Assessment of the Therapeutic Potential of a Dental Probiotic in Orthodontic Patients Affected by Gingivitis: A Randomized Control 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: As fixed orthodontic appliances pose a risk factor for plaque accumulation and gingivitis, a need for an adjunctive aid to the oral hygiene regimen exists. A probiotic complex (Lorodent) has yet to be investigated within the orthodontic context.

Purpose: To investigate the efficacy of Lorodent in reducing gingivitis and selected periodontal pathogens in orthodontic patients.

Research Design: This randomized controlled trial consisted of 60 patients who received either the probiotic or placebo lozenges for 28 days. Gingival indices were assessed. Saliva samples and subgingival plaque were collected for evaluation of probiotic and selected periodontal bacteria levels over the span of 56 days.

Results: Lozenge consumption compliance was over 90% and no adverse events were
observed in the probiotic group. The probiotic test group showed no significant changes in gingival indices. There were no significant differences in the level of pathogenic bacteria between the probiotic or placebo group.

**Conclusions:** The Lorodent probiotic lozenge did not significantly reduce periodontal pathogens or gingival indices in adolescent patients undergoing fixed orthodontic appliance treatment.
ACKNOWLEDGMENTS

I would like to extend my sincerest gratitude to my supervisor, Dr. Siew-Ging Gong, for her guidance and support throughout my thesis and to Dr. Fatima Ebrahim, my co-investigator.

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<th>Full Form</th>
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<tbody>
<tr>
<td>BLIS</td>
<td>Bacteriocin-like inhibitory substance</td>
</tr>
<tr>
<td>BOP</td>
<td>Bleeding on probing</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony forming units</td>
</tr>
<tr>
<td>cMGI</td>
<td>Composite modified gingival index</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>GI</td>
<td>Gingival index</td>
</tr>
<tr>
<td>IL-8</td>
<td>Interleukin-8</td>
</tr>
<tr>
<td>IQR</td>
<td>Interquartile range</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>MGI</td>
<td>Modified gingival index</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative polymerase chain reaction</td>
</tr>
<tr>
<td>RCT</td>
<td>Randomized control trial</td>
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<tr>
<td>rDNA</td>
<td>Ribosomal deoxyribonucleic acid</td>
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INTRODUCTION, DEFINITIONS AND STATEMENT OF THE PROBLEM

The oral cavity hosts abundant and different types of microbes. Many of these bacteria colonize and contribute to the formation of dental plaque. Dental plaque is a biofilm composed of multiple aggregating bacteria and their exopolymer matrix.¹ More than 700 bacterial species are estimated to be capable of colonizing human subgingival plaque and more than 500 species are capable of colonizing the oral cavity as a whole (including the tongue and mucosal surfaces).²-⁴ Most of these bacteria live in harmony with their host but approximately 10-30 species are responsible for gingivitis and the destruction associated with periodontal disease.⁵ When the equilibrium is compromised and an imbalance appears among the indigenous bacteria, pathologies such as gingivitis or periodontitis, occur.

Gingivitis is inflammation of the oral gingival tissues surrounding the cervical aspect of teeth and overlaying the alveolar bone.⁶ Children and adults often develop gingivitis as a result of the accumulation of food and subsequent colonization by potentially deleterious bacteria in the form of plaque. In the general population, gingivitis may often progress to periodontal disease. Periodontitis results in destruction of the periodontal attachment, loss of alveolar bone height and plausible consequential tooth loss.⁷ In contrast to periodontitis, which is not often seen in healthy children, gingivitis is the most commonly occurring periodontal disease in children.⁸ Periodontal disease is now also linked to wider systemic complications such as cardiovascular disease, preterm labour, arthritis and other health conditions.⁹-¹² Although gingivitis is reversible,¹³,¹⁴ the changes that occur during periodontitis are destructive and predominantly irreversible. Since gingivitis most often precedes periodontitis,¹⁵ treatment of gingivitis can help prevent
periodontal disease.\textsuperscript{16}

It is estimated that approximately one third of North Americans suffer from either gingivitis or periodontal disease with a significant microbial component.\textsuperscript{17} For example, studies examining the prevalence and distribution of gingivitis found that 65-97\% of children and adolescents have gingivitis,\textsuperscript{18} and 82\% of individuals aged 12-20 years of age exhibited gingivitis and gingival bleeding in the United States.\textsuperscript{15} Comparing children from various socioeconomic strata (SES) in Brazil, gingivitis and visible plaque were evident in 98.1\% of children from a high SES\textsuperscript{19} and 100\% of children from a low SES.\textsuperscript{20} Many other studies have reported similar results in children and teens of various ethnic backgrounds and populations,\textsuperscript{21-26} pointing to the universality of the problem of periodontal disease.

Recent evidence suggests that probiotic therapy might be applied to the maintenance of oral health.\textsuperscript{27-29} The World Health Organization defines probiotics as bacteria associated with beneficial effects on the host.\textsuperscript{30} The favourable effects of probiotic therapy are mainly achieved through the modulation of existing microbial flora associated with the host, thus attaining a balanced and healthy microbe-host relationship. Classic probiotic strains, such as those that belong to the genus \textit{Lactobacillus}, have been tested for their ability to confer a probiotic effect in the oral cavity.\textsuperscript{31-34} The Lorodent Probiotic Complex (Integra Medical, Inc., London, ON) is a commercially available probiotic lozenge. It is a blend of six probiotic bacteria with \textit{Streptococcus salivarius} BLIS K12 and five probiotic strains of the genus \textit{Lactobacillus}: \textit{L. paracasei}, \textit{L. plantarum}, \textit{L. acidophilus}, \textit{L. salivarius} and \textit{L. reuteri} being the key ingredients.

The purpose of this study is to investigate the efficacy of the Lorodent Probiotic Complex in reducing clinical gingivitis and to examine a possible modification of the oral microflora in favour of commensals versus recognized pathogens in children and
adolescents undergoing orthodontic treatment. The following sections will provide a review of the literature on the development of plaque and gingivitis, and the potential use of oral probiotics to combat periodontal disease.

CHAPTER 1: REVIEW OF THE LITERATURE

1.1 DENTAL PLAQUE

1.1.1 Properties of a Biofilm

Biofilm is a term used to describe a heterogeneous, organized aggregation of microorganisms bound to a surface. Bacteria can exist in two forms, either as a free planktonic form or in a biofilm. Bacteria in the form of biofilms are the major cause of chronic bacterial infections. Treatment of such infections is complicated by the fact that bacteria in a biofilm are 500 to 1000 times more resistant to antibacterial agents compared to planktonic bacteria in a culture. The structure and composition of a biofilm award it a unique set of properties that contribute to its reduced antimicrobial sensitivity (Table 1; described in greater detail in subsequent sections). Oral biofilms are made up of 15-20% bacteria by volume surrounded by a glycocalyx matrix which makes up the remaining 75-80%. The extracellular matrix consists of 50-95% exopolysaccharides produced by the bacteria, in addition to other proteins, salts and cell byproducts. This matrix creates a heterogeneous, non-dense architecture with channels and void spaces that enable nutrients, gases and cell products to diffuse through and circulate. Although this may be the case for the more peripheral biofilm, as depth increases, penetration is less likely and protection from host defenses and antimicrobial agents is granted.
1.1.2 **Structure and Formation of Dental Plaque**

Dental plaque is an oral microbial biofilm that adheres to natural tooth surfaces as well as intraoral restorations and dental prosthesis. It is an aggregation of salivary components, bacterial microorganisms and their exopolymer matrix. Knowing how plaque forms and matures is of clinical importance to help identify therapeutic targets and possible limitations of treatment. Many studies have investigated the formation and molecular structure of dental plaque.\(^{51-61}\) Stages of plaque formation include pellicle formation on a clean tooth surface, colonization of the tooth surface by the primary colonizing bacteria, followed by attachment of secondary colonizers to the primary colonizers to eventually result in mature biofilm (Figure 1).\(^{39}\)

Studies using light and electron microscope technology show that there is order in plaque formation.\(^{60,62}\) The first layer in dental plaque development is formation of the acquired pellicle. Pellicle induction occurs as soon as a cleaned tooth surface is

---

**Table 1. Properties of a biofilm**

<table>
<thead>
<tr>
<th>Protection from host defense mechanisms(^{42,43})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Channels allowing for diffusion and communication(^{41})</td>
</tr>
<tr>
<td>Reduced sensitivity to antimicrobial agents due to</td>
</tr>
<tr>
<td>• Slow growth rate(^{44})</td>
</tr>
<tr>
<td>• Poor penetration(^{45})</td>
</tr>
<tr>
<td>• Altered bacterial properties(^{46})</td>
</tr>
<tr>
<td>• Close bacterial communities confer ability to transfer resistance genes(^{47})</td>
</tr>
<tr>
<td>• Neutralization or inactivation of antimicrobial agents(^{48})</td>
</tr>
<tr>
<td>Spatial and environmental heterogeneity(^{49})</td>
</tr>
<tr>
<td>Spatial organization facilitating metabolic interactions and symbiotic relationships(^{50})</td>
</tr>
</tbody>
</table>
exposed to saliva. The initial acquired pellicle is less than 1µm thick and consists of salivary and gingival crevicular fluid (GCF) proteins and glycoproteins adsorbed to the hydroxyapatite crystals of the tooth surface. Bacteria attach to the pellicle matrix and begin to form microcolonies. Initial pellicle formation and attachment of the primary colonizers comes about via micro-roughness in the tooth surface, free surface energy and hydrophobic interactions. The non-shedding surface of a tooth combined with the presence of a constant food source (via saliva and GCF) make the dentition an ideal site for biofilm formation. A cleverly designed study by Listgarten et al. (1975), used human volunteers requiring crown prosthodontic restorations to examine the formation and structural changes that occur during maturation of dental plaque from 1 day to 2 weeks. Epoxy resin crowns were fabricated for each patient and worn for various designated time periods to provide insight on plaque formation from a consistent anatomic site. The crowns, with their overlaying plaque, were then retrieved and studied under light and transmission electron microscopes to evaluate plaque structure and composition at various stages. It was found that the pioneer colonizers were predominantly Gram-positive facultative cocci and rods that adhere to the acquired pellicle. Initially, the bacteria divide and grow laterally across the tooth surface after which they begin to grow in thickness, in the form of columnar microcolonies. Growth rate of bacteria is fastest during the initial colonization and slows down as the biofilm matures. As the population increases, the initial colonizers begin to compete for nutrients. As plaque matures, there is a shift in the predominating species from Gram-positive facultative cocci to Gram-negative rods and motile species. Around the 3rd day after plaque formation, filamentous bacteria appear on top of the currently present columnar structured plaque. After about 1 week, the filamentous bacteria grow into, and start replacing the initially predominating coccoid microbiota. After about 2 weeks the coccoid microbial colonies have been fully replaced by the filamentous bacteria, which
characterize the supragingival plaque. Left undisturbed, the supragingival plaque mineralizes to form calculus.

Figure 1. Schematic representation of the different stages in the formation of dental plaque: 1. Pellicle forms on a clean tooth surface. 2. Primary colonizing bacteria are transported passively to the tooth surface, where they then attach and colonize. 3. Secondary colonizers attach to primary colonizers. 4. Growth results in biofilm maturation, facilitating interbacterial interactions. 5. Eventually, detachment of the bacteria can occur. Adapted with permission from "Oral Microbiology" by Marsh PD, Martin MV, Lewis MAO, Williams D. Copyright 2009, Elsevier Health Sciences.
1.1.3  The Microbial Shift

Primary/early colonizers are predominantly Gram-positive aerobic and facultative anaerobic cocci and rods consisting mainly of *Streptococcus* and *Actinomyces* species. The initial colonizing *Streptococcus* species include *S. sanguinis*, *S. oralis* and *S. mitis*. *Actinomyces* species that are consistently present are *A. viscosus* and *A. oris* (previously *A. naeslundii*). These early colonizers thrive in oxygen and carbon dioxide containing environments and are able to resist the mechanical threat of salivary and GCF flow, swallowing, chewing and mechanical action of the tongue. Streptococci are saccharolytic and obtain nutrients from saliva and the easily accessible food source consumed by the host. As the population expands, a competition for nutrients develops. In contrast, Gram-negative anaerobes, the secondary colonizers, tend to be proteolytic and obtain their energy from hydrolysis of host proteins found in the GCF.

![Diagram](image)

**Figure 2.** A diagrammatic representation of the spatial and chemical changes that occur as plaque thickens and matures. As the plaque thickens and matures, the pH, nutrients, oxygen and carbon dioxide levels become reduced closer to the tooth surface compared to the outer surface of the plaque. Contrarily, metabolic products are higher closer to the tooth surface.
As the oral biofilm matures, nutritional and chemical gradients develop across the plaque layer (Figure 2). The established oxygen, carbon dioxide and pH gradients allow anaerobic Gram-negative rods and motile bacteria to thrive in the depths of the plaque. If left undisturbed, a microbial shift in the predominating bacteria within the biofilm begins to occur (Figure 3). One week after initiation of plaque formation, streptococci remain the dominant group of organisms. By 2 weeks, they constitute only 15% of the cultivable microflora, and anaerobic species prevail. \( F. \text{nucleatum} \) acts as a bridge by being able to bind to proteins in the pellicle alongside coaggregation with various early and late colonizers. Three to twelve weeks after initial supragingival plaque formation, anaerobic Gram-negative species abound in the subgingival sulcus. These secondary/late colonizers coaggregate via cell-to-cell interactions and begin to form a mature biofilm. Table 2 shows a list of early and late oral plaque colonizers. Once established, the microbial community in a biofilm remains relatively stable.

<table>
<thead>
<tr>
<th>Early Colonizers</th>
<th>Late Colonizers</th>
</tr>
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<tbody>
<tr>
<td>A. viscosus</td>
<td>C. rectus</td>
</tr>
<tr>
<td>A. oris</td>
<td>P. intermedia</td>
</tr>
<tr>
<td>S. sanguinis</td>
<td>P. nigrescens</td>
</tr>
<tr>
<td>S. oralis</td>
<td>Fusobacterium</td>
</tr>
<tr>
<td>S. mitis</td>
<td>P. gingivalis</td>
</tr>
<tr>
<td>S. gordonii</td>
<td>T. forsythia</td>
</tr>
<tr>
<td>S. intermedius</td>
<td>T. denticola</td>
</tr>
</tbody>
</table>
**Figure 3.** A diagrammatic representation of the microbial shift and environmental changes associated with plaque maturation. As plaque matures, the predominating bacteria shift from Gram-positive facultative anaerobic cocci to Gram-negative anaerobic rods and motile species. The nutritional supply also changes, from saccharolytic bacteria utilizing sources from saliva, to proteolytic bacteria obtaining nutrients from the gingival crevicular fluid (GCF). Adapted with permission from “Microbial etiology of periodontitis” by Nishihara T and Koseki T, 2004, *Periodontology 2000*, Copyright 2004, John Wiley and Sons.
Supragingival and subgingival environmental conditions differ and these differences are reflected in their prevailing bacterial communities. There are three variables in the supra- and subgingival habitats that lead to structurally unique plaque formation with an alteration in the predominating microbial species. These factors include the surface to be colonized, the available source of nutrients (saliva vs. GCF) and the environment (such as O₂, CO₂ and pH). Supragingival plaque provides an ideal niche for aerobic/facultative anaerobic, capnophilic, saccharolytic microbial species whereas subgingival plaque is ideal for anaerobic, proteolytic bacteria. Supragingivally, the tooth surface is the only site of colonization but subgingivally there is both the tooth surface and the sulcular epithelial lining, providing two biofilms in one location. Gram-positive cocci and rods dominate the supragingival tooth surface, whereas Gram-negative motile rods and spirochetes are found adjacent to the inner epithelial lining of the gingival pocket where they are able to invade the tissues. Supragingival plaque provides an environment with an ample supply of saliva whereas subgingival plaque is constantly bathed in GCF. In a study to determine the absolute counts and proportion of bacteria in supra- and subgingival plaque samples, respectively, 50 periodontally healthy subjects and 89 subjects diagnosed with chronic periodontal disease were recruited. The supra- and subgingival plaque samples of these patients were assayed for 40 different bacterial species using checkerboard DNA-DNA hybridization (results are summarized in Figures 4 and 5). It was found that in healthy subjects, supragingival plaque had consistently higher counts of bacteria compared to subgingival plaque, with particularly higher counts of Actinomyces and representatives of the purple, yellow and green complexes (species associated with periodontal health; to be discussed in greater detail in section 1.2.2). In terms of proportions, there were few differences between supra- and subgingival plaque in healthy subjects. Significantly higher proportions of F. nucleatum and P. intermedia (P < 0.05) were seen in subgingival plaque, whereas
percentages of *A. naeslundii* 2 (*A. oris*) and *S. sanguinis* were higher in the supragingival plaque in healthy subjects. Comparing plaque from health and periodontitis, a greater absolute count of bacteria was seen in samples from disease sites, with higher percentages of orange and red complex species (those associated with periodontal disease). These findings can be explained in part by the space available for colonization in healthy versus disease sites. The gingival sulcus of healthy sites provides less surface area for colonization compared to the loss of attachment and deeper pockets found in areas demonstrating periodontitis. These findings can also be explained by the distinct supra- and subgingival variances in habitat discussed above, and the corresponding species that thrive in each condition.
Figure 4. Absolute counts of 40 species in supragingival (yellow) and subgingival (red) plaque samples taken from periodontally healthy subjects (left) and subjects with chronic periodontitis (right) using checkerboard DNA-DNA hybridization. \(^* P < 0.05, \quad ** P < 0.01, \quad *** P < 0.001\). Adapted with permission from “Periodontal microbial ecology” by Socransky S and Haffajee A, 2005, *Periodontology 2000*, Copyright 2005, John Wiley and Sons.
Figure 5. Percentage of DNA of 40 species in supragingival (yellow) and subgingival (red) plaque samples taken from periodontally healthy subjects (left) and subjects with chronic periodontitis (right) using checkerboard DNA-DNA hybridization. *P < 0.05, ** P < 0.01, ***P< 0.001. Adapted with permission from “Periodontal microbial ecology” by Socransky S and Haffajee A, 2005, *Periodontology 2000*, Copyright 2005, John Wiley and Sons.
1.1.4 **Periodontal Consequences of Dental Plaque**

Dental plaque is found naturally on the teeth of healthy individuals. There are host mechanisms in place that act to resist plaque accumulation such as the cleansing action of the tongue, saliva and GCF. Additional plaque control and removal is achieved by tooth brushing, flossing and other oral hygiene aids. However, if it is allowed to accumulate beyond the threshold of oral health, there is a shift in the oral microflora and a disposition for oral disease. Dental plaque is a major contributing factor in the etiology of many oral pathologies such as caries, gingivitis, periodontitis and peri-implantitis.

Healthy gingiva is characterized by a pink colour, knife-edge margins and a firm stippled surface (Figure 6). On the other hand, gingivitis is clinically evident as erythema, edema, rolled gingival borders and increased propensity to bleeding upon gentle probing (Figure 7). Various factors influence gingival health such as the presence or absence of systemic disease, medication, genetic predisposition and the qualitative and quantitative characteristics of the biofilm. Although gingivitis is a multifactorial disease, the primary etiological factor is the microbial plaque.
**Figure 6.** A clinical image of healthy gingiva exhibiting a firm stippled texture, pink colour and knife-edge gingival margins.

**Figure 7.** A clinical image of gingivitis showing erythema, edema, rolled gingival borders and bleeding upon gentle probing.
Many studies have shown that the extent and composition of dental plaque correspond well to the health or disease state of the periodontal tissues.\textsuperscript{78-80} Pioneering experiments by Loe and Theilade (1965 and 1966), demonstrate the role and association of dental plaque in the formation of gingivitis.\textsuperscript{14,81} Eleven subjects (ages 21-27) with previously healthy gingiva and good oral hygiene ceased all intentional plaque removal and were monitored regularly until their gingival index score reflected mild gingivitis. Within 9-21 days, all subjects developed thick accumulations of plaque and generalized mild gingivitis. The rate of plaque accumulation was positively correlated with the development of gingivitis. When an oral hygiene regime was reinstated, plaque levels fell 1-2 days after and gingival inflammation was resolved one day after plaque removal. Within 7-11 days, plaque and gingival indices returned to their original values. The clinical development of gingivitis differs between young children and adults. Clinical experiments show that upon withdrawal of oral hygiene, children with primary dentition are more resistant to developing gingivitis compared to adults.\textsuperscript{82,83} The reason for the difference between young children and adults is not fully known but may be related to variance in the bacterial population and the innate host response.\textsuperscript{8}

The macroscopic changes in gingival tissue characteristics are based on cellular alterations. The first clinical sign of gingivitis is redness and loss of texture of the gingival margins. This results from engorgement of blood vessels in the underlying subepithelial connective tissue and a thinning of the outer keratinized layer.\textsuperscript{84} Increased permeability of the vasculature contributes to increased GCF flow.\textsuperscript{85,86} As edema and swelling take place, the once supragingival plaque now becomes subgingival due to development of a pseudopocket created by the engorged cervical gingival tissues. This provides a now anaerobic environment for more pathogenic bacteria. Bleeding upon gentle probing results from micro-ulcerations in the inner epithelial lining of the gingival pocket. Left untreated, gingivitis may progress to periodontitis, with more serious
consequences of attachment and bone loss. Fortunately, in pre-pubertal children, gingivitis rarely evolves to periodontitis.

1.2 **GINGIVITIS**

1.2.1 **Host Factors that Influence Gingivitis**

Bacteria alone will not cause periodontal disease. It is a combination of plaque alongside host and environmental factors. The presence and severity of gingivitis is influenced by factors such as drugs, hormones, smoking and any systemic factors that influence the individual's immune response. By itself, a drug does not cause gingivitis but contributes to a magnified response to the presence of plaque in the form of gingival hyperplasia. Drugs associated with excessive gingival overgrowth include nifedipine (a calcium channel blocker used to control high blood pressure), phenytoin (an anti-epileptic drug) and cyclosporine (an immunosuppressant). Conditions with altered hormone levels such as puberty and pregnancy also influence the development of gingivitis. Cigarette smoking tends to mitigate gingivitis due to its vasoconstrictive effects, resulting in reduced edema, gingival bleeding and GCF. However, this does not mean that smoking is a protective factor against periodontal disease; in fact, smoking is associated with an increased risk of periodontal disease. Knowledge of these host factors is critical and exclusion of such patients from a study is important, as they may be confounding factors when examining gingivitis.

1.2.2 **The Microbiology of Gingivitis**

The bacterial composition of plaque varies in health, gingivitis and periodontitis. In a clinical trial by Loe et al. (1965), it was found that healthy
subjects had 90-100% Gram-positive cocci and rods. Once clinical gingivitis was established, the percentage of Gram-positive bacteria went down to 45-60% and there was an increase in Gram-negative cocci and rods, constituting 11-31%. This is consistent with other studies that have shown the increase of Gram-negative bacteria in clinical gingivitis. Socransky et al. (1998), examined plaque samples from 185 subjects and conducted a cluster analysis of 40 taxa to outline the bacterial communities associated with subgingival plaque in patients with gingivitis and periodontitis as compared to healthy controls (Figure 8). The bacteria within a cluster tend to have similar atmospheric and nutritional preferences. The yellow, green and purple complexes include the species found in periodontal health and are associated with shallow pockets characterized by probing depths (PD) <3mm. A group of bacteria, termed the “orange complex”, predominate in the presence of gingivitis. This group includes *Fusobacterium nucleatum* along with some subspecies, *Prevotella intermedia*, *Prevotella nigrescens*, *Peptostreptococcus micros*, *Campylobacter rectus*, *Campylobacter gracilis*, *Campylobacter showae*, *Eubacterium nodatum*, and *Streptococcus constellatus*. These bacteria release toxins that penetrate the gingival tissues and result in inflammation in the form of gingivitis. The red complex includes species associated with advanced periodontal lesions clinically manifested as PD >4mm and bleeding on probing (BOP). A study by Lee et al. (2012), quantified the levels of specific Gram-negative bacteria in individuals of different stages of chronic periodontal disease. The bacteria tested included *Treponema denticola*, *Aggregatibacter actinomycetemcomitans*, *Porphyromonas gingivalis*, *Tannerella forsythia*, *Campylobacter rectus*, and *Fusobacterium nucleatum*. Subgingival tissue samples were collected and analyzed using real-time PCR. They found that individuals with chronic gingivitis and moderate periodontitis had significantly higher levels of *A. actinomycetemcomitans* and *C. rectus*.  

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It is the biofilm as a community, not single bacteria\textsuperscript{100} or the presence of a specific bacteria\textsuperscript{3} that contributes to gingivitis. However, it is the proportion of a group of bacteria, in the form of a biofilm, that are associated with gingivitis. The predominating anaerobic species in gingivitis show overlap with the microbiota of initial periodontitis.\textsuperscript{101}

For example, \textit{C. rectus} and \textit{P. intermedia} were found to be elevated in active periodontal disease sites (showing >2.5mm attachment loss in 2 months) compared to inactive sites.\textsuperscript{3} The bacterial shift from health to disease is more of a continuum rather than an abrupt change in the prevailing bacterial species. Many of the bacteria associated with gingivitis are found in even greater proportions in periodontitis.\textsuperscript{97,98,101,102} An increased proportion of periopathogenic bacteria produces a greater risk of periodontal disease.\textsuperscript{3}

Not only does plaque cause gingivitis, but the presence of gingivitis in itself has the disposition to increase plaque formation.\textsuperscript{103} It has been found that plaque accumulation is higher at sites exhibiting gingivitis compared to sites of healthy gingiva.\textsuperscript{104,105} This may be partly explained by the increase in GCF flow, along with a source of nutrients, appearing in sites with gingivitis.\textsuperscript{75}
Figure 8. A diagram showing the various clusters of microbial species within and between microbial complexes. Adapted with permission from “Microbial complexes in subgingival plaque” by Socransky SS, Haffajee AD, Cugini MA, Smith C, Kent RL, 1998, Journal of Clinical Periodontology, 25, p140, Copyright 2005, John Wiley and Sons. Note re-classification of A. naeslundii to A. oris, S. sanguis to S. sanguinis, and T. forsythensis to T. forsythia.

1.2.3 *Fusobacterium nucleatum*

*F. nucleatum* is one of the most commonly isolated bacterial species found subgingivally.\textsuperscript{106} Many studies have demonstrated an association between gingivitis and *F. nucleatum*.\textsuperscript{82,97,98,107} Specifically, levels of *F. nucleatum* were shown to be higher in orthodontic patients with gingivitis compared to healthy controls.\textsuperscript{108}

*F. nucleatum* is a Gram-negative, non-motile rod that is anaerobic but can grow in the
presence of up to 6% oxygen.\textsuperscript{109} It does not have any fimbriae, flagellae or pili. \textit{F. nucleatum} plays a crucial role in plaque development due to its ability to adhere to a wide range of bacteria and is often thought of as the link between early and late microbial colonizers (Figure 3).\textsuperscript{110} Its ability to coaggregate with both aerobic and anaerobic bacteria allows \textit{F. nucleatum} to act as a shield so that obligate anaerobes can survive in an aerated environment.\textsuperscript{72}

Not only does \textit{F. nucleatum} adhere to other microbes, it also has the ability to adhere and invade the gingival epithelial tissues.\textsuperscript{111} This results in increased inflammatory markers including interleukin-8, which encourages inflammatory cells to migrate to the site of infection. \textit{F. nucleatum} also produces tissue irritants which contribute to gingival inflammation.\textsuperscript{112-114}

\subsection{1.2.4 \textit{Prevotella intermedia}}

\textit{Prevotella intermedia} (formerly known as \textit{Bacteroides intermedius}) is a black-pigmented, Gram-negative, rod-shaped, obligate anaerobe. Studies show that \textit{P. intermedia} is rarely found in the subgingival plaque of children with healthy gingiva,\textsuperscript{115,116} but is elevated in sites of gingivitis in both children and adults.\textsuperscript{98,117-119} Subgingival plaque samples show that \textit{F. nucleatum} is often accompanied by \textit{P. intermedia}. A study by Ali et al. (1994), showed that and \textit{P. intermedia} was never found without the presence of \textit{F. nucleatum} but \textit{F. nucleatum} could exist without habitation of \textit{P. intermedia}.\textsuperscript{120} \textit{P. intermedia} is non-motile, and is considered weakly invasive or non-invasive in its ability to attach to and invade the epithelial lining of the gingival sulcus.\textsuperscript{111} However, it does stimulate production of inflammatory products (IL-8) and contribute to gingivitis.\textsuperscript{111,121}
1.2.5  **Campylobacter rectus**

*Campylobacter rectus* is a Gram-negative, curved-rod shaped, microaerophilic facultative anaerobe with a single flagellum. Many studies have shown an increased proportion of *C. rectus* in gingivitis compared to health.\(^98,103,119,122\) It was suggested that the presence of *C. rectus* marks a shift from periodontal health to disease.\(^102\) *C. rectus* in plaque samples was also significantly associated with bleeding on probing (P < 0.001) and a higher gingival index score (P < 0.001), two markers of gingival disease.\(^123\)

Many characteristics of *C. rectus* contribute to its ability to produce a strong inflammatory response. Its outer membrane contains lipopolysaccharides (LPS) which elicit a strong inflammatory response in gingival tissues.\(^124,125\) Its flagellum grants it motility to migrate to the epithelium and it has also been implicated as a virulence factor.\(^123\) *C. rectus* also has the rare ability to produce leukotoxins which destroy the white blood cells whose role is to protect the host from bacteria and other pathogens.\(^126\) All of these factors have been proposed but the exact mechanism of pathogenicity is still unknown.

1.2.6  **Clinical Evaluation of Gingivitis**

Many indices exist for the evaluation of gingivitis. In choosing the most appropriate periodontal index, Hazen et al. (1974), have set some guidelines for consideration.\(^127\)

1) An index should be simple and cost effective, to allow for examination of a large number of individuals. 2) The criteria defining each interval in an index should be clear and reproducible. 3) A severity index should indicate meaningful clinical stages of disease progression. 4) The index should be amenable to statistical analysis.

Several indices have been used to quantify gingivitis using visual and tactile means.
Their ability to satisfy the aforementioned criteria make the Gingival Index (GI)\textsuperscript{128} and Modified Gingival Index (MGI)\textsuperscript{129} the most commonly used in clinical research.\textsuperscript{130} Assessment of gingival inflammation using the Gingival Index involves evaluation of gingival bleeding upon probing.\textsuperscript{131} Probing of the gingival tissues may be invasive, does not allow for repeated appraisal within a short period of time and may interfere with the local plaque biofilm.\textsuperscript{132}

Lobene et al. (1986), created a Modified Gingival Index to help overcome the downfalls of the Gingival Index, by evaluating gingival status based on direct observation without probing.\textsuperscript{129} In addition to this benefit, the MGI is more sensitive than the GI at the lower end of the scale.\textsuperscript{132} The MGI employs a scale that ranges from 0-4, representing a gradient from gingival health all the way to severe inflammation (Table 3). An increase in the MGI score corresponds with amassed gingival inflammation.

\begin{table}[h]
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\begin{tabular}{|c|p{12cm}|}
\hline
0 & Healthy: absence of gingival inflammation. \\
\hline
1 & Mild inflammation (partial unit): slight change in colour, little change in texture of any portion of but not the entire marginal or papillary gingival unit. \\
\hline
2 & Mild inflammation (entire unit): criteria as above, but involving the entire marginal or papillary gingival unit. \\
\hline
3 & Moderate inflammation: glazing, redness, edema, and/or hypertrophy of the marginal or papillary gingival unit. \\
\hline
4 & Severe inflammation: marked redness, edema, and/or hypertrophy of the marginal or papillary gingival unit; spontaneous bleeding or ulceration. \\
\hline
\end{tabular}
\caption{Modified Gingival Index (MGI) Scoring Criteria\textsuperscript{129}}
\end{table}
Full mouth assessment of the periodontal condition involves examination of 6 sites on all teeth present, involving up to 192 recordings. This is time consuming and may limit the number of participants included in a research study due to time limitations and resources. Often times, partial recording of the periodontium is used to represent the state of the entire dentition while creating clinical efficiency. Ramfjord teeth (teeth #16, 21, 24, 36, 41, 44), which include a combination of anterior and posterior teeth, have been used to evaluate clinical periodontal status in multiple studies.\textsuperscript{119,133} Although Ramfjord teeth have limitations in diagnosis of probing depth and clinical attachment loss,\textsuperscript{134} it is a reliable and valid method of representing whole mouth gingival inflammation and plaque levels.\textsuperscript{135,136} Use of the Ramfjord teeth has been found to be fairly representative of the entire dentition, with the tendency to underestimate plaque and gingival indices obtained from low scores (<1) and overestimate those obtained from high scores (>2).\textsuperscript{137}

1.2.7 Microbial Evaluation of Periodontal Pathogens

A variety of methods exist to detect and quantify microbes in plaque and saliva. Traditionally, cultivation on selective media would rely on multiplication of the bacteria with manual plating and counting. Many bacteria are difficult to culture and others nearly impossible.\textsuperscript{3} Microbial culture techniques are also sensitive and time consuming. Periodontal pathogens such as \textit{F. nucleatum} and \textit{P. intermedia} are anaerobic and require extraction and culturing in an oxygen-free environment. Due to the sensitivity of the process, researchers have opted for the less problematic method of quantitative polymerase chain reaction (qPCR). Real time qPCR allows for the amplification of DNA through the ability of DNA polymerase to synthesize new strands of the template DNA. The use of 16s rDNA has become the standard for quantification of species associated
with gingivitis. Although C. rectus is microaerophilic, methods using 16S rDNA are the most reliable for its detection. The advent of DNA probes and use of qPCR make it possible to distinguish and quantify anaerobic bacteria.

### 1.3 Plaque Accumulation and Gingivitis in Orthodontics

The presence of fixed orthodontic appliances such as braces have been shown to be a risk factor for plaque accumulation and gingivitis. Orthodontic appliances increase the potential retention of plaque and complicate its adequate removal. A study evaluating children with full bracketing reported increased plaque and gingival index scores compared to untreated controls. Even in the presence of good oral hygiene, children with orthodontic attachments displayed a gingival index consistent with mild inflammatory changes. These alterations in gingival condition were transient and no permanent damage was detected. Kloehn and Pfeifer (1974), evaluated the periodontal condition of patients before, during and after orthodontic treatment with full edgewise appliances. They reported an increased incidence of gingivitis throughout treatment with pronounced gingival hyperplasia in the posterior segments compared to the anterior. A marked decrease in gingival hyperplasia was observed within 48 hours of appliance removal. Histologic samples taken from patients undergoing orthodontic treatment have revealed chronic inflammatory changes associated with increased infiltration of inflammatory cells proportional to the level of inflammation. Although hyperplastic chronic inflammatory changes were observed, there was minimal tissue destruction. The gingival condition returned to normal after treatment was completed and the orthodontic appliance was removed.
If orthodontic brackets increase the risk of plaque retention and gingivitis, then it is logical that they would also cause a shift in the microbial flora. The microbiological changes in subgingival plaque in patients before and during full orthodontic bracketing revealed that the frequency of periodontal pathogens increased within one week after bonding and showed a general trend upwards until the 6 month examination. C. rectus had statistical increased (P < 0.007) from 44.2% prior to bonding to 65% 1 week after bonding and a non-statistical increase thereafter. Many additional studies support the finding of increased periopathogens in the plaque samples of orthodontic patients compared to controls. It appears that ligation method using elastomeric rings or metal ligatures does not influence bacterial levels in orthodontic patients.

It has been demonstrated by multiple investigations that once the risk factors are eliminated and the biofilm is removed, or the bacterial effects controlled, gingivitis can be reversed. Mechanotherapy to remove plaque is important, but in cases where mechanical removal of the bacterial biofilm is complicated or insufficient, adjunctive methods may be considered. Given their increased risk for gingivitis, orthodontic patients with fixed bonded brackets would benefit from adjunctive therapy.

1.4 Adjunctive Oral Hygiene Therapies

There are 3 different treatment approaches that can be taken when aiming to treat gingivitis: 1) prevent biofilm formation or maturation, 2) disrupt the existing biofilm, and/or 3) reduce or disable the pathogenic bacteria. These targets are achieved either by physically removing the biofilm, such as with oral hygiene methods and professional scaling, or altering the bacterial composition to favour the non-pathogenic...
Bacteria display different properties and susceptibilities when contained within a biofilm. Bacteria are more susceptible to antimicrobial agents in their planktonic form than in the coaggregation of a biofilm. Used alone, anti-microbial agents have proven futile in targeting oral biofilms, especially those found subgingivally. Antibiotics attach the most superficial layer, which is not the site of the anaerobic pathogenic bacteria, which dwell deeper in the biofilm. Antimicrobial tolerance is the ability of the disease-causing bacteria to resist the killing activity of the antimicrobial agent. There are many factors that contribute to antimicrobial tolerance (Table 1): 1) Slow growth of bacteria in a biofilm makes them less susceptible to antimicrobial agents compared to rapidly dividing bacteria. 2) Protection from the exopolysaccharide matrix results in reduced ability for antimicrobial agents to penetrate the complex matrix. 3) Altered phenotype of bacteria in a biofilm compared to their planktonic state may render it resistant or less sensitive to the antibacterial agent. 4) The close association between bacteria in a biofilm provides the ideal situation for the transfer of resistance genes, and 5) the environmental changes found in the depths of the biofilm may produce unfavourable environments for the antimicrobial agent resulting in a neutralizing or denaturing effect.

Many adjunctive therapies have been proposed for the treatment of gingivitis in orthodontic patients (Table 4). Although many of these therapies have shown to be beneficial, their long-term effectiveness has not been demonstrated and they often require patient compliance. Many patients, especially children, fail to follow the recommended oral hygiene instructions to brush and floss. As compliance with brushing and flossing has been demonstrated to be low even in patients without orthodontic appliances, it is expected that flossing compliance in orthodontic patients could be similarly low or worse due to the difficulty in flossing in the presence of brackets and
wires. Second to mechanical removal of plaque, chlorhexidine is considered the gold standard in terms of chemical control of plaque and gingivitis.\textsuperscript{152} Chlorhexidine is a commonly used oral antiseptic that is often used as a rinse in a 0.12\% or 0.2\% chlorhexidine gluconate form. It is valuable in reducing plaque and gingivitis but cannot be used for prolonged periods of time due to dental staining and alteration in taste perception.\textsuperscript{153} Although it reduces gingivitis, studies testing the effectiveness of chlorhexidine on biofilm viability have found that exposure of up to 0.2\% chlorhexidine has little effect on the pathogenic bacteria found deep-seated in the biofilm.\textsuperscript{154-156} It is evident that oral hygiene requires a supplementing treatment to help reduce gingivitis and plaque in orthodontic patients. A potential therapeutic agent for the maintenance of oral health is the use of probiotic therapy.

\begin{table}[h]
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\begin{tabular}{|l|l|l|}
\hline
\textbf{Method} & \textbf{Result} & \textbf{Reference} \\
\hline
Physical & & \\
\hline
Electric toothbrush & Better plaque control than with manual toothbrush & \textsuperscript{157} \\
 & No significant difference in plaque and gingival scores & \textsuperscript{158, 159, 160} \\
\hline
Water irrigation & Reduced plaque and gingivitis at sites of irrigation & \textsuperscript{161} \\
 & Reduced plaque and bleeding & \textsuperscript{162} \\
\hline
Floss & Reduced plaque and gingival indices & \textsuperscript{163} \\
\hline
\end{tabular}
\caption{Adjunctive therapies to reduce gingivitis during orthodontic treatment}
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<thead>
<tr>
<th>Chemical</th>
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<tr>
<td>0.12% Chlorhexidine rinse</td>
<td>Reduced plaque index, gingival index and bleeding on probing</td>
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<tr>
<td>Amine fluoride mouthrinse</td>
<td>Reduced in bleeding and gingivitis</td>
</tr>
<tr>
<td>Amine/stannous fluoride mouthrinse</td>
<td>Reduced plaque but no significant reduction in gingivitis</td>
</tr>
<tr>
<td>Fluoride toothpaste</td>
<td>Do difference compared to toothpaste not containing fluoride</td>
</tr>
<tr>
<td>Essential oil mouthrinse</td>
<td>Reduced plaque and gingival indices</td>
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<tr>
<td></td>
<td>Reduced plaque and gingival indices but not as much as chlorhexidine or fluoride rinse</td>
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<tr>
<th>Other</th>
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<tbody>
<tr>
<td>Oral hygiene education program</td>
<td>Reduced plaque and gingival indices</td>
</tr>
<tr>
<td>Oral hygiene with disclosing solution</td>
<td>Reduced plaque and gingival indices</td>
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1.5 **ORAL PROBIOTIC THERAPY**

1.5.1 **Introduction to Probiotics**

Probiotics are live bacteria that when ingested, confer a beneficial effect on the host. Typically, these bacteria belong to the natural microbial flora and function by colonizing the host and competing with pathogenic bacteria. Probiotics were first introduced by Nobel Prize winner Elie Metchnikoff at the beginning of the 20th century when he found
that ingestion of yogurt containing bacteria produced health benefits. Most of what is known about probiotic advantages and mechanisms of action stems from the gastrointestinal field.

The mechanisms of action of probiotics in dentistry have been hypothesized to occur in both a direct and indirect manner (Figure 9). A probiotic can defend a host in many ways. It can interact directly with dental plaque by occupying sites and using nutrients that would have otherwise been employed by the pathogen in addition to inhibiting pathogen adhesion and growth. Probiotics are also capable of producing antimicrobial and virulence factors such as hydrogen peroxide, organic acids, carbon peroxide, bacteriocins and adhesion inhibitors that act to inhibit pathogenic bacteria. In addition to local effects, probiotics also act indirectly via immune modulation (Figure 9). Treatment with probiotics has shown to increase gastrointestinal and circulating levels of IgA and IgG, two immunoglobulins found in high proportions in the oral cavity. Although it is well accepted that the mechanisms of action in the oral cavity mirror that of the gastrointestinal system, the comprehensive mechanisms by which probiotics influence the oral cavity have been investigated but are not yet fully elucidated.

The safety of probiotic use has been demonstrated by many studies for various medical and health related conditions. Probiotic treatment has been found to be safe and efficacious for use in conditions such as antibiotic associated diarrhea, inflammatory bowel disease, upper respiratory tract infections, along with many other bacteria associated conditions. A randomized, double blind, prospective study by Saavendra et al. (2004), demonstrated the safety of probiotics by examining the effects of formulae containing probiotics on growth and gastrointestinal health of 118 infants for at least 200 days. It was found that infants on the probiotic formula exhibited less gastrointestinal irritability and reduced rate of antibiotic use compared to controls. Although the benefits
of probiotics have been clearly demonstrated in many medical conditions, its role and use in the dental field is still in its early stages.

**Figure 9.** Hypothetical mechanisms of probiotic action in the oral cavity. The two-way arrow indicates that the putative action may be to both directions. Adapted with permission from “Probiotics: do they have a role in oral medicine and dentistry?” by Meurman JH, 2005, *European Journal of Oral Sciences*, 113(3), p 190, Copyright 2005, John Wiley and Sons.

Research has shown that probiotics may play a role in improving oral health. The promotion of gingival health has been demonstrated by probiotic strains of *S. salivarius*\(^{180-181}\) and *Lactobacillus*.\(^{34,133,183,184}\) Many probiotic formulations have been tested in the market. An example is the Lorodent lozenge, which is a multi-strain
probiotic lozenge that contains S. salivarius K12 along with the Lactobacillus strains L. paracasei, L. plantarum, L. acidophilus, L. salivarius and L. reuteri. The known roles of these bacteria in their probiotic use are discussed below.

1.5.2  **Streptococcus salivarius** as a Probiotic for Periodontal Health

*Streptococcus salivarius* is a human commensal bacteria present as a member of a “healthy” oral microflora. It is the first bacteria to colonize the human oral cavity as it establishes itself on the oral epithelium within one day after birth and is the primary inhabitant of the back of the tongue and throat. The levels of *S. salivarius* in swab samples taken from newborn infants represent 10% of the total streptococci isolated, increasing to 25-30% by one month of age. *Streptococcus salivarius* K12, a component of the Lorodent lozenge, is a natural strain that was originally isolated from the oral cavity of a healthy New Zealand child who had never developed throat infections. Its antimicrobial characteristic is partly due to the fact that *S. salivarius* K12 produces bacteriocin-like inhibitory substances (BLIS). Bacteriocins are ribosomally synthesized proteinaceous toxins and antibiotics that inhibit growth or kill bacteria of a closely related species. Intraoral transmission of the plasmid DNA coding for the bacteriocins to plasmid-negative strains has also been demonstrated. Its non-pathogenic nature, abundance in the oral cavity, and ability to produce broad-spectrum bacteriocins makes *S. salivarius* K12 a good oral probiotic candidate.

Use of *S. salivarius* K12 as a probiotic has been demonstrated for many health related conditions. It has been shown to reduce levels of *S. mutans* in biofilm formation related to caries, as well as efficacy for the treatment of halitosis, oral candidiasis, and otitis media. However, no clinical data has demonstrated use of *S. salivarius* for gingival health. The safety and human tolerance of high doses of *S. salivarius* K12 (1 x
10^{10} CFU) were evaluated in a randomized, double-blind clinical trial for a period of 28 days. No statistical differences were seen between the probiotic and placebo groups in terms of blood chemistry or adverse events. The researchers concluded that daily oral ingestion of *S. salivarius* K12 for a 28-day period is well-tolerated and safe for clinical use.

In addition to bacteriocins that kill and inhibit growth of oral pathogens, *S. salivarius* K12, also reduces the levels of pro-inflammatory cytokines secreted by gingival fibroblasts when challenged with oral pathogens. This suggests that *S. salivarius* has the potential to both reduce pathogen levels within the oral cavity and reduce tissue-damaging inflammation associated with oral pathogens. Additional anti-inflammatory properties have been shown in human bronchial epithelial cells by down regulating the NF-κB pathway, consistent with an emerging hypothesis that indicates the down regulation of epithelial immune responses by commensal strains of *S. salivarius*. *S. salivarius* K12 is also capable of inducing alterations in gene expression of 660 genes, of which 565 were specifically regulated by this commensal bacterium, as compared to those induced by tested and opportunistic pathogens. Interestingly, the genes up-regulated by *S. salivarius* K12 were those with adhesion and cytoskeleton-related functions, likely acting to strengthen the interactions between commensals and the host to help maintain tight junctions of cells on epithelial surfaces. The genetic analysis with *S. salivarius* K12 thereby underlines the non-inflammatory nature of the induced immune response.

### 1.5.3 *Lactobacillus* as a Probiotic for Periodontal Health

*Lactobacillus* is a genus of Gram-positive, rod-shaped microaerophilic or facultative anaerobic bacteria. *Lactobacillus* species are found in dairy products, vegetables as
well as the human oral cavity, intestines, and vagina. Lactobacilli are transiently present in newborns due to the absence of teeth, and then usually reappear after 2 years of age. They are members of the lactic acid bacteria (LAB) group, which convert sugars into lactic acid. Species are either homofermentive (ferment carbohydrates into lactic acid) or heterofermentive (ferment carbohydrates into various end products). Inhibitory properties of lactobacilli are due to the release of biologically active bacteriocins, antimicrobial factors (hydrogen peroxide) and antifungal peptides. The bacteriocins liberated by LAB are primarily bactericidal towards closely related Gram-positive bacteria. In addition to bio-production, oral lactobacilli are involved in adhesion to the dentition and soft tissues of the oral cavity. This in itself may modify the adhesion and availability of binding sites to other bacteria.

It is interesting to note the lactic acid production from lactobacilli is implicated in caries progression, but their role in initiation of carious lesions is not substantiated. Lactobacilli are found in low levels on surface plaque, but in high levels of already cavitated lesions, corroborating the knowledge that they play a role in progression rather than induction of carious lesions. Furthermore, their cariogenicity is strongly dependent on the presence of carbohydrates in the host diet. Despite their relation to caries, many Lactobacillus species act as probiotics by promoting health. In fact, their acid production may be one of the mechanisms by which they inhibit growth of periodontal pathogens such as P. gingivalis and P. intermedia.

Lactobacilli have long been of interest as probiotics for oral health and are included in the Lorodent Probiotic Complex. Many Lactobacillus species have been studied for their properties and ability to inhibit oral pathogens while improving indices of oral health (Table 5). L. reuteri and L. plantarum are among the most studied probiotics. L. reuteri secretes reuterin, a potent antimicrobial molecule that antagonizes oral pathogens.
inhibits binding of oral pathogens in vivo, and demonstrates downregulation of pro-inflammatory cytokines. Likewise, *L. plantarum* also inhibits common oral pathogens in vivo and in vitro. Other strains of *Lactobacillus* have also exhibited probiotic benefits. *L. salivarius, L. acidophilus,* and *L. paracasei* are all capable of modulating serum and salivary immunoglobin levels. *L. salivarius, L. paracasei,* 

*L. plantarum* and *L. rhamnosus* harvested from the human oral cavity are able to displace and inhibit periodontal pathogens including *P. gingivalis, P. intermedia* and *A. actinomycetemcomitans.* In addition to inhibition, lactobacilli are also able to coaggregate with *F. nucleatum,* which is a major secondary colonizer in dental plaque. To further our knowledge, a more recent study by Köll et al. (2008), aimed to identify lactobacilli strains for potential probiotic use. Characterizations of 67 strains from salivary and subgingival lactobacilli, comprising 10 species, were gathered from healthy human subjects. Each strain was tested for its antimicrobial activity against common oral pathogens. Results showed that most *Lactobacillus* species were able to suppress growth of *P. gingivalis, P. intermedia, A. actinomycetemcomitans,* and *S. mutans.* The strains found to have the most potential for probiotic use were *L. plantarum, L. paracasei, L. salivarius,* and *L. rhamnosus.* Data on strains contained in Lorodent validate that *L. reuteri, L. paracasei,* and *L. salivarius* all have in vitro or in vivo antagonism to common oral pathogens. Although *L. acidophilus* demonstrates probiotic potential for reduction of *S. mutans,* no benefit has been demonstrated against periodontal pathogens. Altogether, research illustrates the potential benefits of many lactobacilli strains for the promotion of oral health.
<table>
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<tr>
<th>Reference</th>
<th>Study Type</th>
<th>Probiotic Species, Strain and Dose</th>
<th>Type of Experiment</th>
<th>Administration Mode of Gums</th>
<th>Duration</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amin et al., 2019</td>
<td>In vitro</td>
<td>L. plantarum</td>
<td>Placebo controlled</td>
<td>Chewing gum</td>
<td>14 days</td>
<td>Produced antimicrobial compounds</td>
</tr>
<tr>
<td>Krasse et al., 2005</td>
<td>In vivo, double-blind, placebo controlled</td>
<td>L. reuteri DSM17938 and ATCC PTA 5289</td>
<td>Split mouth</td>
<td>Placebo controlled</td>
<td>21 days</td>
<td>Improved gingival index in probiotic and placebo groups compared to scaling and root planning</td>
</tr>
<tr>
<td>Vicario et al., 2013</td>
<td>In vivo, double-blind, placebo controlled</td>
<td>L. reuteri DS17938 and ATCC PTA 5289</td>
<td>Lozenge Prodentis</td>
<td>Placebo controlled</td>
<td>30 days</td>
<td>Reduced plaque, gingival and PD</td>
</tr>
<tr>
<td>Krasse et al., 2005</td>
<td>In vivo, double-blind, placebo controlled</td>
<td>L. reuteri DSM17938 and ATCC PTA 5289</td>
<td>Lozenge Prodentis</td>
<td>Placebo controlled</td>
<td>21 days</td>
<td>Improved plaque, gingival, and gingival bleeding indices compared to scaling and root planning</td>
</tr>
<tr>
<td>Vivekananda et al., 2010</td>
<td>In vivo, randomized, double-blind, placebo controlled</td>
<td>L. reuteri DSM17938 and ATCC PTA 5289</td>
<td>Lozenge Prodentis</td>
<td>Placebo controlled</td>
<td>30 days</td>
<td>Reduced plaque and gingival indices, reduced BOP and PD</td>
</tr>
<tr>
<td>Twetman et al., 2009</td>
<td>In vivo, double-blind, placebo controlled</td>
<td>L. reuteri DSM17938 and ATCC PTA 5289</td>
<td>Lozenge Prodentis</td>
<td>Placebo controlled</td>
<td>14 days</td>
<td>Reduced BOP, reduced pro-inflammatory cytokines in GCF</td>
</tr>
</tbody>
</table>

Table 5: Studies examining oral probiotic lactobacilli strains for periodontal health.
<table>
<thead>
<tr>
<th>Year</th>
<th>Study Authors</th>
<th>Treatment</th>
<th>Duration</th>
<th>Outcome Measures</th>
</tr>
</thead>
<tbody>
<tr>
<td>2009</td>
<td>Mayanga et al.</td>
<td>L. reuteri</td>
<td>8 weeks</td>
<td>Reduced periopathogens in subgingival plaque</td>
</tr>
<tr>
<td></td>
<td></td>
<td>2x10^8 CFU</td>
<td></td>
<td>Placebo-controlled, double-blind, placebo-controlled, placebo-controlled, placebo-controlled, placebo-controlled, placebo-controlled, placebo-controlled, placebo-controlled, placebo-controlled, placebo-controlled, placebo-controlled, placebo-controlled, placebo-controlled, placebo-controlled, placebo-controlled, placebo-controlled, placebo-controlled, placebo-controlled, placebo-controlled, placebo-controlled, placebo-controlled, placebo-controlled, placebo-controlled, placebo-controlled, placebo-controlled, placebo-controlled, placebo-controlled, placebo-controlled, placebo-controlled, placebo-controlled, placebo-controlled, placebo-controlled, placebo-controlled, placebo-controlled, placebo-controlled, placebo-controlled, placebo-controlled, placebo-controlled, placebo-controlled, placebo-controlled, placebo-controlled, placebo-controlled, placebo-controlled, placebo-controlled, placebo-controlled, placebo-controlled, placebo-controlled, placebo-controlled, placebo-controlled, 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</tr>
<tr>
<td>Lactobacillus Strain</td>
<td>In Vivo/Dose</td>
<td>Amount Consumed</td>
<td>Outcome</td>
<td></td>
</tr>
<tr>
<td>---------------------</td>
<td>--------------</td>
<td>-----------------</td>
<td>---------</td>
<td></td>
</tr>
<tr>
<td>L. salivarius WB21</td>
<td>Oil drops</td>
<td>4.0 × 10^8 CFU/day</td>
<td>Reduced BOP, Reduced P. intermedia, Reduced P. gingivalis, Reduced BOP, Reduced P. intermedia in subgingival plaque, No significant change in F. nucleatum</td>
<td>15 days, double-blind, placebo controlled, in vivo, double-blind</td>
</tr>
<tr>
<td>L. casei Shirota</td>
<td>Drink</td>
<td>6.5 × 10^8 CFU/ml</td>
<td>Reduced BOP, Reduced P. intermedia, Reduced P. gingivalis, Reduced BOP, Reduced P. intermedia in subgingival plaque, No significant change in F. nucleatum</td>
<td>4 weeks, in vivo</td>
</tr>
<tr>
<td>L. salivarius WB21</td>
<td>Oil drops</td>
<td>4.0 × 10^8 CFU/day</td>
<td>Reduced P. intermedia, Reduced P. gingivalis, Reduced BOP</td>
<td>15 days, double-blind, placebo controlled, in vivo, double-blind</td>
</tr>
</tbody>
</table>
1.6 **STUDY OBJECTIVES AND HYPOTHESIS**

The objective of this study was to investigate the efficacy of oral exposure of *S. salivarius* K12 along with the selected *Lactobacillus* probiotic strains, in the form of Lorodent lozenges in reducing gingivitis and gingivitis-associated bacteria in pediatric patients undergoing fixed orthodontic treatment.

We hypothesized that oral exposure of the Lorodent oral probiotic lozenge for 28 days would show a significant decrease in gingivitis and microbial counts of *C. rectus*, *F. nucleatum* and *P. intermedia* over time in orthodontic patients compared to controls receiving a placebo lozenge.

Research Questions:

Does Lorodent reduce gingivitis compared to the placebo in adolescents with fixed orthodontic appliances?

Does Lorodent reduce the amount of *C. rectus*, *F. nucleatum* and/or *P. intermedia* in subgingival plaque?

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

Can this intervention be practically implemented in a clinical orthodontic setting, as measured by compliance?
CHAPTER 2: METHODS AND MATERIALS

This study consisted of two components focusing on: 1) caries and S. mutans - conducted by principal investigator, Dr. Fatima Ebrahim (FE)\textsuperscript{227} and, 2) gingivitis and associated microbes, the topic of this current thesis, led by principal investigator, Dr. Sarah Habib (SH). The following methods and materials are as outlined in the first component of this study.\textsuperscript{227}

2.1 FACILITIES

This clinical trial was carried out in the Graduate Orthodontic Department at the University of Toronto, Faculty of Dentistry. Microbiological analysis was carried out at the Department of Microbiology and Immunology at the University of Western Ontario.

2.2 LORODENT AND PLACEBO LOZENGES

2.2.1 Lorodent and Placebo Lozenge Components

Lorodent is a blueberry flavored probiotic lozenge (details of product in Appendix 1). Each lozenge is formulated to contain one billion colony forming units (CFU) of active probiotics (\textit{S. salivarius} K12, \textit{L. paracasei}, \textit{L. plantarum}, \textit{L. acidophilus}, \textit{L. salivarius} and \textit{L. reuteri}), along with lactitol, inulin, dicalcium phosphate, blueberry flavor (natural), dextrose, fructose, stearic acid, citric acid, vanilla flavor (natural), and stevia rebaudioside (97\%) as excipients. Manufacturer recommendations for storage are room temperature for 18 months.

The placebo lozenges used in this study contained lactitol, inulin, dicalcium phosphate, blueberry flavor (natural), dextrose, fructose, stearic acid, citric acid, vanilla flavor (natural), and stevia rebaudioside (97\%).
Integra Medical, Inc. provided both the probiotic and placebo lozenges. The lozenges were originally manufactured by Nutraceutix Inc. (Redmond, Washington, USA) in August 2011. Enumeration by Integra Medical Inc. in July 2014 yielded an active probiotic concentration of $1.6 \times 10^9$ CFU/lozenge (Appendix 1). Enumeration assays to test the potency of the probiotic lozenges used in this study were carried out by our collaborators at Western University (Dr. Jeremy Burton and colleagues) in July 2014, immediately prior to initiation of this trial. Assays showed that \textit{S. salivarius} K12 and total lactobacilli levels were each $1.5 \times 10^9$ CFU/lozenge, for a total probiotic concentration of approximately $3 \times 10^9$ CFU/lozenge (Appendix 2).

Once provided to the investigators, all lozenges were securely stored in a -80°C freezer until distributed to the participants. All subjects were instructed to store the lozenges in their fridge at home for the duration of the trial.

2.2.2 Dosage and Administration

Dosage instructions were prescribed based on recommendations from Integra Medical Inc. Participants were instructed to take two lozenges, two times per day (after breakfast and after dinner) for the first 7 days. This was meant to act as a loading dose. Maintenance doses of two lozenges, once a day (after breakfast) were prescribed for the next 21 days. The total administration period was 28 days, in line with other studies examining the use of oral administration of probiotics in the form of a lozenge.\textsuperscript{221,222} This length of administration was chosen based on previous safety assessments with a 28-day administration of \textit{S. salivarius} K12 ($1 \times 10^{10}$ CFU) followed by a 28-day washout period.\textsuperscript{194}

Subjects were instructed to slowly dissolve the lozenge on their tongue for five minutes without chewing or swallowing them. Patients were also instructed not to brush or rinse their mouth for 1 hour following administration of the lozenges.
2.2.3 **Risks of the Lorodent Lozenge**

Oral probiotics have a long history of use without serious side effects.\(^{228}\) When side effects do occur, they are usually mild and digestive in nature. Based on preclinical studies of the probiotic species included in the Lorodent lozenge, no significant side effects were expected.\(^{194,229,230}\) In the rare event of a bacterial infection from Lorodent, the species present would be susceptible to treatment by antibiotics. Patients were cautioned to report any signs of fever, nausea, vomiting, diarrhea or severe abdominal pain. If such symptoms were to occur and persist for 3 or more days, probiotic use was discontinued. If pregnancy, antibiotic use, or a severe medical condition were to arise during the study period, the participant was asked to discontinue lozenge use and was withdrawn from the study.

2.3 **STUDY PARTICIPANTS**

2.3.1 **Subject Recruitment and Eligibility**

All patients were under current orthodontic treatment at the Graduate Orthodontic clinic, University of Toronto, Faculty of Dentistry. One of the two principal investigators then screened the potential subject for eligibility. The principal investigators (SH and FE) screened patients based on recommendations from co-residents in the Graduate Orthodontic Clinic. Recommendations were made if a patient was considered to be compliant (subjectively based on previous experience of their treating orthodontic resident) and was 18 years old or younger. Screening involved a review of medical and dental history along with a dental examination to evaluate the inclusion and exclusion criteria mentioned below. Orthodontic patients meeting the inclusion and exclusion criteria were then recruited for study participation.

The inclusion criteria included subjects who:
• Were male or female between the ages of 11 to 18 years
• Had mild to moderate gingivitis as determined by the principal investigators (MGI index of at least 1 to a maximum of 2)
• Were able to or whose legal guardian had given voluntary, written informed consent to participate in the study and were able to communicate in English
• Were undergoing fixed orthodontic treatment on both arches with attachments on at least 20 teeth including bonded 1st molars
• Had been in full fixed braces for at least 5 months
• Had fully erupted teeth #16, 21, 23, 36, 41, 43
• Were caries inactive prior to study initiation
• Were in a healthy systemic condition
• Had not used any antimicrobial mouth rinses, probiotics, antibiotics or anti-inflammatory drugs within one month prior to the study
• Had the following standard orthodontic bonding procedure: 37% phosphoric acid etch, Transbond™ Plus Self Etching primer (3M Unitek), and Transbond™ light cure adhesive (3M Unitek)

The exclusion criteria excluded subjects who:
• Were unable to make informed consent or communicate fluently in English
• Had an allergy or sensitivity to milk or milk products, gluten, soy or any other ingredient present in the Lorodent probiotic complex
• Were immune compromised or had a major underlying medical condition or ENT problem
• Were pregnant, smoked or consumed alcohol
• Had oral conditions such as existing periodontal disease, dental caries or xerostomia, or any systemic condition that could directly affect gingival condition
• Had recent (within the past 45 days) or planned (within the next 90 days) surgery of any kind (major or minor)
• Were using medications (antibiotics, anti-inflammatories) that may influence the outcome or had ongoing or recent (within 1 month) use of probiotics unrelated to the study
• Had participated in another clinical trial within 30 days prior to randomization
• Had experienced any nausea, fever, vomiting, bloody diarrhea or severe abdominal pain within the past 30 days
• Had orthodontic molar bands

2.3.2 Informed Consent

Once a subject was deemed eligible, one of the two principal investigators, verbally requested subject and/or guardian consent to participate in the study. Study rationale, design, and potential risks and benefits were reviewed both verbally and with written documentation (Appendix 3). The ‘Patient Information and Consent Form’ further outlined details of the procedures, time requirements, the right to refuse participation, compensation, alternatives to study participation and use of data. Potential subjects and guardians were then allowed the chance to review the document and ask any questions in order to ensure voluntary participation. Consent was obtained both verbally and in writing. Written consent was documented from all guardians of participants younger than 18 years of age, or subject if he or she was 18 years old. Consent was given verbally from all subjects younger than 18 years old. Since compliance was a vital factor in this study, consent and enthusiasm to participate were emphasized to all subjects.

In addition to the consent form affiliated with this particular study, each participant had also previously consented to treatment and collection of personal information for the use of health management and research at the University of Toronto, Faculty of Dentistry.
2.3.3 Compensation

This study required attendance at 4 different time points. In order to minimize subject burden, 3 of the 4 time points coincided with the subject’s regular orthodontic visits at the Graduate Orthodontic Clinic, University of Toronto. One additional appointment (T2), 14 days after the initial data collection, did not coincide with orthodontic visits. Each participant received a total of $75 in shopping mall gift cards for their participation in this clinical trial. Compensation was given in installments, with a $25 gift card at T2 and another $50 gift card upon completion of the study, at T4.

2.3.4 Confidentiality

Each participant was assigned a unique numerical subject ID number upon enrollment into the study. All samples and data collected utilized the subject ID while confidentiality of personal identity was maintained. All forms with patient information were locked in a cabinet, which was then locked in a private office at University of Toronto, Faculty of Dentistry.

2.3.5 Subject Withdrawal

Participants were withdrawn from the study if they met any of the following criteria:

1. Personal reasons: as stated in the Informed Consent Form, a subject may withdraw from the study for any reason at any time.

2. Clinical judgment of health care practitioner: a subject may be withdrawn from the study if, in the opinion of the dentist, it is not in the subject's best interest to continue.
This includes, but is not limited to, adverse events related to the ingestion of the product causing clinically significant illness or the need for prohibited concomitant medication (i.e., antibiotics) during the course of the trial.

The clinical data and samples collected from such subjects were withdrawn from the study analysis.

2.4 STUDY DESIGN

This study was designed as a randomized, double-blind, placebo controlled trial which examined the ability of the Lorodent probiotic lozenge to reduce clinical gingivitis and subgingival plaque levels of selected periopathogens (P. intermedia, C. rectus and F. nucleatum) in patients undergoing comprehensive fixed orthodontic treatment.

Following informed consent, eligible participants were randomly allocated to either lozenge Group A or Group B. One group represented the intervention probiotic lozenge group and the other was the control placebo lozenge group. At the time of group allocation, it was not known to the examiners or the participants which group was the intervention and which was the placebo control. The probiotic and placebo lozenges looked and tasted the same and the only difference in packaging was if a lozenge was labeled Group A or B. Lozenges were administered for 28 consecutive days, succeeded by another 28-day follow-up without lozenge administration, for a total trial length of 56 days (Figure 10).227 Previous studies on probiotic lozenges have examined their use with an approximate 1-month period of intervention.221,222 An additional time point midway through the 28-day intervention was added to the present study in attempt to gain a better understanding of any progression of changes that may occur. A fourth time point was added 28 days after cessation of lozenge administration to evaluate if any potential changes would persist after discontinuation of lozenge administration. Data
and sample collection were taken at four time points:

T1 – baseline examination and sample collection at day 0 and initiation of lozenge administration.

T2 – examination and sample collection at day 14.

T3 – examination and sample collection at day 28. Lozenge administration ceased and all remaining lozenges returned to investigators.

T4 – follow up examination and sample collection at day 56.

**Figure 10.** Study design.

### 2.4.1 Randomization and Blinding

Blocked randomization was carried by first generating an online randomization sequence (random.org) for a predetermined 1:1 allocation ratio to ensure each group
was the same size. Participants were given a unique Subject ID based on their sequence of enrollment. This ID, which did not use any personal identifiers, was used throughout the course of the study. The allocation sequence from random.org was 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 5.

2.4.2 Data and Sample Collection

The following measures of dental health and samples were collected at each of the four time points:

1. Modified Gingival Index (MGI)
2. Subgingival plaque sample
3. Unstimulated saliva sample

The parameters of interest were clinical gingivitis and levels of selected periopathogens in subgingival plaque. Data collection was carried out in the form of MGI scores to assess clinical gingivitis. The parameters outlining MGI are defined in the following section.

Use of a partial periodontal recording and sampling was utilized in this study. Modification of the Ramfjord teeth to include canines rather than first premolars was made to allow for examination of the canines, which have the highest incidence of white spot lesions during fixed orthodontic treatment.\textsuperscript{231,232} This change was included to facilitate the first arm of this study, which examined the use of probiotics in relation to supragingival plaque accumulation and caries.\textsuperscript{227}

Subgingival plaque was used to assess the levels of \textit{P. intermedia}, \textit{C. rectus} and
*F. nucleatum* at the various time points along with the presence of *S. salivarius K12* and *Lactobacillus*. Plaque samples were collected using sterile stainless steel Gracey curettes as outlined in previous studies such as Ooshima et al. (2003).\textsuperscript{116} Plaque samples from teeth #16, 21, 23, 36, 41, and 43 were combined at each individual time point and placed in a 1.5mL Eppendorf tube (Figure 11A).

Saliva samples were collected to evaluate the presence of probiotics (*S. salivarius K12* and total lactobacilli) in unstimulated saliva. While seated in the upright position, each subject expectorated approximately 2 mL of unstimulated saliva into a 15 mL Falcon tube (Figure 11B). After collection, saliva and plaque samples were immediately transferred to a -20°C freezer in a secured laboratory. Dry ice was used during the transport of samples to the University of Western Ontario. These methods were similar to the collection and storage of samples seen in other studies examining periodontal pathogens.\textsuperscript{217,233} Each sample of subgingival plaque and saliva was tagged with a unique label identifying the subject ID number, the lozenge group (A or B), visit number and type of sample (Figure 11C).

**Figure 11.** Plaque and saliva sample collection and labeling. (A) Subgingival plaque sample placed in sterile 1.5mL plastic Eppendorf tube. (B) Subject expectorating unstimulated saliva in sterile 15 mL Falcon tube. (C) Example of sample labeling which included subject ID, lozenge group, visit number and type of sample.
2.4.3 **Patient Instructions**

Subjects were given a bottle of either lozenge A or B. All participants were instructed to take the lozenges for 28 days as follows: slowly dissolve on the tongue two lozenges twice a day (morning and evening) for the first 7 days and then two lozenges once a day for the next 21 days. Subjects were advised to take the lozenges after breakfast and dinner and not to brush their teeth for one hour after lozenge administration. A compliance calendar (Figure 12) was provided for each patient to indicate the days which lozenges were taken. The calendars had a magnetic backing that allowed them to be displayed on the subject’s refrigerator at home. Since it was advised that lozenges be stored in the refrigerator, it was logical to place the compliance calendar in a conveniently stationed and accessible location. Honesty in calendar recordings was stressed and patients were made aware that there was no punishment for not taking the lozenges. Participants were instructed not to brush their teeth before the appointment or upon arrival to the clinic. Otherwise, subjects were encouraged to continue their normal regime of oral care including brushing and flossing. Participants were instructed not to use any antimicrobial mouth rinse during the 56-day trial period. In addition to being asked about adverse events at each appointment, patients were asked to report any adverse event immediately if one were to take place.
**Figure 12.** Example of the compliance calendar. A check mark indicates one lozenge taken. An empty box indicates lozenge not taken.

### 2.4.4 Monitoring Compliance and Safety

Compliance calendars were used to both encourage and assess compliance of lozenge administration. Each box represented one lozenge. Patients were instructed to check off one box for each lozenge taken, or to leave the box empty if the lozenge was not taken. Upon study completion, compliance was assessed based on the percentage of boxes/lozenges that were taken from the total number of lozenges that were supposed to be taken (for a total of 70 lozenges). A patient was deemed “compliant” if administration was $\geq 70\%$, and “non-compliant” of lozenge administration was $<70\%$.

Monitoring of lozenge safety was conducted through verbal questioning at each appointment. Participants were asked about any adverse event such as fever or gastrointestinal discomfort including nausea, vomiting, bloody diarrhea or severe abdominal pain. Subjects were also asked about any changes to their medical history or if any antibiotics had been taken since commencement of the study.
2.5 OUTCOMES OF INTEREST

2.5.1 Evaluation of Clinical Gingivitis

The Modified Gingival Index (MGI)\textsuperscript{129} was used to assess clinical gingivitis in this study. The scale ranges from 0-4, representing a gradient from gingival health all the way to severe inflammation (Table 3). An increase in the MGI score corresponds with amassed gingival inflammation. A score of 0-4 was assigned for the buccal, lingual, mesial and distal surfaces of teeth # 16, 21, 23, 36, 41 and 43 at four time points (T1, T2, T3 and T4). A total of 24 MGI scores were documented for each subject at each time point. A composite MGI (cMGI) score was calculated for each participant. The cMGI score was comprised of the sum of all 24 MGI scores (6 teeth with 4 surfaces) and represented the subject’s overall gingival status at each time point.

2.5.2 Microbial Evaluation

Microbial analysis was carried out on the saliva and subgingival plaque samples. The levels of \textit{C. rectus}, \textit{F. nucleatum} and \textit{P. intermedia} were assessed in subgingival plaque. The presence of \textit{S. salivarius} K12 and total \textit{Lactobacillus} were examined in both subgingival plaque and saliva to identify their existence and quantify their presence. Quantitative real-time PCR was used to determine the levels of the bacteria of interest. Samples were first to be tested to compare baseline (T1) and 28 days after lozenge administration (T3) for each subject. If significant results were obtained, samples from T2 and T4 would then also be tested to evaluate bacterial levels after 14 days of lozenge administration (T2) and 28 days after cessation of lozenge administration (T4).
2.5.3 DNA Extraction and Quantification

DNA extraction and quantification are as outlined in the aforementioned counterpart of this investigation, as provided to the principal investigators by their colleagues who conducted the laboratory analysis at the University of Western Ontario:\textsuperscript{227}

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 mMTris [pH 8.0], 1 mM EDTA). The extraction was carried out according to the manufacturer’s protocol (Appendix 10) 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.

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.
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.

<table>
<thead>
<tr>
<th>Bacterial Species</th>
<th>Primer Sequence (5'-3')</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>S. salivarius K12</strong></td>
<td>F: CGGCAAAACCAA AGCTAGAG</td>
</tr>
<tr>
<td></td>
<td>R: ACGTGGTTTTTTGGGGTTAG</td>
</tr>
<tr>
<td><strong>Total lactobacilli</strong></td>
<td>F: TGGAAACAGRTGCTAATACCG</td>
</tr>
<tr>
<td></td>
<td>R: GTCCATTGTGGAAGATTCCC</td>
</tr>
<tr>
<td><strong>C. rectus</strong></td>
<td>F: TTTCGGAGCGTAAACTCCTTTTC</td>
</tr>
<tr>
<td></td>
<td>R: TTTCTGCAAGCAGACACTCTTT</td>
</tr>
<tr>
<td><strong>P. intermedia</strong></td>
<td>F: CGTGGAACCAAAGATTTCATCGTGAGGA</td>
</tr>
<tr>
<td></td>
<td>R: CCGCTTTACTCCCCCAACAAA</td>
</tr>
<tr>
<td><strong>F. nucleatum</strong></td>
<td>F: AGAGTTTTGATCCTGGCTCAG</td>
</tr>
<tr>
<td></td>
<td>R: GTCATCGTGACACAGAATTTGCTG</td>
</tr>
<tr>
<td><strong>Total bacteria</strong></td>
<td>F: TCCTACGAGGGAGGCACAGT</td>
</tr>
<tr>
<td></td>
<td>R: GGACTACCAGGGTATCTAATCCTGTT</td>
</tr>
</tbody>
</table>
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 *C. rectus* DNA, *F. nucleatum* DNA, *P. intermedia* DNA, *S. salivarius* K12 DNA and total lactobacilli DNA were calculated as a percentage of that.

2.6 **EXAMINER ALIGNMENT AND ASSESSMENT**

All data and sample collection was carried out by the two principal examiners (SH and FE). Both examiners were licensed dentists and graduate students in the Department of Orthodontics, Faculty of Dentistry, University of Toronto.

Examiner alignment and assessment were carried out as outlined by Hefti and Preshaw (2012).\(^{132}\) According to these authors, there is no gold standard or infallible gauge for clinical assessment of periodontal condition; therefore, the examiners cannot be “calibrated” to such a standard. Instead, examiners are aligned and assessed so that their appraisal of the periodontal condition can be consistent. This is a two-step process. First, the examiners are aligned to establish mutual agreement, and secondly, they are assessed for reproducibility. The goal was to establish substantial agreement or higher inter-rater reliability\(^{234}\) for recording of the Modified Gingival Index (MGI).

To start off, the examiners discussed the parameters of the MGI scoring system (Table 3). Together, the examiners then evaluated intra-oral photos representing the various
levels of MGI (Sample A). Each examiner screened the buccal, mesial and distal surfaces of teeth #21, 23, 41 and 43 and verbally called out the designated score. Any discrepancies between examiners were discussed and resolved. Teeth #16 and 36 along with all lingual surfaces were not used in this assessment due to their altered visibility in intra-oral photographs. No recording of the measurements was made.

After alignment, an assessment of the inter-rater reliability was conducted using a different set of photos (Sample B), also representing various stages of the MGI scoring scale. This time, examiners evaluated photos separately and a recording of their measurement was made. In addition to photographs, the examiners also scored three orthodontic patients (Sample C) who were not enrolled in the study, and recorded scores of all the surfaces (buccal, lingual, mesial and distal) on all the teeth of interest (#16, 21, 23, 36, 41 and 43) as described in the study protocol. The scoring from Samples B and C were then used to assess inter-rater reliability. Upon study completion, examiners re-scored the intraoral photographs from Sample B to evaluate intra-rater reliability.

2.7 ETHICAL APPROVAL AND FUNDING

Ethical approval was obtained from the University of Toronto Health Sciences Research Ethics Board (#30148) (Appendix 6) and the University of Western Ontario (#101955) (Appendix 7). The clinical trial was registered and conducted in compliance with Health Canada (#185428) (Appendix 8) and funded by the Ontario Centres of Excellence (#20964) (Appendix 9). Probiotic and placebo lozenges were provided at no cost by Integra Medical Inc. as part of the collaboration with the Ontario Centres of Excellence.
2.8 **Statistical Methods**

The statistical methods utilized are as outline in the preceding counterpart to this trial:\textsuperscript{227}

### 2.8.1 Sample Size Determination

Sample size was determined to provide sufficient power to test the hypothesis that an oral probiotic lozenge would significantly reduce clinical gingivitis compared to a placebo lozenge. An *a priori* sample size determination was conducted to assure the study had sufficient statistical power to detect a difference between the two groups, if one truly existed.\textsuperscript{235}

An *a priori* power analysis often involves accepting several assumptions in order to determine the required sample size. Otherwise, determining sample size before the study commencement would be rather complex and involve a large pilot study. A power analysis was conducted using a test of two independent proportions, which tested relative differences between groups rather than absolute changes and thus did not require the standard deviation of the groups to be known.

- Sample size calculations were based on:
  - Primary outcome measure: MGI scores
  - One tail – this study was directional; testing the hypothesis that the probiotic only reduces clinical gingivitis (MGI) and does not increases it.
  - Type I error: $\alpha = 0.05$
  - Type II error: $\beta = 0.20$
  - Statistical power: $1 - \beta = 0.80$
  - Minimum detectable difference between groups (the difference in proportions): 25%
The research committee decided that for the probiotic to be a worthwhile adjunct to orthodontic treatment, it should reduce gingivitis scores by 25% compared to the placebo group. This means that for the 4 surfaces per tooth (buccal, mesial, distal, and lingual), and 6 teeth examined, there were a total of 24 sites. Based on the professional opinion of the dental specialists on the research committee, it was concluded that the probiotic may be beneficial if a minimum of 6 out of 24 sites (25%) would show a reduction in MGI. Improvement of gingivitis was defined as a lowering in the MGI by at least 1 grading level. The probiotic would be deemed effective if MGI were reduced in 25% of the sites. These estimations could have vary well been set at different values, but these are the values that were agreed upon to be tested.

A statistical power analysis program, G*Power 3.1, determined that for a difference in proportion of 0.25 with power set at 0.8 (1-sided, \( \alpha = 0.05 \)), a total of 28 subjects per group would be required (Figure 13).\(^{236}\) An additional 10% of subjects were added to account for dropouts or loss to follow-up. This yielded a sample of 30-31 participants per group, for a total sample size of \( n = 60-62 \).
2.8.2 Baseline Comparisons

Demographic, microbial and clinical data were compared for balance across the probiotic and placebo lozenge groups. This included comparisons for gender, age, MGI and cMGI scores at baseline (T1), along with the proportion of *F. nucleatum* and total lactobacilli DNA compared to total bacterial DNA at T1. Evaluation of the relative proportions of *C. rectus*, *P. intermedia*, and *S. salivarius* K12 were excluded from both baseline comparisons and any testing thereafter due to reasons discussed in section 3.1.
A chi-square test was used to compare the proportions of each gender between the probiotic and placebo groups. The age at enrollment was compared using an independent samples t-test (parametric) and a Mann-Whitney U-test (non-parametric).

Baseline MGI between lozenge groups, and between genders was evaluated using the frequency distribution of the various levels of the MGI scoring scale (0-4) using a chi-square test. The chi-square test was also used to evaluate the frequency distribution of MGI scores between the probiotic and placebo lozenge groups subdivided by tooth type (incisor, canine, molar) and tooth surface (buccal, lingual, mesial/distal).

Comparison of baseline cMGI scores between lozenge groups and between genders was evaluated using median scores and the Mann-Whitney U-test. The Mann-Whitney U-test was also used to compare baseline cMGI scores subdivided by tooth type (incisor, canine, molar) and tooth surface (buccal, lingual, mesial/distal).

The Mann-Whitney U-test was used to evaluate baseline microbial analysis between lozenge groups. Further detail on the baseline evaluation of the selected periodontal pathogens is described in section 3.1.

2.8.3 Gingival Analyses

MGI scores are ordinal, non-parametric data; therefore, averaging the scores can confound the outcome. Frequency analysis is more accurate to evaluate this type of data. The Wilcoxon Signed-rank test was used to evaluate changes in MGI frequency distribution within each group at each time point (T2, T3, and T4) compared to baseline (T1). The Mann-Whitney U-test was used to evaluate changes between groups at each time point compared to baseline.

The improvement, worsening or ties (no change) in MGI and cMGI scores from baseline to each time point were compared within each group and statistically tested using the
Wilcoxon Signed-rank test.

Comparison of the change in MGI and cMGI scores from baseline to each time point between the probiotic and placebo test groups, also subdivided by tooth type (incisor, canine, molar) and tooth surface (buccal, lingual, mesial/distal) was statistically gauged using the Mann-Whitney U-test.

2.8.4 Microbial Analysis

Statistical analysis on the microbial component of this study was only performed on the levels of *F. nucleatum* and total lactobacilli, for reasons discussed in section 3.1. The Wilcoxon Signed-rank test was used to assess changes in the proportions of total lactobacilli DNA relative to total bacterial DNA in unstimulated saliva samples, within each lozenge group, from T1 to T3. The Mann-Whitney U-test was then used to evaluate the change experienced in the proportion of total lactobacilli DNA in unstimulated saliva, between the probiotic and placebo groups from T1 to T3.

The proportion of probiotic (total lactobacilli) and pathogen (*F. nucleatum*) DNA were then assessed in subgingival plaque samples. The Wilcoxon Signed-rank test was used to statistically analyze the changes in proportions of *F. nucleatum* and total lactobacilli DNA in subgingival plaque within the probiotic and placebo groups from T1 to T3. A comparison of the amount of change of these parameters between lozenge groups was evaluated using the Mann-Whitney U-test.

If statistically significant changes in the proportion of bacteria in the saliva and subgingival plaque samples were to be seen between groups at T3 compared to T1, additional tests would be carried out to evaluate the proportion of the selected bacteria at T2 and T4. If no statistical differences in the proportion of probiotic or pathogenic bacteria were seen in the selected samples at T1 compared to T3, then no further
testing would be completed on samples gathered from the T2 or T4 time-points.

2.8.5 **Inter-rater and Intra-rater Reliability**

Inter-rater and intra-rater reliability was assessed using weighted kappa statistics calculated 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 MGI 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).²³⁴

| Table 7. Interpretation of the kappa statistic for inter-rater and intra-rater reliability²³⁴ |
|-----------------------------------------------|---------------------------------------------|
| 0.00 | No agreement |
| 0.0-0.20 | Slight agreement |
| 0.21-0.40 | Fair agreement |
| 0.41-0.60 | Moderate agreement |
| 0.61-0.80 | Substantial agreement |
| 0.81-0.99 | Almost perfect agreement |
| 1.00 | Perfect agreement |

2.8.6 **Assessment of Compliance**

Compliance was appraised at two time points during the study: day 14 (T2) and day 28 (T3, lozenge administration discontinued).

The original study design included a comparison of the proportion of ‘compliant’ (≥70% lozenge consumption) and ‘non-compliant’ (<70% lozenge consumption) participants
between probiotic and placebo group. However, no subjects reported a compliance of less than 70%. Instead, it was decided that a comparison of the proportion of participants with 'perfect compliance' (100% lozenge consumption) was to be made with those with 'less than perfect compliance' (<100% lozenge consumption). This comparison was carried out between the probiotic and placebo groups at T2 and T3 and between males and females at T2 and T3. A 2-sided chi-square test was used for statistical analysis if both of the subgroups consisted of 5 or more subjects. For comparisons with less than 5 subjects in each subgroup, a 2-sided Fisher’s Exact test was used instead a chi-square test since having less than 5 individuals in a group would be a violation of an assumption of the chi-square test.

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

CHAPTER 3: RESULTS

A rolling enrollment was carried out from August to October 2014 by screening patients from the Graduate Orthodontic Clinic, University of Toronto, Faculty of Dentistry, according to the inclusion and exclusion criteria outlined above. A total of 87 patients were screened: 19 did not meet the inclusion criteria (i.e., presence of molar bands, unerupted canines, active caries and fewer than 20 orthodontically bonded teeth); 8 subjects who met the inclusion and exclusion criteria declined participation for undisclosed reasons. The final sample size was 60. Once enrolled, each participant was randomly assigned to either the probiotic or placebo group, with a total of 30 subjects in each group. One participant in each group did not complete the trial. One subject was withdrawn due to reports of adverse events including gastrointestinal discomfort and
diarrhea and later determined to belong to the placebo group. A participant in the probiotic group was withdrawn from the study due to administration of systemic antibiotics after surgery to repair a broken elbow.

After the loss of 1 participant in each group, a total of 58 subjects completed the clinical trial, with total sample sizes of 29 in each of the placebo and probiotic groups. The trial was carried out from August to December 2014. An illustration of the patient enrollment, allocation, follow-up and analysis is portrayed in Figure 14.

**Figure 14.** Participant recruitment and flow in current study.  

\(^{227}\)
3.1 **Baseline Characteristics**

The probiotic group had 16 males and 13 females, whereas the placebo group consisted of 9 males and 20 females, with an overall higher proportion of females (56.9%) compared to males (43.1%) (Table 8 and Figure 15). Although the placebo group had more females (69.0%) than the probiotic group (44.8%), it was not statistically significant ($p = 0.063$).

There was no statistically significant difference between the mean age of the subjects in the probiotic and placebo groups whether the data was compared parametrically ($p = 0.812$) or non-parametrically ($p = 0.828$) (Table 8). Overall, the average age was $15.69 \pm 1.70$ years, with a mean of $15.75 \pm 1.67$ and $15.64 \pm 1.75$ years in the probiotic and placebo groups, respectively. In summary, the baseline characteristics on demographic (gender and age) and clinical presentation (MGI and cMGI) were not statistically different between the probiotic and placebo groups.

<table>
<thead>
<tr>
<th>Table 8. Demographic distribution in the probiotic and placebo groups</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gender</strong></td>
</tr>
<tr>
<td>Male</td>
</tr>
<tr>
<td>Female</td>
</tr>
<tr>
<td><strong>Age (years)</strong></td>
</tr>
</tbody>
</table>

* Mean ± standard deviation
† Chi-square test
‡ Independent samples t-test
§ Mann-Whitney U-test
Figure 15. Gender distribution in the probiotic and placebo groups.

There was no significant difference in the MGI or cMGI scores at baseline (T1) between the probiotic and placebo groups (\( p = 0.823 \) and \( p = 0.560 \), respectively) (Tables 9 and 12, Figures 16 and 19). However, a significant difference in the frequency distribution of MGI was seen between males and females at T1 (\( p = 0.050 \)). At baseline, males showed a greater proportion of higher MGI scores compared to females (Table 9 and Figure 16). No significant difference was seen between each lozenge group when examining the baseline MGI scores subdivided by tooth type for incisor (\( p = 0.984 \)), canine (\( p = 0.981 \)) or molar (\( p = 0.176 \)) (Table 10 and Figure 17). Similarly, when the data was subdivided by surface type, no statistical difference was found for the buccal (\( p = 0.438 \)), lingual (\( p = 0.156 \)) or mesial/distal (\( p = 0.592 \)), between lozenge groups (Table 11 and Figure 18).

Likewise, when the cMGI scores of the two groups were compared, there was no significant difference when the groups were subdivided by tooth type incisor (\( p = 0.74 \)), canine (\( p = 0.82 \)), or molar (\( p = 0.19 \)) (Table 13 and Figure 20) or by tooth surface buccal (\( p = 0.28 \)), lingual (\( p = 0.53 \)) or mesial/distal (\( p = 0.78 \)) (Table 14 and Figure 21).
Table 9. Frequency distribution of the baseline (T1) MGI scores between lozenge groups and between genders

<table>
<thead>
<tr>
<th>MGI Scores</th>
<th>Overall</th>
<th>Probiotic</th>
<th>Placebo</th>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MGI 0</td>
<td>MGI 1</td>
<td>MGI 2</td>
<td>MGI 3</td>
<td>MGI 4</td>
</tr>
<tr>
<td>Overall</td>
<td>142 (10%)</td>
<td>493 (35%)</td>
<td>444 (32%)</td>
<td>264 (19%)</td>
<td>49 (4%)</td>
</tr>
<tr>
<td>Probiotic</td>
<td>68 (10%)</td>
<td>239 (34%)</td>
<td>225 (32%)</td>
<td>138 (20%)</td>
<td>26 (4%)</td>
</tr>
<tr>
<td>Placebo</td>
<td>74 (11%)</td>
<td>254 (36%)</td>
<td>219 (31%)</td>
<td>126 (18%)</td>
<td>23 (3%)</td>
</tr>
<tr>
<td>Male</td>
<td>61 (10%)</td>
<td>187 (31%)</td>
<td>203 (34%)</td>
<td>124 (21%)</td>
<td>25 (4%)</td>
</tr>
<tr>
<td>Female</td>
<td>81 (10%)</td>
<td>306 (39%)</td>
<td>241 (30%)</td>
<td>140 (18%)</td>
<td>24 (3%)</td>
</tr>
</tbody>
</table>

† Chi-square test

Figure 16. Comparison of the baseline (T1) frequency distribution of MGI scores overall, between lozenge groups and between genders (*p < 0.05).
Table 10. Frequency distribution of the baseline (T1) MGI scores by tooth type between lozenge groups

<table>
<thead>
<tr>
<th>Group</th>
<th>MGI 0</th>
<th>MGI 1</th>
<th>MGI 2</th>
<th>MGI 3</th>
<th>MGI 4</th>
<th>Total</th>
<th>P-value†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Incisor</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Probiotic</td>
<td>24 (10%)</td>
<td>77 (33%)</td>
<td>63 (27%)</td>
<td>51 (22%)</td>
<td>17 (7%)</td>
<td>232 (100%)</td>
<td>0.984</td>
</tr>
<tr>
<td>Placebo</td>
<td>22 (9%)</td>
<td>83 (36%)</td>
<td>61 (26%)</td>
<td>50 (22%)</td>
<td>16 (7%)</td>
<td>232 (100%)</td>
<td></td>
</tr>
<tr>
<td>Canine</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Probiotic</td>
<td>18 (8%)</td>
<td>71 (31%)</td>
<td>78 (34%)</td>
<td>58 (25%)</td>
<td>7 (3%)</td>
<td>232 (100%)</td>
<td>0.981</td>
</tr>
<tr>
<td>Placebo</td>
<td>16 (7%)</td>
<td>68 (29%)</td>
<td>83 (36%)</td>
<td>59 (25%)</td>
<td>6 (3%)</td>
<td>232 (100%)</td>
<td></td>
</tr>
<tr>
<td>Molar</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Probiotic</td>
<td>26 (11%)</td>
<td>91 (39%)</td>
<td>84 (36%)</td>
<td>29 (13%)</td>
<td>2 (1%)</td>
<td>232 (100%)</td>
<td>0.176</td>
</tr>
<tr>
<td>Placebo</td>
<td>36 (16%)</td>
<td>103 (44%)</td>
<td>75 (32%)</td>
<td>17 (7%)</td>
<td>1 (0%)</td>
<td>232 (100%)</td>
<td></td>
</tr>
</tbody>
</table>

† Chi-square test

Figure 17. Comparison of the baseline (T1) frequency distribution of MGI scores between the probiotic and placebo groups, subdivided by tooth type (*p < 0.05).
Table 11. Frequency distribution of the baseline (T1) MGI scores by tooth surface between lozenge groups

<table>
<thead>
<tr>
<th>Group</th>
<th>MGI 0</th>
<th>MGI 1</th>
<th>MGI 2</th>
<th>MGI 3</th>
<th>MGI 4</th>
<th>Total</th>
<th>P-value†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buccal</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Probiotic</td>
<td>2 (1%)</td>
<td>56 (32%)</td>
<td>66 (38%)</td>
<td>43 (25%)</td>
<td>7 (4%)</td>
<td>174 (100%)</td>
<td>0.438</td>
</tr>
<tr>
<td>Placebo</td>
<td>3 (2%)</td>
<td>62 (36%)</td>
<td>73 (42%)</td>
<td>33 (19%)</td>
<td>3 (2%)</td>
<td>174 (100%)</td>
<td></td>
</tr>
<tr>
<td>Lingual</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Probiotic</td>
<td>62 (36%)</td>
<td>67 (39%)</td>
<td>39 (22%)</td>
<td>6 (3%)</td>
<td>0 (0%)</td>
<td>174 (100%)</td>
<td>0.156</td>
</tr>
<tr>
<td>Placebo</td>
<td>61 (35%)</td>
<td>80 (46%)</td>
<td>31 (18%)</td>
<td>1 (1%)</td>
<td>1 (1%)</td>
<td>174 (100%)</td>
<td></td>
</tr>
<tr>
<td>Mesial/Distal</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Probiotic</td>
<td>4 (1%)</td>
<td>116 (33%)</td>
<td>120 (34%)</td>
<td>89 (26%)</td>
<td>19 (5%)</td>
<td>348 (100%)</td>
<td>0.592</td>
</tr>
<tr>
<td>Placebo</td>
<td>10 (3%)</td>
<td>112 (32%)</td>
<td>115 (33%)</td>
<td>92 (26%)</td>
<td>19 (5%)</td>
<td>348 (100%)</td>
<td></td>
</tr>
</tbody>
</table>

† Chi-square test

Figure 18. Comparison of the baseline (T1) frequency of MGI scores in the probiotic and placebo groups, subdivided by tooth surface (*p < 0.05).
Table 12. Comparison of the baseline cMGI scores between lozenge groups and between gender

<table>
<thead>
<tr>
<th>Group</th>
<th>Median</th>
<th>Range</th>
<th>IQR</th>
<th>P-value†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Overall (n = 58)</td>
<td>42</td>
<td>19-60</td>
<td>32-49</td>
<td></td>
</tr>
<tr>
<td>Lozenge Group</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Probiotic (n = 29)</td>
<td>45</td>
<td>24-56</td>
<td>33.5-49.5</td>
<td>0.560</td>
</tr>
<tr>
<td>Placebo (n = 29)</td>
<td>42</td>
<td>19-60</td>
<td>31-48.5</td>
<td></td>
</tr>
<tr>
<td>Gender</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male (n = 25)</td>
<td>45</td>
<td>24-57</td>
<td>33.5-50.5</td>
<td>0.260</td>
</tr>
<tr>
<td>Female (n = 33)</td>
<td>41</td>
<td>19-60</td>
<td>31-48</td>
<td></td>
</tr>
</tbody>
</table>

† Mann-Whitney U Test

Figure 19. Comparison of the baseline cMGI scores between lozenge groups and between genders. Box plots show median values (solid vertical line), interquartile range (outlined box), and the highest and lowest values within the upper and lower limits (whiskers).
Table 13. Comparison of the baseline cMGI scores between lozenge groups subgrouped by tooth type

<table>
<thead>
<tr>
<th>Group</th>
<th>Median</th>
<th>Range</th>
<th>IQR</th>
<th>P-value†</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Incisor</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Probiotic (n = 29)</td>
<td>15</td>
<td>7-21</td>
<td>11.5-18.5</td>
<td>0.74</td>
</tr>
<tr>
<td>Placebo (n = 29)</td>
<td>14</td>
<td>6-24</td>
<td>10.5-18</td>
<td></td>
</tr>
<tr>
<td><strong>Canine</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Probiotic (n = 29)</td>
<td>15</td>
<td>9-21</td>
<td>11.5-18</td>
<td>0.82</td>
</tr>
<tr>
<td>Placebo (n = 29)</td>
<td>15</td>
<td>7-22</td>
<td>12-18.5</td>
<td></td>
</tr>
<tr>
<td><strong>Molar</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Probiotic (n = 29)</td>
<td>12</td>
<td>6-19</td>
<td>9-15</td>
<td>0.19</td>
</tr>
<tr>
<td>Placebo (n = 29)</td>
<td>10</td>
<td>2-17</td>
<td>7-15</td>
<td></td>
</tr>
</tbody>
</table>

† Mann-Whitney U-test

Figure 20. Comparison of the baseline cMGI scores between lozenge groups, subdivided by tooth type (incisor, canine, molar). Box plots show median values (solid vertical line), interquartile range (outlined box), and the highest and lowest values within the upper and lower limits (whiskers).
Table 14. Comparison of the baseline cMGI scores between lozenge groups subdivided by tooth surface

<table>
<thead>
<tr>
<th>Group</th>
<th>Median</th>
<th>Range</th>
<th>IQR</th>
<th>P-value†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buccal</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Probiotic (n = 29)</td>
<td>12</td>
<td>6-17</td>
<td>9-14.5</td>
<td>0.28</td>
</tr>
<tr>
<td>Placebo (n = 29)</td>
<td>12</td>
<td>6-17</td>
<td>8.5-13</td>
<td></td>
</tr>
<tr>
<td>Lingual</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Probiotic (n = 29)</td>
<td>6</td>
<td>0-11</td>
<td>3.5-8.5</td>
<td>0.53</td>
</tr>
<tr>
<td>Placebo (n = 29)</td>
<td>5</td>
<td>0-13</td>
<td>2-8</td>
<td></td>
</tr>
<tr>
<td>Mesial/Distal</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Probiotic (n = 29)</td>
<td>25</td>
<td>13-32</td>
<td>20-28.5</td>
<td>0.78</td>
</tr>
<tr>
<td>Placebo (n = 29)</td>
<td>25</td>
<td>13-36</td>
<td>18.5-28</td>
<td></td>
</tr>
</tbody>
</table>

† Mann-Whitney U-test

Figure 21. Comparison of the baseline cMGI scores between lozenge groups, subdivided by tooth surface (buccal, lingual and mesial/distal). Box plots show median values (solid vertical line), interquartile range (outlined box), and the highest and lowest values within the upper and lower limits (whiskers).
Due to the lack of growth and difficulty with culturing *C. rectus* and *P. intermedia*, and the previously documented lack of expression of *S. salivarius* K12, only the levels of *F. nucleatum* and total lactobacilli DNA relative to total bacterial DNA in subgingival plaque were measured. In addition to this, the levels of total lactobacilli DNA relative to total DNA in the saliva samples was used to assay for the persistence of lactobacilli after exposure to a lactobacilli-containing probiotic lozenge compared to the placebo. No significant differences in the baseline proportion of *F. nucleatum* (p = 0.971) and total lactobacilli (p = 0.581) DNA between the probiotic and placebo groups in subgingival plaque samples were detected (Table 15). There was also no significant difference in the baseline proportion of total lactobacilli DNA in saliva between the two lozenge groups (Table 16).

<table>
<thead>
<tr>
<th>Group</th>
<th>% of <em>F. nucleatum</em> DNA at T1</th>
<th>P-value†</th>
<th>% of Lactobacilli DNA at T1</th>
<th>P-value†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Probiotic</td>
<td>0.0013</td>
<td>0.971</td>
<td>0.1165</td>
<td>0.581</td>
</tr>
<tr>
<td>Placebo</td>
<td>0.0013</td>
<td></td>
<td>0.0869</td>
<td></td>
</tr>
</tbody>
</table>

† Mann-Whitney U-test

<table>
<thead>
<tr>
<th>Group</th>
<th>% of Lactobacilli DNA in saliva at T1</th>
<th>P-value†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Probiotic</td>
<td>4.984790</td>
<td></td>
</tr>
<tr>
<td>Placebo</td>
<td>5.430305</td>
<td>0.335</td>
</tr>
</tbody>
</table>

† Mann-Whitney U-test
3.2 **Clinical Gingivitis Scores**

A change in clinical gingivitis was evaluated by the frequency distribution of MGI score from T1-T4 at both the median and mean levels, comparing each time point to the baseline (T1). The frequency distributions of the MGI scores from T1 to T4 are displayed in Table 17 and Figure 22. The median scores for both the probiotic and placebo groups were an MGI of 2 for all time points. Examining the median cMGI provided more detailed information on the general trend of clinical gingivitis than the median MGI. For the probiotic group, there was a trend towards reduced cMGI scores from T1 to T3, then a slight increase at T4, but still remaining below the baseline cMGI value. For the placebo group, there was a decline in the cMGI from T1 to T2, a slight increase at T3 and then back to the original cMGI score at T4. However, none of these trends showed statistical or clinical significance between the two groups.

Within each group, a comparison of an improvement, worsening or tie (no change) in MGI and cMGI score was made, with an improved score being one that is lower down on the scale and a worsening as having a larger value. A comparison of the improvement, worsening or ties in MGI and cMGI scores from baseline to each time point within each lozenge group revealed that both the probiotic and placebo lozenge groups experienced more sites of MGI improvement than worsening (Table 18 and Figure 23). However, the majority of sites did not change. The majority of subjects showed an improved cMGI score, with few subjects experiencing no change in cMGI. Statistical analysis revealed a significant improvement in MGI score for the probiotic at T3 (p = 0.000) and T4 (p = 0.004) and for the placebo group for all time points T2 (p = 0.037), T3 (p = 0.000), T4 (p = 0.024) compared to baseline (T1). A significant improvement was seen for the cMGI score of the placebo group between time points T1 and T3 (p = 0.023). When the magnitude of this improvement was considered, the median improvement in cMGI score was 1 cMGI unit from T1 to T2, and 2 units from T1 to both T3 and T4 (Table 18). Considering that cMGI was scored out of 96, a 1 or 2 unit
difference was not much of an improvement and was unlikely to be clinically significant.

Table 19 and Figure 24 display a comparison of the improvements in MGI and cMGI scores between groups from baseline to each time point. With a p-value set at 0.05, no significant differences were found when evaluating the improvement of MGI or cMGI between groups. The probiotic and placebo groups showed similar improvement in MGI scores at each time point. In attempt to examine the data further, statistical analysis was run to subdivide the groups by tooth (incisor, canine, molar) and by tooth surface (buccal, lingual, mesial/distal). There were no significant differences in the change in frequency of MGI scores between groups even if subdivided by tooth type or by tooth surface (p > 0.05) (Figure 25 and Figure 26).

<table>
<thead>
<tr>
<th>Table 17. Frequency distribution of the MGI scores from T1-T4 for the probiotic and placebo groups.</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
</tr>
<tr>
<td>Probiotic</td>
</tr>
<tr>
<td>T1</td>
</tr>
<tr>
<td>T2</td>
</tr>
<tr>
<td>T3</td>
</tr>
<tr>
<td>T4</td>
</tr>
<tr>
<td>Placebo</td>
</tr>
<tr>
<td>T1</td>
</tr>
<tr>
<td>T2</td>
</tr>
<tr>
<td>T3</td>
</tr>
<tr>
<td>T4</td>
</tr>
</tbody>
</table>
Figure 22. Frequency distribution of MGI scores at each time point for the probiotic and placebo groups.
Table 18. Comparison of the improvement, worsening or ties in MGI scores and cMGI scores from baseline to each time point within each lozenge group.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Change in Gingival Score</th>
<th>P-value†</th>
<th>Median Improvement</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Improved</td>
<td>Worsened</td>
<td>Same</td>
</tr>
<tr>
<td>MGI Scores</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Probiotic from T1 to T2 (n = 696)</td>
<td>119</td>
<td>94</td>
<td>483</td>
</tr>
<tr>
<td>Probiotic from T1 to T3 (n = 696)</td>
<td>167</td>
<td>99</td>
<td>430</td>
</tr>
<tr>
<td>Probiotic from T1 to T4 (n = 696)</td>
<td>176</td>
<td>127</td>
<td>393</td>
</tr>
<tr>
<td>Placebo from T1 to T2 (n = 696)</td>
<td>123</td>
<td>92</td>
<td>481</td>
</tr>
<tr>
<td>Placebo from T1 to T3 (n = 696)</td>
<td>163</td>
<td>101</td>
<td>432</td>
</tr>
<tr>
<td>Placebo from T1 to T4 (n = 696)</td>
<td>153</td>
<td>117</td>
<td>426</td>
</tr>
<tr>
<td>cMGI scores</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Probiotic from T1 to T2 (n = 29)</td>
<td>14</td>
<td>12</td>
<td>3</td>
</tr>
<tr>
<td>Probiotic from T1 to T3 (n = 29)</td>
<td>15</td>
<td>10</td>
<td>4</td>
</tr>
<tr>
<td>Probiotic from T1 to T4 (n = 29)</td>
<td>18</td>
<td>11</td>
<td>0</td>
</tr>
<tr>
<td>Placebo from T1 to T2 (n = 29)</td>
<td>17</td>
<td>12</td>
<td>0</td>
</tr>
<tr>
<td>Placebo from T1 to T3 (n = 29)</td>
<td>20</td>
<td>8</td>
<td>1</td>
</tr>
<tr>
<td>Placebo from T1 to T4 (n = 29)</td>
<td>17</td>
<td>11</td>
<td>1</td>
</tr>
</tbody>
</table>

† Wilcoxon Signed-rank test
* p < 0.05
**Figure 23.** A comparison of the improvement, worsening or ties in MGI and cMGI scores from baseline (T1) to each time point, within each lozenge group (* p < 0.05).

**Table 19.** Comparison of the change in MGI and cMGI scores from baseline to each time point between the probiotic and placebo groups.

<table>
<thead>
<tr>
<th>Outcome</th>
<th>Probiotic Mean, Median</th>
<th>Placebo Mean, Median</th>
<th>P-value†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MGI</td>
<td>cMGI</td>
<td>MGI</td>
</tr>
<tr>
<td>T1 to T2 improvement</td>
<td>0.04, 0</td>
<td>1.03, 0</td>
<td>0.05, 0</td>
</tr>
<tr>
<td>T1 to T3 improvement</td>
<td>0.11, 0</td>
<td>2.66, 1</td>
<td>0.11, 0</td>
</tr>
<tr>
<td>T1 to T4 improvement</td>
<td>0.08, 0</td>
<td>1.83, 3</td>
<td>0.06, 0</td>
</tr>
</tbody>
</table>

† Mann-Whitney U-test
Figure 24. Comparison of the MGI scores from baseline to each time point, between lozenge groups (* p < 0.05).
Figure 25. Comparison of the change in MGI scores from baseline to each time point, compared between lozenge groups, subdivided by tooth type (* p < 0.05).
Improvements in PI Scores from Baseline to Each Time Point Compared Between Lozenge Groups Subgrouped By Surface

Figure 26. Comparison of the change in MGI scores from baseline to each time point, compared between lozenge groups, subdivided by tooth surface (buccal, lingual, mesial/distal) (* p < 0.05).

3.3 PROBIOTIC LEVELS IN UNSTIMULATED SALIVA

The relative proportion of lactobacilli DNA from total DNA was tested at T1 and T3 to evaluate the presence of this probiotic family in unstimulated saliva using qPCR. The proportion of total lactobacilli DNA in unstimulated saliva decreased from T1 to T3 in...
both the probiotic (\(p = 0.826\)) and placebo (\(p = 0.096\)) groups, with no significant difference within groups (Table 20). When examining the change in proportion of lactobacilli DNA between groups, a positive median value denotes an increase in proportion and a negative value indicates a reduction in the proportion of total lactobacilli DNA (Table 21). Although both groups showed a small reduction in the median percentage of lactobacilli (-0.384% for the probiotic and -2.373% for the placebo), there was no significant difference in the amount of reduction of lactobacilli DNA between groups (\(p = 0.154\)). Since there was no significant difference in the proportion of total lactobacilli between T1 and T3, no additional tests were performed to assay the level of probiotic bacteria at T2 or T4.

<table>
<thead>
<tr>
<th>Table 20. Changes in the proportions of total lactobacilli DNA in unstimulated saliva samples, within the probiotic and placebo groups</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample</td>
</tr>
<tr>
<td>Probiotic % of lactobacilli DNA in saliva</td>
</tr>
<tr>
<td>Placebo % of lactobacilli DNA in saliva</td>
</tr>
</tbody>
</table>

† Wilcoxon Signed-rank test

<table>
<thead>
<tr>
<th>Table 21. Comparison of the amount of change experienced between the probiotic and placebo groups in their proportions of total lactobacilli DNA in unstimulated saliva from T1 to T3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample</td>
</tr>
<tr>
<td>Probiotic</td>
</tr>
<tr>
<td>Placebo</td>
</tr>
</tbody>
</table>

† Mann-Whitney U-test
The proportions of *F. nucleatum* and total lactobacilli DNA relative to total bacterial DNA was measured in each subgingival plaque sample using qPCR. Samples that had undetectable levels of DNA were excluded from statistical analysis. The numbers of samples included in the analyses are indicated in Table 22.

The median values for the proportion of *F. nucleatum* DNA relative to total bacterial DNA were similar for both the probiotic and the placebo group at T1 and T3, ranging from 0.0012% to 0.0013% (Table 22). The proportion of *F. nucleatum* DNA stayed the same for both groups from T1 to T3. Overall, there were no significant changes in the proportion of *F. nucleatum* DNA in the probiotic (p = 0.820) or placebo (p = 0.575) groups from T1 to T3 (Table 22). There was no change in the median value of *F. nucleatum* DNA from T1 to T3 and no significance between groups (p = 0.761) (Table 23).

The proportion of lactobacilli DNA relative to total bacterial DNA tended to increase in the probiotic group, from 0.1165% to 0.1752%, but this increase was not statistically significant (p = 0.527) (Table 22). In the placebo group, the proportion of lactobacilli DNA showed a small insignificant decrease from 0.0869% to 0.0147% (p = 0.976). The median change of lactobacilli DNA was a 0.0626% increase in the probiotic group, and a decrease of 0.0001% in the placebo group, with no significant difference in the percentage of change between groups (p = 0.749).

Since there were no significant differences in the proportions of *F. nucleatum* or total lactobacilli in subgingival plaque between T1 and T3, no additional tests were performed to assay the microbial levels at T2 or T4.
### Table 22. Changes in the proportions of *F. nucleatum* DNA and total lactobacilli DNA in subgingival plaque 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>Probiotic</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% of <em>F. nucleatum</em> DNA</td>
<td>0.0013</td>
<td>0.0012</td>
<td>same</td>
<td>0.820</td>
</tr>
<tr>
<td>(n=27)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% of Lactobacilli DNA</td>
<td>0.1165</td>
<td>0.1752</td>
<td>increase</td>
<td>0.527</td>
</tr>
<tr>
<td>(n=25)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Placebo</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% of <em>F. nucleatum</em> DNA</td>
<td>0.0013</td>
<td>0.0013</td>
<td>same</td>
<td>0.575</td>
</tr>
<tr>
<td>(n=26)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>% of Lactobacilli DNA</td>
<td>0.0869</td>
<td>0.0147</td>
<td>decrease</td>
<td>0.976</td>
</tr>
<tr>
<td>(n=23)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

† Wilcoxon Signed-rank test

### Table 23. Comparison of the amount of change experienced by the probiotic and placebo groups in their proportions of *F. nucleatum* DNA and lactobacilli DNA from T1 to T3

<table>
<thead>
<tr>
<th>Sample</th>
<th>Change in % of <em>F. nucleatum</em> DNA from T1 to T3</th>
<th>Change in % of lactobacilli DNA from T1 to T3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Median P-value†</td>
<td>Median P-value†</td>
</tr>
<tr>
<td>Probiotic</td>
<td>0.0000</td>
<td>0.761</td>
</tr>
<tr>
<td>Placebo</td>
<td>0.0000</td>
<td>-0.0001</td>
</tr>
</tbody>
</table>

† Mann-Whitney U-test

### 3.5 Adverse Events

One subject reported adverse events including gastrointestinal pain and diarrhea after 2 weeks of taking the lozenges on a regular basis. The subject was advised to discontinue use of the lozenges immediately. The gastrointestinal symptoms continued for a couple of days after discontinuation of lozenge administration. The subject was followed for another 6 weeks, with no recurrence of symptoms. At the time, it was not known which lozenge the participant was taking. After the study was complete, it was...
revealed that this subject had in fact been taking the placebo lozenges and that the incident was likely coincidental.

None of the participants in the probiotic group reported any adverse events.

3.6 Compliance

Compliance assessment was based on self-report using the tracking calendars. All subjects reported compliance over 90% (Figure 27 and Figure 28). The median lozenge consumption at T2 (day 14) was 42 for both the probiotic and placebo groups, which was at the level of perfect compliance. At T3 (day 28), the median lozenge consumption was 70 for both groups, with 72 lozenges being the perfect compliance level. The mean values between groups were also very similar (Table 26). At T2 and T3, 89.7% and 72.4% of subjects reported a perfect compliance, respectively. There was no significant difference concerning the compliance between the two lozenge groups at either T2 or T3 (Table 24 and Figure 29).

When considering gender, a significant difference in the proportion of males and females with perfect compliance was seen at T2 (p = 0.032), with females showing significantly less perfect compliance than males (Table 25). However, by T3, no gender difference was seen in the proportion of perfect compliance (p = 0.595). Although the median number of lozenges taken by T2 and T3 (42 and 70 respectively) were the same for both males and females, females showed a significant reduction in the number of lozenges consumed at T2 (p = 0.026) (Table 26). Although there was a difference, it was of a small magnitude, with females having a mean lozenge consumption of 41.58 and males showing a mean of 42 at T2. By T3, there was no significant difference in the number of lozenges consumed by males or females (p = 0.617).
Figure 27. Lozenge consumption of all subjects combined, at T2 (day 14).

Figure 28. Lozenge consumption of all subjects combined, at T3 (day 28).
### Table 24. Compliance by lozenge group

<table>
<thead>
<tr>
<th>Compliance</th>
<th>Probiotic group</th>
<th>Placebo group</th>
<th>Total cohort</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n = 29 (50%)</td>
<td>n = 29 (50%)</td>
<td>n = 58 (100%)</td>
<td></td>
</tr>
<tr>
<td>T2: 14 days (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>T3: 28 days (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 25. Compliance by gender

<table>
<thead>
<tr>
<th>Compliance</th>
<th>Male</th>
<th>Female</th>
<th>Total cohort</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n = 25 (43.1%)</td>
<td>n = 33 (56.9%)</td>
<td>n = 58 (100%)</td>
<td></td>
</tr>
<tr>
<td>T2: 14 days (42 lozenges)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<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>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>T3: 28 days (70 lozenges)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<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>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
Figure 29. Comparison of the proportion of subjects with ‘perfect compliance’ to ‘less than perfect compliance’ in probiotic vs. placebo groups and male vs. female at 14 days (T2) and 28 days (T3) (*p < 0.05).
Inter-rater reliability, the degree of agreement between different examiners, was assessed before and after the study (Table 27). Inter-rater reliability was measured for the MGI scores assigned to Sample B (intraoral photos) and Sample C (clinical patients). Examiner alignment prior to reliability evaluation resulted in ‘substantial agreement’ (Table 7) between examiners for Sample B (kappa = 0.7942) and ‘almost perfect agreement’ for Sample C (kappa = 0.8344) prior to commencement of the study. Similar kappa scores were seen for the intraoral photos and clinical evaluations. The post-study inter-rater reliability was higher (kappa = 0.8938), showing ‘almost perfect agreement’ between the two examiners.

### Table 26. Comparison of number of lozenges taken between lozenge groups and between genders at T2 (14 days) and T3 (28 days)

<table>
<thead>
<tr>
<th>Lozenge Group</th>
<th>Mean and median # of lozenges taken @ T2</th>
<th>Mean and median # of lozenges taken @ T3</th>
<th>P-value†</th>
<th>P-value†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Probiotic (n = 29)</td>
<td>Mean = 41.79 Median = 42</td>
<td>Mean = 69.17 Median = 70</td>
<td>0.967</td>
<td>0.721</td>
</tr>
<tr>
<td>Placebo (n = 29)</td>
<td>Mean = 41.72 Median = 42</td>
<td>Mean = 69.10 Median = 70</td>
<td>0.026*</td>
<td>0.617</td>
</tr>
<tr>
<td>Male (n = 25)</td>
<td>Mean = 42 Median = 42</td>
<td>Mean = 69.20 Median = 70</td>
<td>0.026*</td>
<td>0.617</td>
</tr>
<tr>
<td>Female (n = 33)</td>
<td>Mean = 41.58 Median = 42</td>
<td>Mean = 69.09 Median = 70</td>
<td>0.026*</td>
<td>0.617</td>
</tr>
</tbody>
</table>

† Mann-Whitney U-test
*p < 0.05

### 3.7 Inter-rater and Intra-rater Reliability

Inter-rater reliability, the degree of agreement between different examiners, was assessed before and after the study (Table 27). Inter-rater reliability was measured for the MGI scores assigned to Sample B (intraoral photos) and Sample C (clinical patients). Examiner alignment prior to reliability evaluation resulted in ‘substantial agreement’ (Table 7) between examiners for Sample B (kappa = 0.7942) and ‘almost perfect agreement’ for Sample C (kappa = 0.8344) prior to commencement of the study. Similar kappa scores were seen for the intraoral photos and clinical evaluations. The post-study inter-rater reliability was higher (kappa = 0.8938), showing ‘almost perfect agreement’ between the two examiners.
Intra-rater reliability, the degree of agreement between repeated administrations of the same test by the same examiner, was measured using the MGI scores assigned to Sample B (intraoral photos), before and after the study (Table 28). Both examiners exhibited ‘almost perfect agreement’ individually (Examiner 1 kappa = 0.8781, Examiner 2 kappa = 0.8681) and overall (kappa = 0.8731). The intra-rater reliability was not significantly different between examiners.

### Table 27. Inter-rater reliability for MGI scores

<table>
<thead>
<tr>
<th>Sample</th>
<th># of values</th>
<th>Agreement</th>
<th>Expected Agreement</th>
<th>Kappa</th>
<th>Std. Error</th>
<th>Z</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample B (intraoral 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>88.33%</td>
<td>43.31%</td>
<td>0.7942</td>
<td>0.0822</td>
<td>9.66</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Post-Study</td>
<td>60 pairs</td>
<td>94.17%</td>
<td>45.07%</td>
<td>0.8938</td>
<td>0.083</td>
<td>10.77</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Pre- &amp; Post-Study Combined</td>
<td>120 pairs</td>
<td>91.25%</td>
<td>44.16%</td>
<td>0.8433</td>
<td>0.0584</td>
<td>14.44</td>
<td>&lt; 0.001</td>
</tr>
<tr>
<td>Sample C (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>91.67%</td>
<td>49.68%</td>
<td>0.8344</td>
<td>0.0749</td>
<td>11.14</td>
<td>&lt; 0.001</td>
</tr>
</tbody>
</table>
3.8 SUMMARY OF RESULTS

A summary of the results from this clinical trial are as follows:

- Baseline MGI and cMGI were not significantly different between the probiotic and placebo groups.

- There was a significant difference in baseline MGI between males and females, with males displaying higher MGI scores compared to females.

- A statistically significant improvement in MGI was observed within the probiotic group at T3 and T4, and within the placebo group at T2, T3 and T4 compared to T1 (baseline). However, the improvement was small and was unlikely to be of any clinical significance. When changes in gingivitis throughout the study were examined between groups, there was no statistical significance between the improvement seen in the probiotic and placebo groups.

- The quantitation of S. salivarius K12, C. rectus and P. intermedia levels in the subgingival plaque samples was not possible due to lack of growth and therefore not included in study.

- There was no significant difference in the proportion of lactobacilli DNA in
unstimulated saliva within or between the probiotic and placebo groups.

- There were no significant changes in the proportions of \( F. \text{nucleatum} \) DNA and total lactobacilli DNA in subgingival plaque within or between the probiotic and placebo lozenge groups.

- No adverse events were reported in the probiotic lozenge group.

- All subjects reported a lozenge consumption compliance of >90%. There was a small but significant difference in lozenge compliance and lozenge consumption between males and females at T2, with females showing significantly less perfect compliance and less lozenge consumption. Overall, both the probiotic and placebo groups exhibited a high level of compliance, with no significant difference between the two groups at T3.

- Inter-rater reliability ranged from ‘substantial’ to ‘almost perfect agreement’ before and after the study. Intra-rater reliability was ‘almost perfect agreement’, with no difference in agreement level between examiners.
CHAPTER 4: DISCUSSION

According to the published literature, this clinical trial is the first of its kind to assess the effect of oral probiotic therapy on gingivitis in orthodontic patients. The results of this trial reject the hypothesis that oral exposure of the Lorodent oral probiotic lozenge for 28 days would show a significant reduction in gingivitis in orthodontic patients compared to controls receiving a placebo lozenge. Although evaluation of the proportion of *C. rectus* and *P. intermedia* was not feasible due to inadequate culturing, microbial counts of *F. nucleatum* were not reduced over time, thereby rejecting the hypothesis that use of the Lorodent lozenge would result in a reduction in the proportion of periodontal pathogens associated with gingivitis.

The following discussion will review the results of this trial and compare it to findings of other studies examining various modalities of probiotic administration and its effect on gingivitis and associated periodontal pathogens.

4.1 COMPARISON OF THIS STUDY TO CURRENT PROBIOTIC LITERATURE

4.1.1 Subject Selection and Study Design

A number of key considerations are critical when choosing appropriate subjects for study participation. The inclusion and exclusion criteria were based on knowledge from previous literature not limited to the probiotic field.

Factors such as the duration of orthodontic treatment and the presence of orthodontic bands were considered during patient selection. Inclusion criteria required that patients have orthodontic brackets for a minimum of 5 months. This time period was chosen based on the knowledge that gingival inflammation, plaque and periopathogens show a
spike in the first 3 months after bonding, with a decrease afterwards.\textsuperscript{237} Patients with orthodontic bands were excluded from the present study because bands are associated with increased plaque retention, inflammation\textsuperscript{238,239} and a different microbial flora compared to teeth with bonded brackets.\textsuperscript{144} It is also possible that mechanical irritation by the band on the gingiva or inflammation caused by chemical irritation of the cement in the gingival sulcus may contribute to localized gingivitis in the area of band placement.\textsuperscript{240} Patients with both self-ligating, steel ligature, and elastomeric ligatures were included in this study since no statistical difference in periodontal health has been demonstrated between these ligation methods.\textsuperscript{241}

For reasons discussed in the section on host factors that influence gingivitis (section 1.2.1), subjects were excluded from study participation if they were pregnant, taking medications known to influence gingivitis, or had a smoking history. Although the altered hormone levels associated with puberty are known to increase gingivitis,\textsuperscript{87,88} subjects were not excluded based on this factor since all participants ranged from the ages of 11 to 18 years. It was assumed that random allocation of subjects to each group, with the addition of a large sample size, balanced out the subjects undergoing puberty in each group. This was supported by the fact that there was no significant difference in the mean age between the probiotic and placebo groups, with means of 15.75 ± 1.67 and 15.64 ± 1.75 years in the probiotic and placebo groups, respectively.

Although there are no orthodontic probiotic studies examining gingivitis, there is a published probiotic study that included orthodontic patients, with a primary clinical outcome of white spot lesions rather than gingivitis. The mean age of children in the study by Gizani et al. (2016) was 15.9 years,\textsuperscript{242} which was similar to the overall mean age of 15.7 years in the present study. In that study, subjects were deemed eligible for inclusion if they had braces on at least 8 maxillary teeth. In contrast, the present study required patients to have at least 20 teeth bonded with orthodontic brackets to maximize
uniformity in the orthodontic treatment rendered between subjects included in the study. The decision to include a minimum of 20 bonded teeth was based on the fact that 4 premolar extractions are common in orthodontic treatment, where a full bonding from first molar to first molar in the maxilla and mandible includes a total of 24 teeth.

The statistically significant greater proportion of higher MGI scores in males compared to females (Table 9 and Figure 16) is consistent with the current literature that demonstrates a gender difference in adolescent children ages 13-15 years old, with males showing a greater proportion of plaque and gingivitis compared to females. However, some studies have not detected a gender difference in gingival health. It is possible that gender differences likely depended on age and the population at hand. In terms of the effect of the statistical difference in MGI, it is unlikely that this gender difference influenced the statistical analysis with regard to improvement in gingivitis between the probiotic and placebo groups since there was no significant difference in the baseline characteristics of gender, age and MGI between lozenge groups.

4.1.2 The Effect of Lorodent on Clinical Gingivitis

When examined within groups, both the probiotic and placebo groups showed improvement in MGI scores compared to baseline. Significance in the probiotic and placebo groups is consistent with the findings of other studies which also reported improved clinical parameters in both test and control groups when examining improvement within groups. Use of the Lorodent probiotic lozenge displayed a significant improvement in MGI at T3 and T4 compared to T1, but no significant changes in cMGI. The placebo group demonstrated significant improvement in MGI scores at T2, T3 and T4 compared to T1 but a significant improvement in cMGI score only at T3 compared to T1. Significance in MGI, but not in cMGI at T2 and T4 in the placebo group may be due to a reduced number of units of comparison. When
comparing MGI, there are 696 units of comparison per time point (29 subjects in each lozenge group, multiplied by 6 teeth, multiplied by 4 surfaces). In contrast, there are only 29 units of comparison in each lozenge group for the cMGI scores, creating a smaller sample for statistical analysis. When the magnitude of this improvement was considered, the median improvement in cMGI score was 1 cMGI unit from T1 to T2, and 2 units from T1 to both T3 and T4 (Table 18). Considering that cMGI was scored out of 96, a 1 or 2 unit difference was not much of an improvement and unlikely to be clinically significant. There were no statistical significances when MGI or cMGI were evaluated between lozenge groups at any of the time points (Table 19). Possible reasons for significance in the placebo group, and no significance between groups will be reviewed in sections 4.2 and 4.3.

There are only a few trials that examine the effect of probiotic therapy in relation to oral health and gingivitis in particular (Table 5), none of which include orthodontic patients. In addition to not including orthodontic patients, all of the published studies test only one probiotic species, unlike Lorodent, which is an amalgamation of various probiotics including S. salivarius BLIS K12, L. paracasei, L. plantarum, L. acidophilus, L. salivarius and L. reuteri. The lactobacilli strains used in Lorodent have not been revealed. In the present study, there was no significant difference in the improvement of gingivitis between lozenge groups. This is consistent with the findings of Burton et al. (2013), who demonstrated no difference in the gingival health of children taking probiotic lozenges daily for 3 months. Instead of the BLIS K12 strain used in the present study, Burton and colleagues used S. salivarius M18. S. salivarius M18, like strain K12, is a megaplasmid carrying probiotic which produces many bacteriocins that inhibit several streptococcal pathogens. Various strains of S. salivarius are notably beneficial in combatting S. mutans, the caries-associated bacteria. However, the major periodontal pathogens associated with gingivitis are not derived from the Streptococcus genus. Although S. salivarius K12 has demonstrated anti-inflammatory properties as

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reviewed in section 1.5.2, at present there is no clinical evidence to demonstrate a
cenefit of *S. salivarius* K12 in relation to improved gingival health. However, in vitro
studies report the potential for *S. salivarius* K12 and M18 to reduce periodontal-
pathogen induced inflammation. As discussed earlier, gingivitis is not solely due to
infection with pathogenic bacteria, but rather, it is the host’s inflammatory response that
causes gingival destruction. Gingival fibroblasts exposed to periodontal pathogens
*P. gingivalis, A. actinomycetemcomitans* and *F. nucleatum* were found to show reduced
expression of pro-inflammatory cytokines when co-incubated with *S. salivarius* K12 or
M18.\textsuperscript{180} Although there is no clinical data to support the use of *S. salivarius* for the
treatment of gingivitis, in vitro experiments do show a potential. Further research
exploring this avenue is warranted.

At present, there are three studies that evaluate gingivitis after administration of a
lactobacilli-containing oral probiotic in the form of a lozenge, two of which support
clinical improvement,\textsuperscript{221,222} and one that reports no clinical impact.\textsuperscript{184} All of these studies
were randomized, double-blind, placebo controlled trials. Prodentis (GUM, Sunstar) is
an oral probiotic lozenge that contains two strains of *L. reuteri* (ATCC 55730 and
ATCCPTA 5289) with a live bacterial count of $2 \times 10^8$. Testing Prodentis, Vivekananda
et al. (2010), compared 4 groups: Prodentis alone, scaling and root planing (SRP) +
Prodentis, placebo lozenge alone, and SRP + placebo lozenge. Results showed
reduced plaque, gingivitis, and gingival bleeding indices in the following ranking order:
SRP + Prodentis > Prodentis > SRP + placebo > placebo alone, with all differences
being statistically significant between groups.\textsuperscript{221} All the subjects in this study were
adults that had been diagnosed with chronic periodontitis, which differs from the present
study that examined pediatric subjects with gingivitis, a less destructive disease
compared to periodontitis.

Similarly, Vicario et al. (2012), evaluated the use of Prodentis in 20 adults with chronic
periodontitis. Analogous to the present study design, subjects were randomly allocated to either the probiotic or placebo lozenge group and the intervention period lasted for a total of 30 days. Subjects were instructed to take 1 lozenge in the evening after brushing their teeth. After 30 days of lozenge administration, a significant reduction in plaque and BOP was seen in the Prodentis group compared to the placebo. This differs from the findings of previous component of the present study, which found no significant change in plaque accumulation with use of the Lorodent probiotic lozenge in orthodontic patients. A lack of clinical impact has also been demonstrated by another L. reuteri-containing probiotic lozenge.

Other research has examined the oral administration of L. reuteri in the form of a chewing gum, rather than a lozenge. Twetman and colleagues (2009), assessed the beneficial effect of two strains of L. reuteri (ATCC 55730 and ATCCPTA 5289, 1 x 10^8 CFU/gum respectively), which are the same strains used in the Prodentis lozenge. Forty-two healthy adults with moderate gingivitis were randomly allocated to either the probiotic or placebo gum group. Subjects were instructed to chew the gum for 10 minutes twice a day, morning and evening, for a total of two weeks. After two weeks of intervention, reduced BOP was seen in both the probiotic and placebo group, with a statistically significant decrease in the probiotic gum group. The probiotic gum group also demonstrated a significant reduction in pro-inflammatory markers in GCF. The use of a probiotic gum did not show any long lasting effects. Follow-up 2 weeks after discontinuation of gum chewing, BOP was no longer significantly different than baseline.

Krasse et al. (2005), also tested the use of an L. reuteri containing chewing gum. Subjects received one of two L. reuteri formulations (1 x 10^8 CFU, strains not specified) indicated as LR-1 and LR-2, or placebo gum for 14 days. Similar to the present study, a significant reduction in gingivitis was seen in the placebo group after 14 days. The gingival index significantly improved in both the probiotic and placebo groups, as seen
in our study. However, between-group analysis revealed a larger improvement in the subjects receiving the LR-1 probiotic, but not the LR-2. They concluded that the LR-1 strain of *L. reuteri*, in the form of a chewing gum, was effective in reducing gingival inflammation in patients with moderate to severe gingivitis.

There are two other studies, with similar methodology to ours, that show no change in gingival index with the use of probiotics.\textsuperscript{133,226} Shimauchi et al. (2008), examined the use of *L. salivarius* WB21 (6.7 x 10\textsuperscript{8} CFU) in the form of a tablet.\textsuperscript{133} Similar to our study design, all clinical parameters and sample collections were obtained utilizing the Ramfjord teeth to represent the entire dentition. This study showed a statistically significant reduction in gingival index for both the probiotic and placebo groups when examined within groups. Similar to the findings of the present study, when the improvement in gingival index was evaluated between groups, no statistical significance was seen. This is corroborated by the findings of Imran et al. (2015), who also found no change in gingival index with the use of an *L. casei* containing probiotic drink.\textsuperscript{226}

The studies presented show mixed results in terms of detecting a statistically significant difference between the gingival improvements in the probiotic vs. placebo groups. Even when a statistical significance was identified, the clinical relevance of this difference was not ascertained. There are a number of critical distinctions between the studies that have demonstrated a statistical improvement in gingivitis compared to the present study. These differences include the probiotic strain(s) used, the length and dose of probiotic administration, and of critical importance, the population at hand, all of which will be reviewed in further detail in the sections below.

Although the published studies presented reviewed thus far demonstrate the potential of oral administration of probiotics for propagation of gingival health in a population without orthodontic appliances, further research is required to demonstrate a potential use in the orthodontic population. Of all the strains included in the Lorodent complex, only
L. reuteri and L. salivarius have been clinically shown to be beneficial in improving gingival health. Although microbiological studies support the potential use of the other probiotic strains included in the Lorodent lozenge, clinical data is certainly lacking.

4.1.3 The Effect of Lorodent on Periodontal Pathogens in Subgingival Plaque

Although other pathogens contribute to gingivitis, our study focused on C. rectus, P. intermedia and F. nucleatum since they are known to predominate in gingivitis. In this study, oral administration of the Lorodent probiotic complex for 28 days did not reduce the proportion of F. nucleatum at T3 compared to T1. Due to inadequate culturing of C. rectus and P. intermedia, their evaluation using qPCR was not reliable.

Looking at the present literature, many studies have demonstrated inhibition of periopathogens with various probiotic species. While aiming to identify the Lactobacillus species with the highest potential to suppress periodontal pathogens, Köll-Klais et al. (2005), reported that L. rhamnosus, L. paracasei, L. plantarum, and L. salivarius (the last three being also present in the Lorodent lozenge) as having the highest antimicrobial activity. These lactobacilli species showed inhibition of P. gingivalis and P. intermedia 82% and 65%, respectively. However, since the strains found in the Lorodent lozenge have not been revealed, it is unknown whether the strains that have demonstrated potential for improvement of gingival health are actually the ones in the Lorodent lozenge. Although these were the species identified as having the most potential for oral probiotic use in vitro, the clinical research available demonstrates potential benefits with certain strains of L. reuteri, L. salivarius and L. casei in reducing the levels of pathogens associated with gingivitis.

In the study on the L. reuteri lozenge (Prodentis) discussed above, Vivekananda et al. (2010) also evaluated the level of periopathogens in subgingival plaque samples.
Microbial evaluation revealed that the level of *P. intermedia* along with other pathogens associated with more severe periodontal disease, were significantly reduced following use of Prodentis, with or without SRP. Another study, with a similar design and finding as ours, tested the use of a tablet containing two strains of *L. reuteri* (DSM-17938 and ATCC PTA 5289) with a dose of $2 \times 10^8$ CFU/tablet on subjects with gingivitis. After taking the tablets for 28 days, no significant difference in the subgingival plaque levels of *P. intermedia*, *C. rectus* or *F. nucleatum* was seen, along with no change in gingival index between the probiotic and placebo groups.

Different strains of *L. salivarius* demonstrate varied efficacy in combating periodontal pathogens. *L. salivarius* TI 2711 in tablet form has been shown to kill *P. intermedia*, but other *L. salivarius* strains such as ATCC 11741 and ATCC 11742 were not as effective. An *L. salivarius* WB21-containing tablet, taken by healthy subjects, resulted in a significant reduction of *P. intermedia* after 4 weeks of administration, but no significant difference at 8 weeks. Like the present study, this study utilized qPCR and subgingival plaque samples to obtain its results. Another study tested *L. salivarius* WB21 in the form of oral oil drops for a period of 15 days. Although they found a significant reduction in levels of *P. intermedia*, similar to our results, there was no change in *F. nucleatum*. These studies demonstrate the importance of knowing the strain of bacteria used, since not all strains in the same species have the same efficiency against oral pathogens. Although the Lorodent complex contains *L. salivarius*, that particular strain may or may not be potent against the pathogens at hand.

An *L. casei* probiotic drink, given to subjects with chronic periodontitis for the span of 1 month has also shown potential for reduction of *P. intermedia* although no clinical differences in gingival index was seen.

Overall, various studies have reported the capacity of certain lactobacilli strains to inhibit the growth of gingivitis associated pathogens, including *P. intermedia*. Current evidence
supports the findings of this study, showing no reduction in *F. nucleatum* with the use of oral probiotics. There is also no evidence to support the inhibition of *C. rectus* with oral probiotic use.

4.1.4 Probiotic Levels and Colonization with use of Lorodent

In order for a probiotic to have a clinical or microbial effect on the host, it must adhere to the oral surfaces and become incorporated into the biofilm. Subgingival plaque is not a typical site for lactobacilli, but it is expected to be found in saliva and on the surface of the teeth, tongue and gingival mucosa. However, low levels in subgingival plaque are consistent with previous findings. An increase in the proportion of total lactobacilli in saliva and subgingival plaque would be a reflection of oral colonization of the *Lactobacillus* species from the Lorodent lozenge. Testing for total lactobacilli DNA relative to total bacterial DNA showed no significant difference in the level of total lactobacilli in saliva or subgingival plaque for both the probiotic and placebo lozenge groups, which indicates no significant colonization. Theoretically, there is a possibility that the probiotic lactobacilli replaced the indigenous lactobacilli, leaving the total lactobacilli DNA unchanged. To test for this possibility, the specific species of *Lactobacillus* would have to be individually assayed. Due to the cost associated with such testing, it was not feasible to test for each of the 5 species of *Lactobacillus* in the Lorodent lozenge. Although this is a possibility, it is highly unlikely seeing as how there were no significant changes in clinical or microbial parameters, which is consistent with lack of probiotic activity.

Two studies have evaluated the level of lactobacilli in orthodontic patients treated with oral probiotics. After 4 weeks of consuming a *L. paracasei* SD1 probiotic milk powder, subjects had increased levels of *L. paracasei* in saliva samples analyzed with PCR. This differs from the findings of Gizani et al. (2016), which found no difference
in the salivary levels of *L. reuteri* in orthodontic patients being treated with a probiotic lozenge for a mean intervention period of 17 months.\textsuperscript{242} Although this study's dependent variable was white spot lesions, its high sample size and long duration of treatment, along with a saliva-collection methodology similar to ours makes it a reliable comparison.

When assessing the colonization of lactobacilli in other dental studies not involving orthodontic patients, many results are consistent with our findings, showing no significant increase in the level of lactobacilli in saliva or plaque after treatment with a lactobacilli-containing probiotic lozenge.\textsuperscript{32,183,248-251} However, there are studies that do demonstrate *Lactobacillus* colonization.\textsuperscript{34,184,252} Due to the fact that the species used in the Lorodent lozenge have not been divulged, it is impossible to compare colonization of species in the literature with those in the Lorodent lozenge.

Not all strains of the same species have the same level of colonization. Krasse et al. (2005), tested two strains of *L. reuteri* which they termed “LR-1” and “LR-2”. Similar to our study design, saliva was collected in a sterile plastic tube and analyzed for *L. reuteri* and total lactobacilli. After 14 days of probiotic administration, 65% of the subjects taking LR-1 were colonized with *L. reuteri*, compared to the 95% colonization seen in the LR-2 group.\textsuperscript{34} A study comparing the levels of *L. reuteri* ATCC-PTA-5289 with DSM-17938 using PCR found 27.5% and 10.6% detection respectively in saliva samples and even lower proportions in subgingival plaque.\textsuperscript{184} More information on the strains in the Lorodent probiotic lozenge is needed in order to evaluate its potential for colonization in the oral cavity.
4.2 Possible Reasons for Negative Findings in the Probiotic Lozenge Group

Possible reasons for the negative findings in the probiotic lozenge group include the use of ineffective strains, inadequate concentration or mode of administration, inability for the specific probiotic strains to colonize the oral cavity and finally, the possibility that the pathogenic and plaque load for orthodontic patients is simply too high for probiotics to alter the clinical and microbiological setting.

4.2.1 Ineffective Probiotic Bacterial Strain

The antimicrobial properties of lactobacilli are strain-specific. As mentioned earlier, since the precise strains of lactobacilli have not been disclosed, it is impossible to compare them to strains shown to have a beneficial outcome on gingivitis or periodontal pathogens. According to clinical data, the Lactobacillus strains that show the greatest potential for the treatment of gingivitis include L. reuteri DSM17938, ATCC PTA 5289 and TCC 55730, L. paracasei, L. salivarius TI 2711 and WB21 (Table 5).

Two of the species in the Lorodent lozenge, S. salivarius K12 and L. acidophilus, lack complete relevance to clinical gingivitis. Although these probiotics have shown potential value to other aspects of oral health (sections 1.5.2 and 1.5.3), to date, clinical research has not demonstrated their benefit to the treatment or prevention of gingivitis.

Other species, not included in the Lorodent lozenge, show some potential for treatment of gingival inflammation. Findings from Riccia et al. (2007), suggests that L. brevis’ anti-inflammatory properties may be useful for the treatment of gingivitis by down regulation of inflammatory factors. A yogurt drink containing L. casei strain Shirota was able to change the oral bacterial population by reducing subgingival plaque levels of


\[ P. \text{intermedia}, P. \text{gingivalis} \text{ and } A. \text{actinomycetemcomitans} \text{ in subjects with mild to moderate periodontitis.}^{226} \text{ Also not included in the probiotic complex, } L. \text{rhamnosus}^{203} \text{ and } Bifidobacterium \text{ species have a suggested association with periodontal health.}^{254} \]

### 4.2.2 Inappropriate Dose or Mode of Administration

Various modalities of oral probiotic ingestion have been employed, including a chewing gum, lozenge, tablet, oil drops and drink. As outlined in Table 5, all of these delivery vehicles have demonstrated clinical or microbial benefit in the treatment of gingivitis in non-orthodontic patients. The test duration of probiotic ingestion for gingival health ranges from 14 days\textsuperscript{34,223} to 8 weeks\textsuperscript{133,182}, with both spectrums demonstrating positive and negative findings. Based on this data, it is unlikely that the precise mode of administration is the defining factor in colonization. Plausibly, it may be the dose or duration that impact probiotic colonization. Burton et al., (2013) support this with their findings that the dose of administration of \( S. \text{salivarius} \) M18 was the defining factor in probiotic persistence.\textsuperscript{255} Overall, the ideal delivery and concentration of each specific oral probiotic has yet to be determined.\textsuperscript{28}

To our knowledge, the ability and time it takes for the Lorodent lozenge to dissolve has not been tested. Although subjects were instructed to slowly dissolve the lozenge on their tongue, it is possible that subjects chewed or swallowed the lozenges, thereby quickly clearing the probiotic from the oral cavity. Using a vehicle such as a chewing gum may have prevented this from happening.

It is generally accepted in the gastrointestinal field that the industry standard for the counts of viable bacteria is \( 1 \times 10^9 \) CFU and should not be lower than \( 1 \times 10^6 \).\textsuperscript{226} There are no studies that test the minimum dosage required for probiotic use in the oral cavity. It may be possible that the dose required for oral probiotic therapy is less than that of
gastrointestinal use since it does not have to pass through the aversive environment of the gastrointestinal system. The majority of probiotic studies evaluating gingival health parameters have tested concentrations in the range of $1 \times 10^8$ CFU (Table 5). Enumeration of the Lorodent lozenge immediately prior to initiation of this trial showed that *S. salivarius* K12 and total lactobacilli levels were each $1.5 \times 10^9$ CFU/lozenge, for a total probiotic concentration of approximately $3 \times 10^9$ CFU/lozenge (Appendix 2). Based on the manufacturer’s recommendations, participants took two lozenges, two times per day for the first 7 days followed by two lozenges once a day for the next 21 days. This equates to a daily dose of $12 \times 10^9$ CFU for the first 7 days and $6 \times 10^9$ CFU for the remaining 21 days. Although these doses are not based on any specific evidence, they certainly exceed the industry standard.

As previously mentioned, different bacterial strains of the same species often have different potentials for oral colonization. Therefore, the dose required may be strain-specific. The ideal dose may even be subject-specific. For example, the optimal dose of *S. salivarius* and the dosing regimen to treat halitosis has been suggested to differ between patients.\(^{181}\) It is possible that although the concentration of probiotic in Lorodent is above the industry standard, the dose required for those specific strains may have to be higher in order to allow for colonization of the bacteria.

### 4.2.3 Viability and Colonization

The viability and adhesion of probiotics is crucial to its efficacy as a therapeutic modality. In order for an oral probiotic to be successful, it must first be viable, then be able to attach to and colonize the surfaces of the oral cavity.\(^ {27}\) This is the reason why many of the oral probiotics, such as *S. salivarius* K12, are oral commensals harvested from a healthy human source, with prior evidence of natural colonization. As mentioned in the previous section, viability testing of the Lorodent lozenge was carried out.
immediately prior to initiation of the study. *S. salivarius* K12 and total lactobacilli counts were above the industry standard at the time of testing. Although it is unlikely that the viability was substantially reduced during the course of the study (since subjects were instructed to store the lozenges in the fridge), there is still a possibility that the viability of this lozenge was diminished.

Studies on the oral colonization of various *Lactobacillus* species have demonstrated a transient colonization that wanes soon after discontinued use.\(^{31,246,256}\) In addition to this, the ability to colonize differs between strains of the same species.\(^{34}\) Since the strains present in Lorodent are unknown, a comparison is infeasible. Reasons for lack of colonization may include inability of a bacterial strain to adhere to oral tissues or host characteristics such as a mature biofilm that is difficult to penetrate or an oral pH and temperature not compatible with bacterial viability.\(^{224}\) More research is required on the persistence of various oral probiotic species and their ability to colonize oral tissues and penetrate the microbial biofilm.

### 4.2.4 Unsuitable use for the Orthodontic Population

Another potential explanation for the negative clinical findings in the probiotic group is the test population at hand. Orthodontic patients are uniquely challenged with excess plaque retention and complication of plaque removal. It is possible that the outstanding retention of plaque and subsequent gingivitis due to presence of orthodontic appliances provides a load that is too large for the probiotic to influence clinical or microbial parameters.

All the studies that have demonstrated reduced gingivitis or microbial counts have only included non-orthodontic subjects. Conceivably, the use of oral probiotics at the tested concentration may be more suited to children without braces. In order to overcome the
higher plaque load and microbial challenge in orthodontic patients, a higher concentration of probiotics may be required. Whether probiotic use for enhanced gingival health is beneficial to the orthodontic population is yet to be validated.

4.3 **POSSIBLE REASONS FOR POSITIVE FINDINGS IN THE PLACEBO LOZENGE GROUP**

In our study, a within-group analysis revealed improved MGI scores in both the probiotic and placebo groups. Positive clinical results within the placebo group have also been observed in other probiotic studies evaluating gingival parameters.\(^{34,133}\) An improvement in both test groups is consistent with the well documented Hawthorne effect, which results in a behavioural change due to knowledge that a certain parameter is being observed.\(^{257,258}\) Subjects may have altered their oral hygiene regimen after being enrolled in the study, resulting in improvement in the measurement of clinical gingivitis. In fact, intentional use of the Hawthorne effect has been used to improve oral hygiene in orthodontic patients.\(^{259}\) This is similar to the placebo effect, which results in a change in behavior or perception due to the belief in the efficacy of an intervention.

Another possible explanation for the positive findings in the placebo group is an observer or recording bias. Although the examiners did not know which group was the probiotic or placebo, they did know if a subject was in Group A or Group B. Although unlikely, a bias towards the placebo group could have lead the examiners to unknowingly favour improvements in MGI recording. In hindsight, examiners should have been blinded as to which participants were taking lozenges A or B.
4.4 **SAFETY AND COMPLIANCE OF LORODENT**

Our study supports the safety of the Lorodent probiotic lozenge, as no adverse events were reported in the probiotic test group. This is consistent with previous publications testing high doses of *S. salivarius* K12 (1 x 10^{10} CFU) for a period of 28 days,\(^{194}\) and various species of *Lactobacillus* for up to 8 weeks.\(^{34,133,184,221,222}\) A 4-year study exploring the potential for *Lactobacillus* to cause infection was carried out on a large population in Finland.\(^{230}\) Data showed that *Lactobacillus* species are highly unlikely to be pathogenic. However, there have been rare cases of bacteremia associated with probiotic use in individuals who suffer from a chronic disease, are immunocompromised, or have short-gut syndrome.\(^{260}\) This highlights the importance of patient selection when considering use of probiotics.

Compliance was exceptional in both the probiotic and placebo test groups, with all subjects exhibiting greater than 90% compliance. Since there was no difference in the compliance between test groups, it is unlikely that poor compliance in the probiotic group was the reason for negative findings. Our study demonstrates that flavored lozenges are a well-accepted delivery modality that can be practically and easily implemented within the orthodontic context and is likely to result in good compliance.

4.5 **LIMITATIONS OF THE CURRENT STUDY**

Every study has limitations in its design and implementation. These limitations include inherent challenges of sample collection and microbial analysis. The physical constraint of the gingival sulcus make collection of subgingival plaque challenging without the inclusion of contaminating species from supragingival sources.\(^{3}\) Likewise, although saliva samples are the method used for evaluation of probiotic colonization in the oral cavity, the validity of this method compared to oral swabs has not been evaluated in the
probiotic field.

In this study, the change in total lactobacilli DNA was measured as a proportion of total bacterial DNA to allow for comparison across the various time points. Testing the proportion of each of the specific *Lactobacillus* strains rather than total lactobacilli DNA would have given a more accurate view of the change or lack of change in the lactobacilli community.

Although our study evaluated the level *F. nucleatum* over time, it is possible that other bacteria, not assayed for, experienced a change in the probiotic test group. One way to test for large-scale changes would be deep sequencing to generate a population of the sample microbial community. Such a task would require extensive funds with substantial data processing and statistical capability.

An assumption of this study is the continued viability of the probiotic once dispensed to the participants. In an ideal situation, a “test lozenge” remaining from the lozenges given to each of the subjects in the probiotic group could have been evaluated for the CFU at the end of lozenge administration (T3). However, this is a large task that would have required additional funds and time not available to us.

### 4.6 Future Studies

The study of probiotics for oral health is a flourishing field that is gaining momentum. Further research is needed in order to optimize probiotic use and quantify the extent of clinical or microbiological benefit. In order to use probiotics to their full extent, further knowledge concerning the adhesion and colonization capabilities of the various strains must be determined. More information is needed to define the ideal concentration, dosing regimen and mode of administration for each probiotic strain. After testing the
efficacy of each strain individually, it would be logical to then move forward in evaluating the potential synergistic effects of combining probiotics into a single entity.

This study is the first of its kind to evaluate the use of oral probiotics to improve gingival health in orthodontic subjects. Orthodontic patients represent a unique subset of the population that require particular attention in the maintenance of gingival health. Instead of testing the use of probiotics to combat established gingivitis, research could be conducted on the possibility of preventing gingivitis using probiotics prior to bonding of orthodontic brackets. Another avenue for research would be the idea of conducting a “microbial transplant” by evaluating the use of probiotics to re-colonize a patient’s microbiome after a sanative phase consisting of a scaling and root planning and possible use of an antimicrobial rinse.

Research is required on the ideal methodology to evaluate colonization of oral probiotics. Although the current literature uses saliva to evaluate the level of probiotics, it may not be the most accurate approach. A comparison should be conducted between stimulated saliva, unstimulated saliva, tongue swabs and mucosal cheek swabs to assess the best method to appraise probiotic colonization. Although much is known about probiotics in the gastrointestinal field, there is a great deal of knowledge to be learned pertaining to probiotics for oral health.
Based on this clinical trial, oral administration of the probiotics in the Lorodent lozenge were ineffective in the treatment of gingivitis in orthodontic patients. Evidence supporting the use of probiotics for the treatment of gingivitis is questionable. Even though some studies show a benefit in terms of reduced gingivitis and plaque, the findings are inconsistent, are of a small magnitude, have a debatable clinical significance, and may be limited to a population without orthodontic appliances. The conclusion of this study is consistent with a systematic review that examined the use of probiotics for periodontal health and concluded that evidence is lacking to make a conclusive statement regarding the efficacy of probiotics for gingival health.\(^{261}\) The benefit and use of probiotics in managing dental health is a flourishing field and requires additional research.

In terms of compliance, flavored lozenges are a well-accepted delivery modality that can be practically and easily implemented within the orthodontic context and is likely to result in good compliance in a short time period similar to the one undertaken by this study.


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Appendix 1. Product Information on Lorodent and Placebo Lozenges

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 2. Probiotic Potency Report

Probiotic Potency Report
for the Probiotic Complex “Lorodent®”
(batch P2237 intended for use in the Clinical Trial LG-001)

Referenced Protocol / Ethics Approval Numbers:
- Health Canada CTA Submission No. 185428
- University of Toronto REB Protocol ID No. 30148
- University of Western Ontario REB Protocol ID No. 101955

Lorodent Probiotic Complex is a novel oral health product formulated to provide optimal efficacy and following all the corresponding parameters and guidelines. The high efficacy of Lorodent resides on its active ingredients: probiotic bacteria. The strains included in Lorodent are the following: *Streptococcus salivarius* K12, *Lactobacillus paracasei* Lpc-37, *L. plantarum* Lp-2001, *L. salivarius* Ls-33, *L. acidophilus* La-14, and *L. reuteri* Lru-1038.

Efficacy of probiotic products relies not only on the beneficial effects of the strains contained in them, but also on administering them in efficacious doses. The minimum amounts required and the optimal period of administration of probiotics needed to elicit a health effect depend on the form in which the probiotic is ingested as well as the strain used. Most studies determining the effects of probiotics within the oral cavity have used dosages of $10^{8}$ – $10^{10}$ per day and often involve multiple strains. However, even though high doses do not entail any specific risk, they are also not necessary if the same benefits can be achieved with just enough amounts. This is the reason that Lorodent is expected to be efficacious at doses of at least $10^{9}$ cfu/lozenge, especially with the prescribed treatment regimen as per the Clinical Study LG-001 protocol, for gingivitis induced by braces in pediatric patients. This dosing regime was developed based on colonization and efficacy data published on *S. salivarius* K12 and the lactobacilli strains.

An enumeration assay was performed on the Lorodent batch (P2237) approved by Health Canada for use in the clinical trial LG-001. Probiotics are live organisms and they experience a decline in their viability after production. To ensure that the active ingredients are still present in an efficacious dose in the Lorodent lozenges, colony-forming units (cfu) were measured in the product. The packages of Lorodent that will be used in the clinical trial have been stored at -20 °C since production and an enumeration assay was performed on them prior to the start of the study following Integra's SOP #002 “Enumeration of *Streptococcus salivarius* K12 and lactobacillus strains in Lorodent probiotic Complex” version 3. This is an industry standard selective spread plate method.

Fig. 1 demonstrates that the probiotic strains in Lorodent are found in efficacious doses in the batch to be used in the clinical trial. Over $1.5 \times 10^9$ cfu/lozenge were found of *S. salivarius* K12, as well as of total lactobacilli after performing the enumeration assay. These numbers make for a total of approximately $3 \times 10^9$ cfu/lozenge of probiotic strains in Lorodent, which is considered to be highly efficacious and representative of a high-quality product.
In conclusion, the enumeration assay that was performed on the Lorodent batch P2237 intended for use in the clinical trial LG-001 demonstrated that its lozenges contain a highly efficacious dose of probiotic strains, within the parameters submitted and accepted by Health Canada (approval notice for the CTA Submission No. 185428).

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CONFIDENTIAL
Appendix 3. Patient Information and Consent Form

Orthodontics
UNIVERSITY OF TORONTO

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 probiotic 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.
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.

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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.

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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.
**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
Appendix 4. University of Toronto, Faculty of Dentistry Privacy and Treatment Consent Forms

Office of the Assistant Dean, Clinics
Faculty of Dentistry
University of Toronto

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
• 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
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 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 that 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
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 pre-determinations 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 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: ___________________________
## Appendix 5. Randomization Schedule

### Orthodontics

**University of Toronto**

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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
Appendix 6. University of Toronto Health Sciences Research and Ethics Board Approval

PROTOCOL REFERENCE # 30148

June 25, 2014

Dr. Siew-Ging Gong          Dr. Fatima Ebrahim
FACULTY OF DENTISTRY        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
Appendix 7. 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:

<table>
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<th>Document Name</th>
<th>Comments</th>
<th>Version Date</th>
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<td>Health Canada Acknowledgement of Notification-Received for Information</td>
<td>2014/08/11</td>
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<tr>
<td>Revised Western University Protocol</td>
<td>Received Sept 17, 2014</td>
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<td>Revised Study End Date</td>
<td>REB approval from Sept 17, 2014-Jun 30, 2015 NOTE: Lapse in REB approval from Jan 1, 2014-Sept 16, 2014</td>
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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 (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

Ethics Officer to Contact for Further Information

<table>
<thead>
<tr>
<th>Erika Basile</th>
<th>Grace Kelly</th>
<th>Mina Mekhail</th>
<th>Vikki Tran</th>
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<td><a href="mailto:vikkitrinh@uwo.ca">vikkitrinh@uwo.ca</a></td>
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This is an official document. Please retain the original in your files.
Appendix 8. Health Canada Approval

NOTICE OF AUTHORIZATION (NOA)

TO:
Ms. Natalya Ioudina
cc: Dr. Peter Cadieux

FROM:
Dana Wang

COMPANY:
London Health Research Institute,
St. Joseph’s Health Centre and The University of Western Ontario

DATE:
February 25, 2013

FAX NUMBER:
519-858-5142
cc: 519-646-6031

PHONE NUMBER:
519-858-5088

SUBJECT:
Clinical Trial Application - Name

FILE NO.:
185428

PAGE(S):
1

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.deed-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/produits/legislation/docs/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 nhpd-cta.dce-dpmr@hc-sc.gc.ca.

Yours sincerely,

[Signature]

Sara O'Connor
Assistant Director, Bureau of Product Review and Assessment
Natural Health Products Directorate
2936 Baseline Rd. (A.L. 3302C), Ottawa, ON K1A 0K9
TO: Natalya Ioudina

FROM: Nalini Balram

COMPANY: Integra Medical Inc.

DATE: AUGUST 11, 2014

NUMBER OF PAGES(S): 1

FAX NUMBER: 1-519-858-5142

SENDERS FAX NUMBER: (613) 946-0174

PHONE NUMBER: 1-519-858-5088

SENDER’S TELEPHONE NUMBER: (613) 948-9266

SUBJECT: CTA for Lrodent Probiotic Complex

COMPANY CODE: 30134

FILE NUMBER: 185428

SUBMISSION NUMBER: 204059

Dear Ms. Ioudina,

We received on July 21, 2014, your email dated July 21, 2014, and information regarding the clinical trial for Lrodent 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 note 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.
Appendix 9. Ontario Centres of Excellence Funding Agreement

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; or
   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 Jouidina
Title: President/CEO
I have authority to bind the Corporation

Governor Council of the University of Toronto

[Signature]

Print Name: Lino DeFacendis
Title: Director, Partnerships
Innovations & Partnerships Office
I have authority to bind the Corporation

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

Application Information

Applicant: Peter Cadieux-University of Western Ontario

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<th>Project Title</th>
<th>Assessment of therapeutic potential of a novel dental probiotic in pediatric patients affected by gingivitis</th>
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<td>Jessie Maggard</td>
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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
Appendix 10. PowerSoil®-htp 96 Well Soil DNA Isolation Kit 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.