effective combination for dental probiotic therapy.\textsuperscript{111,121,127,145}

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<th>Non-Dairy Delivery Vehicle</th>
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<tr>
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<td>13</td>
<td>18</td>
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<td>Total</td>
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\textsuperscript{†} Chi-square test.

The number of active probiotics per lozenge may have also been suboptimal. One concern in particular was that the probiotic lozenges used in this study were manufactured in August 2011. However, bacterial counts from July 2014 confirmed that each probiotic lozenge contained \(10^9\) active CFUs. In comparison to the literature, this is well within the range of the other probiotic studies that assessed clinical outcomes like caries experience or plaque levels, ranging from \(10^9\) CFU,\textsuperscript{121,127,136,145} to \(10^8\) CFU (which was slightly less than our study),\textsuperscript{111,137,142,144} to \(10^{10}\) CFU (which was slightly more).\textsuperscript{139} It is important to note that ‘one billion CFUs of bacteria’ is the status quo for probiotic therapy in the gastrointestinal literature and has similarly been adopted as the standard in the dental literature. These numbers have been shown to be effective \textit{in vitro},\textsuperscript{90,98,99} but as of yet, no studies have been undertaken with
the purpose of establishing the optimal number of CFU for probiotic therapy in vivo within the dental context and/or orthodontic context.

As well, the optimal daily dose and dosing regimen of probiotic treatment is fairly unknown, making it difficult to ascertain the dose-response relationship of probiotic therapy. Only 1 of the 38 probiotic studies considered the effect of dosage on the effectiveness of the probiotic. This study evaluated the effect of varying dosages in two probiotic groups, and found significant changes in S. mutans and lactobacilli levels only when the daily dosage of the probiotic was doubled. Thus, the literature is generally lacking with regard to optimal oral probiotic dosing and without any previous clinical studies using the Lorodent probiotic complex, our study used the manufacturer recommended dosing as there was no evidence-based one and this dose and duration of probiotic therapy may not have been appropriate for an orthodontic population.

Similarly, the literature is very heterogeneous in terms of the optimal duration of probiotic therapy. For example, 5 of the 6 orthodontic probiotic studies were 4 weeks or less in duration while the remaining study was 17 months long (Table 3) – the lack of intermediary studies makes it difficult to assess the appropriate treatment duration in this patient subset. On the other hand, 8 of the 9 studies that assessed clinical outcomes such as caries experience or plaque accumulation had longer durations than our study ranging from 3 to 23 months (Table 2). Thus, perhaps a longer duration of probiotic treatment may have been required to establish the Lorodent probiotic strains in the oral cavity. It should be kept in mind, however, that while the long-term effects of the Lorodent probiotic complex are still largely unknown, our findings are in agreement with the 17-month study by Gizani et al.

Another potential explanation for the results observed could be that in the orthodontic patient population the levels of intraoral plaque were simply too high for the probiotic to have any meaningful effect. It is well observed that oral hygiene in adolescent orthodontic patients is poor and mechanical debridement around the orthodontic bracket is arduous resulting in oral biofilms that are thicker, denser and generally more pathogenic. Previous studies have shown that S. mutans levels increased about four times in patients undergoing active orthodontic
treatment compared to controls. Therefore, the probiotic may not have been able to compete with these thick, mature biofilms of cariogenic plaque and find a niche for itself. In a study by Aminabadi et al., it was shown that complete mouth disinfection with chlorhexidine prior to probiotic usage increased the ability of the probiotic strains to compete and establish themselves whereas probiotic therapy on its own was ineffective. Conversely, in orthodontic patients there is seldom ever a time in which plaque is completely removed resulting in a lack of available niche sites for colonization. Thus, the amount of plaque may have overcome any potential beneficial action of the probiotic strains and probiotic therapy may not be an effective treatment modality in the orthodontic patient subset. It is important to note that there is a significant difference between delivering the probiotic strains early in an individual’s life when the oral microbiome is initially being established, rather than later on when the probiotic strains would have to overcome and outcompete a long-standing pathogenic oral microbiome.

4.3 POSSIBLE EXPLANATIONS FOR POSITIVE FINDINGS IN THE PLACEBO GROUP

In our study, we observed that the placebo group had a significant improvement in plaque scores compared to the probiotic group. One potential reason could be that the participants may have improved their oral hygiene behavior in response to being observed as part of a study. This so-called Hawthorne effect is well-documented in clinical trial literature. Specifically, the participants in this study were counseled that they were participating in a trial about probiotic lozenges to decrease plaque levels and white spot lesions. Therefore, knowledge about the outcome being tested may have consciously or subconsciously caused these participants to take greater care in their oral hygiene routines as they knew beforehand that these were the outcomes they were being evaluated on. Furthermore, by selecting orthodontically compliant participants for our study and then motivating them with compensation 2-weeks into the study with the promise of additional compensation by the end of the study, this may have resulted in the participant’s improving their oral hygiene and performing better plaque removal. These reasons could have contributed to the placebo group
having a decrease in plaque scores. However, this does not explain why there was a difference between the two lozenge groups as both groups should have been equally predisposed to the Hawthorne effect given the randomization and blinding process.

Another potential source of error could be due to the effect of sample size on randomization. While the randomization process attempts to balance the groups in the trial such that they represent identical populations, this may not have been the case due to the small number of participants in each lozenge group. Although the study had a sufficient number of observation sites (n = 1392 total, n = 696 per arm) and an adequate power to detect a change of 25% between the probiotic and placebo groups, the number of subjects was much lower (n = 58 total, n = 29 per arm). Therefore, although randomization was performed so that the effect of confounding variables would be evenly distributed between both groups, there is a chance that the groups were not completely identical.

For example, because the baseline data for sex, age, plaque levels, and oral microbial levels of S. mutans and total lactobacilli showed that the probiotic and placebo groups were not significantly different in any measured respect, this indicated that there was effective randomization of the study participants into each group. However, upon closer inspection, the p-value for the proportions of the sexes between the two groups was p = 0.063, which while not significant is borderline and demonstrates that it is not inconceivable that there may have been systematic differences between the groups. Thus, although the sample size was large enough to detect a significant difference in the measured outcome, it may not have been large enough to drown out potential confounders.

When specifically considering sex as a potential confounder, it is important to note that while there were also significant differences in the baseline PI scores between sexes – with females having a lower proportion of plaque scores than men, there were no significant differences in the amount of improvement in PI and cPI scores at any time frame between sexes or in the overall compliance between sexes. Thus, it is debatable if the borderline significance of having more females (with lower plaque scores) in the placebo group than the probiotic group affected the outcome.
Other potential confounding variables that may have affected the outcome are frequency and pattern of brushing and flossing, past caries incidence, diet, frequency of eating, fluoride exposure, type of orthodontic appliances in the mouth, and stage of orthodontic treatment. Each of these variables exert a large influence on plaque buildup and bacterial levels. While the two groups were assumed to be identical in terms of these variables, if this was not the case and if one of these groups had attributes that predisposed them to plaque buildup making it harder for them to clean their teeth, this may have contributed to the results observed. For example, elastomeric ties are porous and plaque retentive as compared to stainless steel ligatures and self-ligating brackets. Further, common auxiliaries such as power chains and coil springs can harbor plaque and food and affect the ability of individuals to adequately clean their teeth. The ligation method and type of auxiliaries used in each participant was not controlled and may have varied during the study – for instance, it is quite plausible that a participant may have had a power chain placed at some point as part of their orthodontic treatment and this could have negatively affected their plaque scores. Thus, if significantly more participants in the probiotic group had power chains compared to the placebo group, this could have attributed to the results observed.

In this study, the allocation of participants into each group was not concealed from the examiners and this may have introduced ascertainment bias into the results. Although the lozenge groups were blinded and the examiners were not aware whether a participant had been randomly assigned to the probiotic or placebo group, they were aware that the participant had been assigned to group ‘A’ or ‘B’. Thus, the examiners were blinded but not masked. This was unavoidable given the constraints of the university setting in which the study was conducted as the examiners had to screen and recruit participants, then coordinate the appointments and organize the intervention with the participants, parents, and orthodontic providers, in addition to recording the observed clinical results, collecting the biological samples and sending them offsite for microbial analysis. Further, any clinical differences observed between the groups may have worked to form or strengthen these biases within the minds of the examiners. For example, if the examiner saw three subjects in a row, each part of group A, with immaculate oral hygiene then this examiner may be biased into thinking that...
this random occurrence was due to the lozenges that group A was taking. Therefore, any preconceived, conscious or unconscious notions about which group was getting the intervention may have caused the examiners to be biased one way or the other. Ideally, the participants should have been randomized and allocated into each lozenge group by a third party separate from the examiners, and the examiners sole job should have been to record the clinical findings observed.

4.4 POSSIBLE EXPLANATIONS FOR UNDETECTABLE S. MUTANS LEVELS

Many participants did not have detectable levels of S. mutans in their collected plaque or saliva samples at baseline, 28 days, or both time points. The fact that the levels of S. mutans were below the detectable limits for many of the plaque samples is likely related to the relatively low average DNA concentrations extracted from the supragingival plaque samples (1.2 ng/µL). The vast number of samples in this study made it necessary to use a multiplex DNA extraction kit to extract DNA from plaque and saliva using a well-established protocol that was expected to yield a sufficient amount of DNA for downstream processing (qPCR, microbiome analysis, etc.). It is possible that individual DNA extraction columns would have been more suitable and efficient at extracting DNA from these kinds of plaque samples, but the time it would take to process each sample individually made it unfeasible. Another possible way to increase the yields of DNA from plaque would be to place the plaque samples in a smaller amount of TE buffer (i.e., 100 µL to 200 µL instead of the 400 µL that was used in the current study) and with more rigorous efforts undertaken to completely homogenize the sample (i.e., through the use of a bead beater apparatus) before undertaking the extraction process.

An examination of the starting DNA concentration of each sample and its final expression values of S. mutans from qPCR found no correlation. Although there was a correlation between some plaque samples with an extremely low DNA concentration and those in which S. mutans was below detectable limits, there were also several cases of samples with relatively high DNA concentrations in which S. mutans levels were low. This is not surprising as it is
very possible that these samples had a low fraction of *S. mutans* in their total bacterial composition. Likewise, samples with a low DNA concentration where *S. mutans* did amplify may have had a high abundance of this bacterial species. It is important to note that the use of each sample's total bacterial DNA as an internal reference standard allowed direct comparison between samples even with varying amounts of extracted DNA.

The DNA extractions from saliva appear to have been much more efficient with over four and a half times the average DNA concentration from saliva samples than from the plaque samples. As a result, future studies may want to focus on examining changes in specific bacterial species concentrations in participants’ saliva over time. However, saliva is more representative of the oral microbiome as a whole, and as such important changes at the tooth and gingival surfaces may be masked.

Sample collection and storage was unlikely a source of the low DNA yields from the plaque samples, as they were immediately stored at -20°C until transfer to the Burton lab, where they were then stored at -80°C until processing. This is supported by the fact that the saliva samples were collected and stored in the exact same manner.

In the current study, validated qPCR primers were chosen from previously published literature and validated against DNA isolated from pure *S. mutans* and *S. salivarius* K12. A more stringent and thorough approach would have been to develop and test primers that are more effective at detecting *S. mutans* in the collected samples, such as outlined in a previous study. However, this would require screening of a substantial number of primers and amplification times and temperatures to find an optimal combination, at a huge investment of time and funds and with no guarantee of better results.

### 4.5 LIMITATIONS

An inherent weakness in this study was that surrogate endpoints such as plaque and *S. mutans* levels were used to assess the probiotic effect on white spot lesions. The ideal primary outcome of any intervention aimed at preventing a disease should be a decrease in the
incidence of the disease itself, and assessing any surrogate measures other than the disease may lead to false conclusions if the effects of the intervention on the surrogate do not reliably predict the overall effect on the disease.\textsuperscript{199,200} That is, using surrogate endpoints like short-term reductions in plaque and \textit{S. mutans} levels may not necessarily be associated with a decreased incidence of white spot lesions or even a reduced risk of white spot lesion formation. Ideally, while spot lesions should have been used as the final clinical endpoint and participants should have been monitored over a longer course of time in order to observe differences in white spot lesion development between the groups.

There are three main drawbacks to Silness and L\öe’s Plaque Index which may have led to our results not being as accurate or reliable as possible. First, this index was developed in 1967 for use in periodontology to assess plaque in the area of the marginal gingiva, which is very important in assessing periodontal risk but less directly relevant to caries and white spot lesions as these often occur in the direct vicinity of the bracket. Second, the scale only has four possible scores, with the score of 0 indicating no plaque which is a rarity in patients with fixed orthodontic treatment. Therefore, this scale essentially classified individuals into three groups making it only slightly better than saying ‘low’, ‘medium’, ‘high’ level of plaque. Given the broad categories into which plaque levels were graded, the scale may have been too blunt to detect less noticeable changes in plaque levels produced by the probiotic. Thus, discontinuous scales can be difficult to interpret, particularly if the values are incorrectly treated as parametric and averaged as is done in some studies. Lastly, this scale is inherently subjective which can lead to discrepancies in accuracy and reproducibility.

Therefore, the traditional plaque indexing scales have not been created for populations with orthodontic appliances, and thus may not be completely relevant in the orthodontic. An alternative is the Orthodontic Plaque Index (OPI), recently developed to measure plaque levels specifically in an orthodontic population.\textsuperscript{201} The OPI grades plaque levels in the immediate vicinity of the bracket on a scale from 0 to 4. While still suffering from a lack of specificity similar to the Silness and L\öe scale, the OPI may be a more appropriate plaque index to use in the study as it may allow for a more accurate detection of changes in plaque levels around the
orthodontic appliances. However, the OPI is a relatively new plaque indexing system that has not been thoroughly used and validated in orthodontic literature. The original OPI article has only been cited by 4 other articles versus the Silness and Löe index that has been cited by over 2,391 articles.

An alternative method to objectively assess plaque levels in the dental population is by a digital plaque image analysis (DIPA) using a digital camera, UV flash units and software evaluation. This technique has been used in the orthodontic population and has been proven to have very good reproducibility. This method measures plaque as a percentage of tooth coverage and gives the results as a continuous variable ranging from 0-100% based on digital image manipulation. Hence, this method is more specific and objective allowing for more subtle differences in plaque buildup between the groups to be detected. However, DIPA requires expensive technology, technical expertise, time-consuming image analysis, and has also not been extensively used in the literature.

From a microbial perspective, there were several limitations. In this study, the levels of S. mutans and total lactobacilli were calculated as a proportion of each sample’s total bacterial DNA to allow for comparison across samples. However, knowing the amount of each species DNA present in a sample does not allow determination of the number of CFU of each species in a sample. To do this, a standard curve would need to be generated based on DNA extracted from a known quantity of each bacterial species. While this was not done in this study due to time constraints, it would be useful in future studies to have actual bacterial numbers to help add context to clinical parameters observed.

To examine microbiological changes in plaque and saliva samples in this study, qPCR was used to examine possible shifts in several bacterial species of interest. While this method allowed very precise monitoring levels of our specific species of interest, it is possible that there were clinically important shifts in microbial levels of species that were not tested. One potential way to avoid missing such changes would be to conduct full 16S rRNA microbiome sequences of the samples to get a broad overview of any shifts that are occurring in bacterial composition of the plaque or saliva. However, one issue with this method is that it is often
not possible to distinguish differences at the species level, as results are typically reported at the genus or even family taxonomic level. If this were the case then *S. mutans* and *S. salivarius* would be indistinguishable from one another and indeed grouped together. Future studies may want to first screen samples with 16S microbiome sequencing to identify broad trends that may otherwise be missed, then use this information to track changes more precisely with qPCR for identified genus' of interest.

### 4.6 FUTURE STUDIES

Future studies on oral probiotics are required to evaluate the true clinical significance of probiotic therapy in terms of clinical outcomes such as decreases in white spot lesions and caries during fixed orthodontic treatment. While no beneficial effects of probiotic therapy on plaque and *S. mutans* levels were seen in the adolescent orthodontic population in this study, it would be imprudent to dismiss oral probiotics based on the results of this study alone as previous studies have shown their potential efficacy under specific conditions. The body of literature on oral probiotics is rapidly increasing in quantity but it is still very scattered. Therefore, methodical and systematic investigations into situations in which probiotics have shown promise are necessary in order to prove or disprove clinical efficacy.

From the results of this study, it can be seen that future probiotic studies should focus on the bacterial strains that have been shown to be efficacious, for instance *L. rhamnosus*. Further, studies should focus on dairy delivery vehicles, such as milk, as it has been seen that dairy products may act synergistically with the probiotics. Targeted investigations should be undertaken to determine the most effective dosage and frequency of administration for their strain and delivery vehicle, as the existing literature does not provide any beneficial insight in this regard. It is important to keep in mind that studies like ours that use multistrain probiotics are not designed to investigate which specific strains are effective, and further *in vitro* studies would also be required to investigate which combination of strains have synergistic and symbiotic activities towards each other. Ideally, future studies should also use more sensitive
and more objective outcome measurements such as the DIPA protocol in addition to endpoint clinical outcomes such as white spot lesions themselves.

Microbial pilot studies should also be undertaken to develop techniques to optimize and increase DNA yields from supragingival plaque (i.e., less dilution, more homogenization), develop custom qPCR primers that are highly effective at detecting *S. mutans*, use specific qPCR primers for specific strains of lactobacilli, use standard curves that can specifically quantify the CFU of each bacteria assayed, and use microbiome sequencing to screen for identify broader shifts in the overall oral microbiome.

Future probiotic studies could also look into combination therapy with chlorhexidine or fluoride to increase the probiotic’s effectiveness. Given the encouraging results of Aminabadi et al. using chlorhexidine pretreatment in facilitating the colonization of the probiotic bacteria and the tremendous increase in bacterial levels seen during orthodontic treatment, future studies investigating the effectiveness of oral probiotic should incorporate a pretreatment phase of oral disinfection using chlorhexidine to reduce the oral bacterial load prior to probiotic administration favor probiotic colonization of the oral microbiome. Stecksén-Blicks et al. and Petersson et al. both demonstrated that a combination of *L. rhamnosus* with fluoride in milk resulted in long-term decreases in caries experience in infants and increases in root caries reversals in elderly adults, respectively. Future studies may want to investigate this potentially effective combination in the orthodontic population.

## 4.7 CONCLUSIONS

The results from this a randomized, double-blind, parallel-group, placebo-controlled trial do not support the use of the Lorodent probiotic lozenge as an adjunctive treatment to decrease plaque or *S. mutans* levels in adolescent patients undergoing fixed orthodontic appliance therapy. Future studies evaluating the incidence of white spot lesions using larger sample sizes, more efficacious bacterial strains and delivery vehicles, different dosages and frequencies of administration, and possible combinations with chlorhexidine and/or fluoride are needed to investigate the potential benefit of probiotics in the prevention of white spot lesions in the adolescent orthodontic population.
5. REFERENCES


31. Marsh PD, Bradshaw DJ. Dental plaque as a biofilm. Journal of Industrial


72. Løe H, Schiott CR. The effect of mouthrinses and topical application of chlorhexidine


6. APPENDICES
June 25, 2014

Dr. Siew-Ging Gong  
FACULTY OF DENTISTRY

Dr. Fatima Ebrahim  
FACULTY OF DENTISTRY

Dear Dr. Gong and Dr. Fatima Ebrahim,

Re: Your research protocol entitled, "Assessment of therapeutic potential of a novel dental probiotic in pediatric patients affected by gingivitis"

ETHICS APPROVAL

Original Approval Date: June 25, 2014  
Expiry Date: June 24, 2015  
Continuing Review Level: 2

We are writing to advise you that the Health Sciences Research Ethics Board (REB) has granted approval to the above-named research protocol, for a period of one year. Ongoing research under this protocol must be renewed prior to the expiry date.

Any changes to the approved protocol or consent materials must be reviewed and approved through the amendment process prior to its implementation. Any adverse or unanticipated events in the research should be reported to the Office of Research Ethics as soon as possible.

Please ensure that you submit an Annual Renewal Form or a Study Completion Report 15 to 30 days prior to the expiry date of your current ethics approval. Note that annual renewals for studies cannot be accepted more than 30 days prior to the date of expiry.

If your research is funded by a third party, please contact the assigned Research Funding Officer in Research Services to ensure that your funds are released.

Best wishes for the successful completion of your research.

Yours sincerely,

Elizabeth Peter, Ph.D.  
REB Chair

Daniel Gyewu  
REB Manager

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Appendix 2. University of Western Ontario Ethics Approval

Western University Health Science Research Ethics Board
HSREB Amendment Approval Notice

Principal Investigator: Dr. Peter Cadieux
Department & Institution: Schulich School of Medicine and Dentistry/Surgery, Western University

HSREB File Number: 101955
Study Title: Assessment of therapeutic potential of a novel dental probiotic in pediatric patients affected by gingivitis
Sponsor: Ontario Centres of Excellence Inc.

HSREB Amendment Approval Date: September 17, 2014
HSREB Expiry Date: June 30, 2015

Documents Approved and/or Received for Information:

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The Western University Health Science Research Ethics Board (HSREB) has reviewed and approved the amendment to the above named study, as of the HSREB Amendment Approval Date noted above.

HSREB approval for this study remains valid until the HSREB Expiry Date noted above, conditional to timely submission and acceptance of HSREB Continuing Ethics Review. If an Updated Approval Notice is required prior to the HSREB Expiry Date, the Principal Investigator is responsible for completing and submitting an HSREB Updated Approval Form in a timely fashion.

The Western University HSREB operates in compliance with the Tri-Council Policy Statement Ethical Conduct for Research Involving Humans (TCPS2), the International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use Guideline for Good Clinical Practice Practices (ICH E6 R1), the Ontario Personal Health Information Protection Act (PHIPA, 2004), Part 4 of the Natural Health Product Regulations, Health Canada Medical Device Regulations and Part C, Division 5, of the Food and Drug Regulations of Health Canada.

Members of the HSREB who are named as Investigators in research studies do not participate in discussions related to, nor vote on such studies when they are presented to the REB.

The HSREB is registered with the U.S. Department of Health & Human Services under the IRB registration number IRB 00000940.

Ethics Officer, on behalf of Dr. Joseph Gilbert, HSREB Chair

This is an official document. Please retain the original in your files.
# Appendix 3. Health Canada Approval

## NOTICE OF AUTHORIZATION (NOA)

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Dear Ms. Ioudina:

Please find attached, your Notice of Authorization for the above-identified clinical trial amendment.

Please also be reminded of your requirement to file a commencement notice at least 15 days prior to the start of the trial.

We wish you all the best in your clinical study.

Sincerely,

Dana Wang
Email: nhpd-cta.deo-dpsm@hc-sc.gc.ca
Submission Coordinator, Clinical Trial Unit
Bureau of Clinical Trials and Health Sciences
February 25, 2013

Ms. Natalya Ioudina (or c/o Dr. Peter Cadieux)
Integra Medical Inc.
Stiller Centre for Technology Commercialization, 700 Collip Circle, Suite 120
London, Ontario
N6G 4X8

Dear Ms. Ioudina,

Re: CLINICAL TRIAL APPLICATION for Lorodent Probiotic Complex
(LG-001) Natural Health Products Regulations Section: 67

The Natural Health Products Directorate, is pleased to inform you that the information and material provided to support the above Clinical Trial Application, have been assessed and we have no objection to your proposed study. Please consider this as your notice of authorization to sell or import this natural health product for the purposes of this clinical trial in Canada.

Please note that you are responsible for ensuring the appropriate considerations are taken into account in order to comply with the requirements set out in the Natural Health Products Regulations (NHPR) and its associated guidance documents. For more information on the expectations and approaches relating to quality requirements and Good Manufacturing Practices for natural health products, please consult the Quality of Natural Health Products Guide and the Good Manufacturing Practices guidance document (http://www.hc-sc.gc.ca/dhp-mps/prodrrhau-legalaut/index-eng.php).

I would remind you of the necessity of complying with the NHPR, Part 4, in the sale of this product for clinical testing. In addition, the Regulations (Part 4) impose responsibilities, including commencement notice, record keeping and reaction reporting, on those conducting clinical trials. Please ensure that all systems are compliant in order to meet these responsibilities.

Please note that as of November 27, 2012, all serious adverse reactions and or serious unexpected adverse reactions need to be reported to the Biologics and Genetic Therapies Directorate (BGTD). Please fax your report(s) to the following number: 613-946-9520.

You are also reminded that all clinical trials should be conducted in compliance with the Health Canada Guidance for Industry: Good Clinical Practice: Consolidated Guideline ICH Topic E6.

Should you have any questions concerning this letter, please contact the submission coordinator at nhps-cet-dec-dpmn@hc-sc.gc.ca.

Yours sincerely,

[Signature]

Sara O'Connor
A Director, Bureau of Product Review and Assessment
Natural Health Products Directorate
2936 Baseline Rd. (A.L. 3302C), Ottawa, ON K1A 0K9
HEALTH CANADA  
NATURAL AND NON-PRESCRIPTION HEALTH PRODUCTS DIRECTORATE

SANTÉ CANADA  
DIRECTION DES PRODUITS DE SANTÉ NATURELS ET SANS ORDOINNANCE

ACKNOWLEDGEMENT OF NOTIFICATION

<table>
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<tr>
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<tbody>
<tr>
<td>Natalya Ioudina</td>
<td>Nalini Balram</td>
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Dear Ms. Ioudina,

We received on July 21, 2014, your email dated July 21, 2014, and information regarding the clinical trial for Lorodent Probiotic Complex File # 185428, Submission # 204059.

RE: Updated CTSI form, QIU form and REB approval.

Thank you. This information will be added to your file.

Best regards,

Nalini Balram  
Senior Regulatory Affairs Officer  
Natural and Non-prescription Health Products Directorate (NNHPD)  
Email <nhpd-cta.dec-dpsn@hc-sc.gc.ca>

The information in this fax is confidential. If you are not the named recipient, or have otherwise received this communication in error, please notify the sender immediately and destroy the communication. Its content should not be disclosed to any other person, be used for any purpose, stored or copied in any medium.
This Agreement is made between Integra Medical Inc. ("Client"), University of Western Ontario and Governing Council of the University of Toronto ("Research Partner"), (Client and Research Partner collectively referred to as the "Participants"), and Ontario Centres of Excellence Inc. ("OCE"), each herein individually referred to as a "Party" and collectively the "Parties".

WHEREAS the parties wish to engage in the project entitled: "Assessment of therapeutic potential of a novel dental probiotic in pediatric patients affected by gingivitis" as set out in the Application attached hereto as Schedule "C" and represent and warrant that they have the unencumbered rights to use the "background" Intellectual Property (IP) required for use in the project, and commercialize any "foreground" IP or have entered into separate agreement(s) with respect to the intellectual property rights relating to the Project;

THEREFORE, recognizing the foregoing recitals and in consideration of the mutual promises set forth in this agreement, the Parties agree as follows:

1. **Definitions.** Capitalized terms used and not otherwise defined herein shall have the meanings attributed thereto in Schedule "A".
2. **General Terms.** The general terms that apply to and form part of this Agreement are attached as Schedule "B".
3. **Schedules.** The following schedules are attached to and form a part of this Agreement:
   - Schedule "A" Definitions
   - Schedule "B" General Terms
   - Schedule "C" Application (including budget)
   - Schedule "D" Intellectual Property Agreement/Term Sheet
4. **Project.** The Project shall be performed in accordance with this Agreement, including the Schedules attached hereto.
5. **Term.** The term of this Agreement (the "Term") shall commence on 2013-12-16 (the "Start Date") and end on 2014-08-31 (the "End Date").
6. **OCE Right to Terminate.** All obligations of OCE hereunder may be immediately suspended, terminated or revoked, in whole or in part, at any time by OCE giving written notice to the other Parties, where OCE determines, in its sole and unfettered discretion, that:
   a. the Project will likely not be completed on schedule or on budget;
   b. interim results are unsatisfactory and demonstrate low likelihood of achieving anticipated outcomes, or one or more Milestones cannot be met or has not been met within the timeframe set out in the Application;
   c. the conclusion reached by OCE through a Project review process organized by OCE is that the overall goals of the Project will likely not be met, or
   d. a Participant has defaulted on its obligation to make any Contribution at the time and in the manner required under this Agreement.
7. **Contributions and Eligible Expenses.** OCE and the Participant(s) shall make the Contributions toward the cost of the Project as set out in the Application and Budget (Schedule "C"). Notwithstanding anything else in this Agreement, the Parties acknowledge and agree that all Contributions to be made by OCE, and OCE's obligations to pay such
Contributions, are entirely conditional on OCE receiving sufficient allocated government funding to enable it to make payment thereof, and that OCE may terminate, suspend or revoke such obligations, in whole or in part, at any time by giving written notice to the other Parties should it not receive or possess funds sufficient for such purposes. The Participant(s) shall use Contributions only in accordance with the Application and Budget for reimbursement of eligible Project expenses in accordance with OCE’s then current published program expense guidelines.

8. Ethical Investments. The Client shall not, directly or indirectly, through a subsidiary or otherwise, engage in:
   a. the sale, marketing or provision of gambling, gambling services or pornography;
   b. the production, sale or marketing of tobacco smoking products;
   c. the manufacture, sale, distribution or promotion of goods or services that are not legal in the Province of Ontario.

For greater certainty, the Client shall not be considered to be directly or indirectly engaged in the foregoing merely as a result of selling products to persons engaged in such activities, provided (i) that such products are not principally related to gambling, gambling services, pornography, tobacco smoking products or goods or services that are not legal in the Province of Ontario and (ii) the Client does not have a material interest in such persons.

9. Reviews and Reporting.
   a. projections, as required by OCE, in such form and content and at such times as specified by OCE in writing from time to time including, without limitation, a final report after Project completion, annual surveys for a period of 5 years following the term of this Agreement, and any other follow-up reporting reasonably required by OCE following the Term of this Agreement.
   b. The Participant(s) agree to cooperate with OCE in the collection of performance metrics relevant to the Project, which shall be used by OCE to evaluate the success of its programs and shall be reported to the Government of Ontario in aggregate, omitting any Confidential Information.

10. Indemnity. Each Party will severally indemnify and save harmless all other Parties including their respective officers, directors, employees, agents and students from and against any and all suits, claims, demands, costs, damages, expenses, losses or injuries (including death) to persons or property, caused by: (A) any default or breach by the indemnifying Party of any of its obligations under this Agreement; and (B) the wilful or negligent act or omission of the indemnifying Party or its officers, directors, employees and agents during the performance or arising out of this Agreement or the Project.

11. Limitation of Liability. No Party shall be liable to the other Parties for loss of business or profit or for any special, indirect, punitive or consequential loss or damage, regardless of whether such loss or damage arises under contract, tort, or based upon strict liability or other theory of law or equity, where such loss or damage arose in connection with the Project. In no case shall the liability of OCE to the other Parties exceed the amount of Contribution theretofore contributed and paid by OCE with respect to the Project. Except as expressly provided herein, OCE or Research Partner, including their respective fellows, directors, trustees, officers, employees and agents, make no representations, warranties, undertakings, promises, inducements or agreements of any kind, whether direct, indirect, express or implied, including, without limitation, the merchantability or fitness for a particular purpose of any research results or intellectual property; and except as expressly provided herein, OCE or Research Partner assume no responsibility whatsoever with respect to design, development, manufacture, use, sale or other disposition of research results or intellectual property by any Client. Provided the foregoing limitations on liability shall not apply to
breach of the confidentiality obligations provided for in Schedule "B".

12. **Intellectual Property (IP).** The Participant(s) represent and warrant that they have the unencumbered rights to use the "background" IP required for use in the project, and commercialize any "foreground" IP or have entered into and are bound by one or more separate agreements governing intellectual property matters relating to or arising from the Project and which shall remain in place during the term of this Agreement, the terms of such agreement(s) to be noted in Schedule "D" – Intellectual Property Term Sheet.
IN WITNESS WHEREOF the Parties have duly executed this Agreement as of the 13 day of February 2014

ONTARIO CENTRES OF EXCELLENCE INC.

[Signature]

Name
Title
I have authority to bind the Corporation

University of Western Ontario

[Signature]

Name Dan Sinal
Title Associate Vice-President, Research
I have authority to bind the Corporation

Integra Medical Inc.

[Signature]

Name NATALYA JOUDINA
Title PRESIDENT/CEO
I have authority to bind the Corporation

Governor Council of the University of Toronto

[Signature]

Print Name Lina DeFontis
Title Director, Partnerships
Innovations & Partnerships Office
I have authority to bind the Corporation

www.oca-ontario.org
SCHEDULE "C"
APPLICATION (including Budget)

Applicant Information

**Application Information**

**Applicant:** Peter Cadieux - University of Western Ontario

**Project Title:** Assessment of therapeutic potential of a novel dental probiotic in pediatric patients affected by gingivitis

**Business Development:** Jessie Maggard

**OCE Managing Region:** South Western Ontario

**Application Type:** Voucher for Innovation and Productivity (VIP)

**Program Partner(s):** Connect Canada,

**Referring Agency**

**Project Finance**

**Source of Funds**

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**Leverage - Cash Only (OCE and Industry):** .65

**Leverage - Cash+In-Kind (OCE and Industry):** 1.79

**Project Contacts**

**Applicant**
Peter Cadieux
University of Western Ontario
(519)646-6000
pcadieux@uwo.ca

**Co-Applicant**
Dr. Siew-Ging Gong
University of Toronto

**Project Partner(s)**
1. Integra Medical Inc. Ms. Natalya loudina nioudina@integra-medical.com

**Summary of Proposal for Public release**

Ontario Centres of Excellence >> www.oce-ontario.org
ASSessment of Therapeutic Potential of a Novel Dental Probiotic in Pediatric Patients Affected by Gingivitis

Patient Information & Consent Form

The pronouns ‘you’ and ‘your’ should be read as referring to the participant rather than the parent/guardian who is signing the consent form for the participant.

Purpose of Study
The purpose of the study is to determine whether a probiotic complex when administered to the mouth can reduce plaque, inflammation and other clinical and microbiological parameters (change in pathogens that are naturally present in oral cavity) in a population of orthodontic patients from 11 to 17 years of age. Probiotics are live microorganisms which provide health benefits when administered in adequate amounts. This study will use a mixture of probiotics that are expected to have a number of health benefits. The study will be administered by two investigators – Dr. Fatima Ebrahim and Dr. Sarah Habib – who are dentists and graduate orthodontic residents at the Department of Orthodontics, Faculty of Dentistry at the University of Toronto.

Procedures
You have been asked to participate because you are an orthodontic patient who has been diagnosed with gingivitis and are between the ages of 11-17 years. This research study will be run involving only those who choose to take part. This patient information and consent form describes the study so you can make an informed decision on participating. Please take time to make a decision and if necessary, discuss this proposal with your doctor, family members and friends, as you feel inclined. Please feel free to ask questions if anything is unclear or there are words or phrases you do not understand.

This study has two treatment groups – a probiotic group and a placebo group. The study has a double-blind design to eliminate any potential bias; that is, it is hidden from both the participants and the investigators which treatment group each participant belongs to. The sample size is a total of up to 60 randomized participants; that is, up to 30 in each treatment group (probiotic or placebo). Participants must meet the inclusion criteria and be deemed eligible by the investigators in order to participate in the study. The treatment that each participant gets will be determined by chance (ie. like the flip of a coin) to the priobiotic or placebo group in a 1:1 ratio. That is, each participant will be randomly allocated to either the probiotic or placebo treatment group.

If you agree to participate, you will be provided with either the probiotic complex or placebo (contains no active medication) and instructions on how to take it daily. We ask that you take a
probiotic lozenge for 30 days as follows: **Slowly dissolve on the tongue two lozenges twice a day for the first 7 days. That is, for the first 7 days take two lozenges in the morning and two lozenges in the evening. Then for the next 21 days, take two lozenges once a day in the morning. If you forget, please take the lozenges as soon as you remember.** You will be asked to otherwise continue your normal healthy oral care including brushing twice per day and flossing once per day. Mouth rinse is not considered the standard of care for oral hygiene and is optional. Therefore, please **do not use any mouth rinse during the study** as it may interfere with the study outcomes and if so, you will be excluded from the study.

You will be given a pillbox to help keep track of the lozenges and a calendar to fill out how many lozenges were taken each day.

At each visit, the following measures of dental health will be taken and biological samples will be collected by one of the two investigators:

- Plaque score
- Gingival health score
- Plaque samples
- Saliva sample

At the conclusion of the study, you will be asked to fill in a questionnaire, possibly with the assistance of a parent or guardian.

**Time Requirements**

The appointments will happen during your regularly scheduled monthly orthodontic visits (*except for one). There will be four appointments in total:

- Visit 1: Day 1
- Visit 2: Day 14* (extra appointment)
- Visit 3: Day 28
- Visit 4: Day 56

The dental exam and collection of biological samples should take approximately 15 minutes.

**Number of Participants**

This study will require up to 60 participants, up to 30 in the probiotic treatment group and up to 30 in placebo group.

**Participant Eligibility**

We are seeking participants undergoing orthodontic treatment (braces) at the Undergraduate or Graduate Orthodontics Clinics at the Faculty of Dentistry, University of Toronto.

Participants will be included who:

- Are male or female between the ages of 11 to 17 years
- Have mild to moderate gingivitis
- Are undergoing fixed orthodontic therapy on both arches with attachments on at least 20 teeth including bonded 1st molars for a minimum of 5 months
- Have fully erupted teeth #16, #21, #23, #36, #41, and #43
- Are caries inactive prior to study initiation
probiotic use should be discontinued and the researchers informed. You will be observed closely at each visit for any ill effect on your oral health including dental caries. If you develop any severe medical condition, become pregnant, please discontinue taking the probiotic and inform the researchers of your withdrawal and condition.

**Benefits**
Participants in the treatment group may experience reduction in plaque, inflammation, and/or decreases in oral pathogenic bacterial levels which may, in turn, lower the risk of gum disease, formation of white spot lesions, staining and cavities.

**Right to Refuse**
Your participation in this study is voluntary. You may refuse to participate, refuse to answer any questions or you may withdraw from the study at any time with no effect on your future healthcare. The study investigators may also withdraw you from the study if you do not follow the instructions you receive, if the investigators feel it is in your best interests to be withdrawn, if the study is discontinued, or for administrative reasons. You may be withdrawn without your consent, but the investigators will explain to you why. Samples and information collected before the date of withdrawal will not be excluded from the study. You can refuse for the samples and information to be included in the study up to and until the point where the data is analyzed. You do not waive any of your legal rights by signing the consent form.

**Compensation for Participation**
You will be given a total compensation of $75 for participating in the study. A $25 gift card to the mall will be provided at the additional appointment at day 14 (visit 2), and a $50 gift card to the mall will be provided upon study completion at the regularly scheduled orthodontic appointment at day 56 (visit 4).

**Alternatives to Study Participation**
If you do not wish to participate in the study, you will continue to receive standard orthodontic care from your orthodontic resident. Choosing not to participate in this study will not affect your regular orthodontic treatment in any way. That is, you will still be seen by your orthodontic resident to have your braces adjusted at your regularly scheduled orthodontic appointments and they will continue to monitor your oral hygiene.

**Participation in Concurrent or Future Studies**
While the likelihood of this study interfering with other studies is minimal, please inform Dr. Ebrahim or Dr. Habib immediately to determine if it is appropriate for you to continue participation in this study if you are involved in another study or plan to be involved in another study.

**Use of Data**
The saliva and plaque samples will become the property of the researchers and once you have provided them you will not have further access to them. They will be used by Dr. Ebrahim and Dr. Habib for research purposes. Specimens will be retained for microbiological analysis. Data will be kept for 7 years in a secured office at the Department of Orthodontics, Faculty of Dentistry, University of Toronto and will then be securely destroyed.
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Consent

I have been given enough time and opportunity to read and understand the information in this patient information and informed consent document and ample time and opportunity to ask questions. All my questions have been answered to my satisfaction. I have had sufficient time to consider whether to participate in the study "Assessment of therapeutic potential of a novel dental probiotic in pediatric patients affected by gingivitis". I understand that my participation in this study is entirely voluntary and that I may withdraw from the study at any time without penalty.

The study orthodontist/dentist has my permission to tell my regular doctor about my being in this study:

YES    NO

I voluntarily consent to participate in this study and will be given a signed copy of this form to take home with me.

Participant Name (Print Name): __________________________________________________________

Legal Guardian Name (Print Name): ______________________________________________________

Legal Guardian Signature: ______________________________ Date: ________________

Person Obtaining Informed Consent (Print Name): __________________________________________

Signature: _______________________________ Date: ________________

GRADUATE ORTHODONTICS
Faculty of Dentistry, 124 Edward Street, Toronto, ON M5G 1G6 Canada
Tel: +1 416 979 4912 ext. 2 • Fax: +1 416 979-4755
PATIENT CONSENT FORM:
FOR COLLECTION, USE AND DISCLOSURE
OF PERSONAL INFORMATION

Privacy of your personal information is an important part of our Faculty providing you with quality dental care. We understand the importance of protecting your personal information. We are committed to collecting, using and disclosing your personal information responsibly. We also try to be as open and transparent as possible about the way we handle your personal information. It is important to us to provide this service to our patients.

In this office, Dr. Luciano Valenzano, Assistant Dean, Clinics acts as the Privacy Information Officer.

All staff members who come in contact with your personal information are aware of the sensitive nature of the information that you have disclosed to us. They are all trained in the appropriate uses and protection of your information.

Attached to this consent form, we have outlined what our office is doing to ensure that:
• only necessary information is collected about you;
• we only share your information with your consent;
• storage, retention and destruction of your personal information complies with existing legislation, and privacy protection protocols;
• our privacy protocols comply with privacy legislation, standards of our regulatory body, the Royal College of Dental Surgeons of Ontario, and the law.

Do not hesitate to discuss our policies with me or any member of our staff.

Please be assured that every staff person in our office is committed to ensuring that you receive the best quality dental care.

How Our Office Collects, Uses and Discloses Patients’
Personal Information

Our office understands the importance of protecting your personal information and will at all times protect it in accordance with applicable privacy legislation. To help you understand how we are doing that, we have outlined here how our office is using and disclosing your information.

This Faculty will collect, use and disclose information about you for the following purposes:
• to deliver safe and efficient patient care
• to identify and to ensure continuous high quality service
• to assess your health needs
• to provide health care
• to advise you of treatment options
• to enable us to contact you
• to establish and maintain communication with you
• to offer and provide treatment, care and services in relationship to the oral and maxillofacial complex and dental care generally
• to communicate with other treating health-care providers, including specialists and general dentists who are the referring dentists and/or peripheral dentists
• to allow us to maintain communication and contact with you to distribute health-care information and to book and confirm appointments
• to allow us to efficiently follow-up for treatment, care and billing
• for teaching and demonstrating purposes on an anonymous basis
• for research and publication purposes on an anonymous basis
• to complete and submit dental claims for third party adjudication and payment

124 Edward Street    Toronto Ontario    M5G 1G6    FAX (416) 979-4936
Appendix 6. Faculty of Dentistry, University of Toronto Privacy & Treatment Consent Forms

- to comply with legal and regulatory requirements, including the delivery of patients’ charts and records to the Royal College of Dental Surgeons of Ontario in a timely fashion, when required, according to the provisions of the Regulated Health Professions Act
- to comply with agreements/undertakings entered into voluntarily by the member with the Royal College of Dental Surgeons of Ontario, including the delivery and/or review of patients’ charts and records to the College in a timely fashion for regulatory and monitoring purposes
- to deliver your charts and records to the dentist’s insurance carrier to enable the insurance company to assess liability and quantify damages, if any
- to prepare materials for the Health Professions Appeal and Review Board (HPARB)
- to invoice for goods and services
- to process credit or debit card payments
- to collect unpaid accounts
- to assist this office to comply with all regulatory requirements
- to comply generally with the law

The Faculty of Dentistry, University of Toronto, and its students and residents may use anonymous patient treatment records and other patient clinic information, including, for example, diagnostic information, x-rays and photos of treatment outcomes for academic and accreditation purposes such as teaching, publication and examinations, including those undertaken after graduation and/or outside the University of Toronto. Photos of treatment outcomes may show the patient’s face.

By signing the consent section of this Patient Consent Form, you have agreed that you have given your informed consent to the collection, use and/or disclosure of your personal information for the purposes that are listed. If a new purpose arises for the use and/or disclosure of your personal information, we will seek your approval in advance.

Your information may be accessed by regulatory authorities under the terms of the Regulated Health Professions Act (RHPA) for the purposes of the Royal College of Dental Surgeons of Ontario fulfilling its mandate under the RHPA, and for the defence of a legal issue.

Our Faculty will not under any conditions supply your insurer with your confidential medical history. In the event this kind of a request is made, we will forward the information directly to you for review, and for your specific consent.

When unusual requests are received, we will contact you for permission to release such information. We may also advise you if such a release is inappropriate.

You may withdraw your consent for use or disclosure of your personal information, and we will explain the ramifications of that decision, and the process.

**Patient Consent**

I have reviewed the above information that explains how your Faculty will use my personal information, and the steps your Faculty is taking to protect my information.

I know that your Faculty has a Privacy Code, and I can ask to see the Code at any time.

I agree that the Faculty of Dentistry, University of Toronto can collect, use and disclose personal information about ________________________ as set out above in the information about the office’s privacy policies.

______________________________
Signature of Patient or Parent/Guardian

______________________________
Print Name

______________________________
Date

______________________________
Signature of Witness
Appendix 6. Faculty of Dentistry, University of Toronto Privacy & Treatment Consent Forms

PRIVACY CODE FOR THE FACULTY OF DENTISTRY, UNIVERSITY OF TORONTO

INTRODUCTION

Privacy of personal information is an important principle in the provision of quality dental care to our patients. We understand the importance of protecting your personal information. We are committed to collecting, using and disclosing your personal information responsibly. We also try to be as open and transparent as possible about the way we handle your personal information.

We have tried to make our office Privacy Code as easy to understand as possible. To ensure that you see how we are complying with the federal privacy legislation, the Personal Information and Protection and Electronic Documents Act (PIPEDA), our Privacy Code is organized to follow the Act’s ten interrelated principles that are the foundation of PIPEDA.

DEFINITIONS

Collection – The act of gathering, acquiring or obtaining personal information from any source, including third party sources by any means

College – Royal College of Dental Surgeons of Ontario

Consent – A voluntary agreement with what is being done or is being proposed to be done. Consent can either be express or implied. Express consent may be given explicitly, either orally or in writing.

Disclosure – Making personal information available to others besides the dentist or the dental team.

Legislation – The Regulated Health Professions Act (RHPA), Schedules attached, Dentistry Act, Regulations made under these Acts, and By-laws of the College, and the Personal Information Protection and Electronic Documents Act (PIPEDA)

Member – A member of the Royal College of Dental Surgeons of Ontario and this includes a health profession corporation

Faculty – The Faculty of Dentistry and when referencing access to information, to the Privacy Information Officer, and the Faculty of Dentistry

Patient – An individual about whom the dentist collects personal information in order to carry out diagnosis, treatment, including controlled acts

Personal Information – Information about a patient that is recorded in any form, and this includes: the patient’s name, address, telephone number, social insurance number, fax number, e-mail address, gender, marital status, children, date of birth, occupation, medical records, health records, insurance company, insurance coverage, history, occupation, place of work, employer

RHPA Procedural Code - The Health Professions Procedural Code, Schedule 2 to the Regulated Health Professions Act (RHPA) PIPEDA PRINCIPLES

Principle 1: Accountability

Any dentist in this Faculty is responsible for information collected by him/her, or under his/her direction, and under his/her control.

Accountability for this Faculty’s compliance rests with the designated individual or individuals, even though others in the Faculty may be responsible for the day-to-day collection and processing of personal information.

The identity of the individual designated by the Faculty to oversee the compliance, the Privacy Information Officer, will be made known upon request.

This Faculty is responsible for information in our possession or custody, including information that has been transferred to a third party for processing. We will use contractual or other means to provide a comparable level of protection while the information is being accessed and/or processed by that third party.

Our Faculty will implement policies and practices to give effect to the principles, including:

- implementing policies to protect personal information;
- establishing procedures to receive and respond to complaints and inquiries;
- training staff about privacy policies and practices;
- developing information to explain privacy policies and procedures.

Principle 2: Identifying Purposes for Collecting Information

The purposes for which personal information is collected in this Faculty will be identified before or at the time the information is collected.

This Faculty collects personal information for the following purposes:

- to deliver safe and efficient patient care
- to identify and to ensure continuous high quality service
- to assess your health needs
- to provide health care
- to advise you of treatment options
- to enable us to contact you
to establish and maintain communication with you
• to offer and provide treatment, care and services in
relationship to the oral and maxillofacial complex and
dental care generally
• to communicate with other treating health-care
providers, including specialists and general dentists
who are the referring dentists and/or peripheral
dentists
• to allow us to maintain communication and contact
with you to distribute health-care information and to
book and confirm appointments
• to allow us to efficiently follow-up for treatment, care
and billing
• for teaching and demonstrating purposes on an
anonymous basis
• for research and publication purposes on an
anonymous basis to complete and submit dental
claims for third party adjudication and payment
• to comply with legal and regulatory requirements,
including the delivery of patients' charts and records to
the College in a timely fashion, when required,
according to the provisions of the Regulated Health
Professions Act
• to comply with agreements/ undertakings entered into
voluntarily by the member with the Royal College of
Dental Surgeons of Ontario, including the delivery
and/or review of patients' charts and records to the
College in a timely fashion for regulatory and
monitoring purposes
• to deliver your charts and records to
the dentist's insurance carrier to enable
the insurance company to assess liability and quantify
damages, if any
• to prepare materials for the Health Professions Appeal
and Review Board (HPARB)
• to invoice for goods and services
• to process credit and debit card payments
• to collect unpaid accounts
• to assist this office to comply with all
regulatory requirements
• to comply generally with the law

This Faculty will identify the purposes for which personal
information is collected, at or before the time of collection.
We will only collect that information necessary for the
identified purposes.

When personal information has been collected and is to be
used or disclosed for a purpose not previously identified,
the new purpose will be identified prior to its use or the
disclosure. Your consent is required before the information
can be used or disclosed for that purpose.

Faculty staff collecting personal information will be able to
explain to you the purpose for which the information is
being collected.

When you sign the Patient Consent Form, you will be
deemed to understand it and accept this office’s collection,
use and disclosure of your information for the specified
purposes.

**Principle 3: Consent**
This Faculty will seek informed consent for the collection,
use and/or disclosure of personal information, except
where it might be inappropriate to obtain your consent,
and subject to some exceptions set out in law.

Consent is required for the collection of personal
information and subsequent use or disclosure of that
information.

In order for the principles of consent to be satisfied, our
office has undertaken reasonable efforts to ensure that
you are advised of the purposes for which information is
being used, and that you understand those purposes.
Once consent is obtained, we do not need to seek your
consent again, unless the use, purpose or disclosure
changes.

Existing protocols for electronic submissions of dental
claims require a signature on file. Specific consent may be
required for additional requests from insurers. This shall
be collected at the time, or in conjunction, with
generations for extensive services, providing the
scope of information released is disclosed. If there is any
doubt, information shall be released directly to you for
review and submission.

Consent for the collection, use and disclosure of personal
information may be given in a number of ways, such as:
• signed medical history form;
• signed introductory questionnaire;
• taken verbally over the telephone and then charted;
• e-mail;
• written correspondence.

You may withdraw consent upon reasonable notice.

**Principle 4: Limiting Collection of Personal
Information**
The collection of personal information by our office shall
be limited to that which is necessary for the purposes
identified in this Privacy Code.

**Principle 5: Limiting Use, Disclosure
and Retention**
Personal information shall not be used or disclosed for
purposes other than those for which the information is
collected, except with your express consent, or as
required by law.

Our Faculty has protocols in place for the retention of
personal information.

Retention of information records is defined and referenced
in College's Guidelines on Dental Recordkeeping.

In destroying personal information, our Faculty has
developed guidelines to ensure the secure destruction in
accordance with the College’s Guidelines on Dental
Recordkeeping.
Principle 6: Accuracy of Personal Information
This Faculty endeavours to ensure that your personal information is as accurate, complete, and as up-to-date as necessary for the purposes that it is to be used.

The extent to which your personal information shall be accurate, complete and up-to-date will depend upon the use of the information, taking into account the interest of our patients.

Information shall be sufficiently accurate, complete and up-to-date to minimize the possibility that inappropriate information is used to make a decision about you as our patient.

Principle 7: Safeguards for Personal Information
Our Faculty has taken appropriate measures to safeguard your personal information from unauthorized access, disclosure, use or tampering.

Safeguards are in place to protect your personal information against loss or theft, as well as unauthorized access, disclosure, copying, use or modification.

Your information is protected, whether recorded on paper or electronically.

Our staff and students are aware of the importance of maintaining the confidentiality of personal information.

Care is used in the care and destruction of personal information to prevent unauthorized access to the information even during disposal and destruction.

Principle 8: Openness about Privacy
Our Faculty will make readily available to you specific information about our Faculty policies and practices relating to the management of personal information.

This information includes:
• a Patient Information Sheet that outlines the name of the Privacy Information Officer who is accountable for our Faculty privacy policies. This is the person to whom you can direct any questions or complaints. The Information Sheet also describes how to access your personal information held in this office;
• a copy of our Patient Consent Form that explains how this Faculty collects, uses and discloses your personal information;
• our office Privacy Code

Principle 9: Patient Access to Personal Information
Upon written request and with reasonable notice, you shall be informed of the existence, use and disclosure of your personal information, and shall be given access to that information.

Upon written request and with reasonable notice, our Faculty will advise you whether or not we hold personal information about you.

Our Faculty shall allow you access to this information. Upon written request and with reasonable notice, our Faculty shall provide you with an accounting of how your personal information has been used, including third party disclosures. In providing this information, we will attempt to be as specific as possible.

When it is not possible to provide a list of the organizations or individuals to which there has been disclosure about you, we will provide you with a list of such organizations or individuals to which we may have disclosed information about you. Disclosure of probabilities in these cases would satisfy this requirement.

We will respond to your request within a reasonable period of time, and at minimal or no cost to you. The request for information will be provided or made available in a form that is generally understandable.

The dentist will comply with the regulations of his/her College that define patient access to records.

You are free to challenge the accuracy and completeness of the information and seek to have it altered, amended, or changed. This process is explained in the Patient Information Sheet.

When a challenge is not resolved to your satisfaction, we will record the substance of the unresolved challenge.

When appropriate, the existence of the unresolved challenge shall be transmitted to third parties having access to the information in question. This disclosure may be appropriate where a dentist has been challenged about a change to a service date or services rendered under consideration for insurance benefits.

Principle 10: Challenging Compliance
You shall be able to challenge compliance with these principles with the Faculty's Privacy Information Officer who is accountable within the dental office for the dentist's compliance. Our Faculty has in place procedures to receive and respond to your complaints or inquiries.

This information, including the name of our Faculty's Privacy Information Officer, is included in the Patient Information Sheet, available on request.

The procedures are easily accessible and simple to use.

Our Faculty has an obligation to inform our patients who make inquiries about how to access the privacy complaint process in our Faculty, and about how to access that process. This information is outlined in the Patient Information Sheet.

The Privacy Information Officer in our Faculty will investigate each and every complaint made to the office in writing.

If a complaint is found to be justified, the Privacy Information Officer will take appropriate measures, including, if necessary, amending any office policies and practices.

Patients will be provided with information about how to contact the Privacy Commissioner of Canada to forward any unresolved complaint. This information is included in the Patient Information Sheet, available on request.
CONSENT FOR TREATMENT

I hereby give consent to the Faculty of Dentistry, University of Toronto, to provide basic preliminary dental care, the need for and the cost of which will be explained to me before it is delivered. This may include teeth cleaning, specific investigations, preventive advice and the treatment of decayed or infected teeth. This may also include the taking of records, radiographs and photographs (which may be used for teaching and publication purposes and may not be left anonymous) and the administration of necessary anaesthetics and medications. I also understand that this treatment will be done by students only, as part of their learning process.

I hereby give consent for the Faculty of Dentistry, University of Toronto, and its students and residents to use patient treatment records and other patient clinic information, including, for example, diagnostic information, x-rays and photos of treatment outcomes for academic and accreditation purposes such as teaching, publication and examinations, including those undertaken after graduation and/or outside the University of Toronto. Photos of treatment outcomes may show the patient’s face.

I have also read and understand the Clinic Policies and Regulations printed on the previous page and agree to abide by them.

As to fees for these services, I agree to make payments as treatment progresses except for those procedures requiring laboratory services. For these services, I shall pay at least one-half the total fee before the treatment is begun and the balance before insertion of the restoration. I am also aware that there may have to be revisions in costs for treatment of long duration. These revisions will be discussed with me before the treatment is begun.

Signature of Patient: ___________________________ Date: ___________________________

(Parents or guardian must sign for dependents or patients under 18 years of age)

Signature of Witness: ___________________________ Date: ___________________________
Manufactured: Lorodent and placebo lozenges were produced by Nutraceutix Inc. (Redmond, Washington, USA) in August 2011.

Lorodent lozenge ingredients: Active Lorodent probiotic complex and lactitol, inulin, dicalcium phosphate, blueberry flavor (natural), dextrose, fructose, stearic acid, citric acid, vanilla flavor (natural), and stevia rebaudioside A (97%) as excipients. Lorodent lozenges can be stored at room temperature for 18 months.

Lorodent CFU: Probiotic strain stability was confirmed throughout the batch's shelf life using Integra Medical standard enumeration protocols. In July 2014, enumerations confirmed there were $1.6 \times 10^9$ total active CFUs per lozenge.

Placebo lozenge ingredients: Lactitol, inulin, dicalcium phosphate, blueberry flavor (natural), dextrose, fructose, stearic acid, citric acid, vanilla flavor (natural), and stevia rebaudioside A (97%).

Lozenge Identity: Following completion of the study, the same enumeration protocols were followed to confirm that Lozenge A was the Placebo, with Lozenge B being Lorodent.

Lorodent Enumeration Protocol

• Place 5 lozenges aseptically into a 50 mL conical tube and fill to 25 mL with PBS.
• Secure tube in an orbital shaker and leave shaking at 300 rpm and 37 °C for 2 hours.
• Pipette up and down with a 10 mL pipette several times and proceed to make serial dilutions in PBS (up to $10^{-7}$).
• Aseptically pipette 0.1 mL of dilution $10^{-5}$ on the surface of a CABK12 agar plate (Streptococcus salivarius) and a Rogosa agar plate (Lactobacillus strains). Use sterile glass dip spreader to evenly distribute the solution and continue spreading gently until solution dries on surface. Repeat twice for each to generate triplicates using new plates for each sample. Repeat with all other dilutions.
• Place plates upside down in anaerobic jar and set up CO$_2$ tube or packet. Seal jar and place in 37°C incubator for 48 hours.
• Calculate CFUs/lozenge by enumerating colonies, taking the average, dividing by the appropriate dilution factor, then multiplying by 10 (due to plating 100 µL) and then multiplying by 5 (since 1 lozenge is diluted in 5 mL). Note: Use the colony counts from plates with a dilution that yields colony numbers between 20 and 200.
Appendix 8. PowerSoil®-htp 96 Well Soil DNA Isolation Kit Protocol

POWERSOIL®-HTP 96 WELL SOIL DNA ISOLATION KIT - DETAILED PROTOCOL

- Remove the Square Well Mat from the PowerSoil®-htp Bead Plate and set aside.
- Add 200 µL of saliva or suspended plaque sample.
- Add 750 µL of PowerSoil®-htp Bead Solution.
- Add 60 µL of Solution C1. Secure the Square Well Mat tightly to the plate.
- Incubate the plate for 10 minutes in a 65°C bead bath.
- Place Bead Plate with mat securely fastened between 2 adapter plates and place on the 96 Well Plate Shaker.
- Shake at maximum speed for 20 minutes.
- Centrifuge at room temperature for 15 minutes at 3200 x g.
- Remove and discard the Square Well Mat. Transfer the supernatant to a clean 1 mL Collection Plate.
- Add 250 µL of Solution C2 and apply Sealing Tape to plate. Incubate at 4°C for 10 minutes. Centrifuge the plate at room temperature for 15 minutes at 3200 x g. Remove and discard Sealing Tape.
- Avoiding the pellet, transfer entire volume of supernatant to a new 1 mL Collection Plate.
- Apply Sealing Tape to plate. Centrifuge the plate again at room temperature for 15 minutes at 3200 x g. Transfer entire volume of supernatant to another new 1 mL Collection Plate.
- Add 200 µL of Solution C3 and apply Sealing Tape to plate. Incubate at 4°C for 10 minutes. Centrifuge at room temperature for 15 minutes at 3200 x g. Remove and discard Sealing Tape.
- Avoiding the pellet, transfer entire volume of supernatant to a new 1 mL Collection Plate.
- Apply Sealing Tape to plate. Centrifuge the plate again at room temperature for 15 minutes at 3200 x g.
- Transfer no more than 650 µL of supernatant to a 2 mL Collection Plate avoiding any residual pellet.
- Add 650 µL of Solution C4 to each well of the plate.
- Add a second 650 µL of Solution C4 to each well of the plate.
- Pipet samples “up and down” to mix.
- Place Spin Plate onto a new 0.5 mL Collection Plate.
- Load approximately 650 µL into each well of the Spin Plate and apply Centrifuge Tape.
- Centrifuge at room temperature for 6 minutes at 3200 x g. Discard the flow through and place the Spin Plate back on the same 0.5 mL Collection Plate. Discard the Centrifuge Tape.
• Repeat until all the supernatant has been processed. Discard the final flow through.
• Place the Spin Plate back on the same 0.5 mL Collection Plate.
• Confirm that ethanol has been added to Solution C5-D. Add 500 µL of Solution C5-D to each well of the Spin Plate. Apply Centrifuge Tape to the Spin Plate.
• Centrifuge at room temperature for 6 minutes at 3200 x g. Discard the flow through and place the same 0.5 mL Collection Plate beneath the Spin Plate.
• Centrifuge again at room temperature for 10 minutes at 3200 x g. Discard the flow through.
• Carefully place the Spin Plate onto a Microplate. Remove Centrifuge Tape and discard.
• Allow to air dry for 10 minutes at room temperature.
• Add 100 µL of Solution C6 to the center of each well of the Spin Plate. Apply Centrifuge Tape.
• Centrifuge at room temperature for 6 minutes at 3200 x g. Remove Centrifuge Tape and discard.
• Cover wells of Microplate with the Elution Sealing Mat provided. DNA is now ready for any downstream application. No further steps are required.
### End of Study Questionnaire

1. How satisfied were you with the taste of the lozenges in the study?

<table>
<thead>
<tr>
<th>Option</th>
<th>Scale</th>
</tr>
</thead>
<tbody>
<tr>
<td>Very satisfied</td>
<td>Satisfied</td>
</tr>
<tr>
<td>Satisfied</td>
<td>Neutral</td>
</tr>
<tr>
<td>Dissatisfied</td>
<td>Very dissatisfied</td>
</tr>
</tbody>
</table>

2. During the 28 day administration period of the study, how successful were you taking 2 lozenges per day?

<table>
<thead>
<tr>
<th>Option</th>
<th>Scale</th>
</tr>
</thead>
<tbody>
<tr>
<td>Completely Successful (rarely missed taking a lozenge)</td>
<td>Mostly Successful (missed taking a few to several lozenges each week)</td>
</tr>
<tr>
<td>Mostly Successful (missed taking about half of my lozenges each week)</td>
<td>Somewhat Successful (missed taking most of my lozenges)</td>
</tr>
<tr>
<td>Completely Unsuccessful (stopped taking the lozenges)</td>
<td></td>
</tr>
</tbody>
</table>

3. It was difficult to take 2 lozenges per day.

<table>
<thead>
<tr>
<th>Option</th>
<th>Scale</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strongly agree</td>
<td>Agree</td>
</tr>
<tr>
<td>Agree</td>
<td>Neither</td>
</tr>
<tr>
<td>Disagree</td>
<td>Strongly disagree</td>
</tr>
</tbody>
</table>

4. Remembering to take the lozenges every day was difficult.

<table>
<thead>
<tr>
<th>Option</th>
<th>Scale</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strongly agree</td>
<td>Agree</td>
</tr>
<tr>
<td>Agree</td>
<td>Neither</td>
</tr>
<tr>
<td>Disagree</td>
<td>Strongly disagree</td>
</tr>
</tbody>
</table>

5. The length of the study (2 months) was too long.

<table>
<thead>
<tr>
<th>Option</th>
<th>Scale</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strongly agree</td>
<td>Agree</td>
</tr>
<tr>
<td>Agree</td>
<td>Neither</td>
</tr>
<tr>
<td>Disagree</td>
<td>Strongly disagree</td>
</tr>
</tbody>
</table>

6. I did not feel the need to prevent white spots, decay or gum disease.

<table>
<thead>
<tr>
<th>Option</th>
<th>Scale</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strongly agree</td>
<td>Agree</td>
</tr>
<tr>
<td>Agree</td>
<td>Neither</td>
</tr>
<tr>
<td>Disagree</td>
<td>Strongly disagree</td>
</tr>
</tbody>
</table>

7. I lost interest in the study.

<table>
<thead>
<tr>
<th>Option</th>
<th>Scale</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strongly agree</td>
<td>Agree</td>
</tr>
<tr>
<td>Agree</td>
<td>Neither</td>
</tr>
<tr>
<td>Disagree</td>
<td>Strongly disagree</td>
</tr>
</tbody>
</table>

8. In your opinion, how effective are the lozenges?

<table>
<thead>
<tr>
<th>Option</th>
<th>Scale</th>
</tr>
</thead>
<tbody>
<tr>
<td>Very effective</td>
<td>Effective</td>
</tr>
<tr>
<td>Effective</td>
<td>Neutral</td>
</tr>
<tr>
<td>Ineffective</td>
<td>Very ineffective</td>
</tr>
</tbody>
</table>

9. What type of lozenges do you think you were getting?

<table>
<thead>
<tr>
<th>Option</th>
<th>Scale</th>
</tr>
</thead>
<tbody>
<tr>
<td>Probiotic</td>
<td>Placebo</td>
</tr>
</tbody>
</table>

10. If the lozenges were shown to be effective in reducing white spots and gum disease, how likely would you be to use the lozenges in the future?

<table>
<thead>
<tr>
<th>Option</th>
<th>Scale</th>
</tr>
</thead>
<tbody>
<tr>
<td>Very likely</td>
<td>Moderately likely</td>
</tr>
<tr>
<td>Moderately likely</td>
<td>Somewhat likely</td>
</tr>
<tr>
<td>Somewhat likely</td>
<td>Somewhat not likely</td>
</tr>
<tr>
<td>Not likely</td>
<td>Very unlikely</td>
</tr>
</tbody>
</table>
### Randomization Schedule

<table>
<thead>
<tr>
<th>Random Integer&lt;sup&gt;1&lt;/sup&gt;</th>
<th>Subject ID&lt;sup&gt;2&lt;/sup&gt;</th>
<th>Lozenge Group&lt;sup&gt;3&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>20</td>
<td>1</td>
<td>A</td>
</tr>
<tr>
<td>37</td>
<td>2</td>
<td>B</td>
</tr>
<tr>
<td>58</td>
<td>3</td>
<td>B</td>
</tr>
<tr>
<td>7</td>
<td>4</td>
<td>A</td>
</tr>
<tr>
<td>46</td>
<td>5</td>
<td>B</td>
</tr>
<tr>
<td>35</td>
<td>6</td>
<td>B</td>
</tr>
<tr>
<td>22</td>
<td>7</td>
<td>A</td>
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<tr>
<td>14</td>
<td>8</td>
<td>A</td>
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<td>B</td>
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<td>B</td>
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<tr>
<td>8</td>
<td>37</td>
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1. Random allocation sequence generated by random.org
2. Assignment of subject ID based on sequence of enrollment
3. Random #1-30 = Lozenge Group A
   Random #31-60 = Lozenge Group B