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THESIS APPROVAL PAGE FOR MASTER OF SCIENCE IN ORAL BIOLOGY

Title of Thesis: Effect of prebiotic inulin and initial probiotic optical density variations on *Lactobacillus reuteri* and *Bifidobacterium animalis* subsp. *lactis* sustainability in an in-vitro plaque biofilm model

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Effect of prebiotic inulin and initial probiotic optical density variations on *Lactobacillus reuteri* and *Bifidobacterium animalis* subsp. *lactis* sustainability in an *in vitro* biofilm model

by

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Thesis submitted to the Faculty of the
Oral Biology Graduate Program
Uniformed Services University of the Health Sciences
In partial fulfillment of the requirements for the degree of
Master of Science 2021

ACKNOWLEDGMENTS

Insofar as any pursuit of greater knowledge through scientific inquiry is futile without the guiding wisdom and obliging charity of innumerable devoted mentors, it is a privilege to acknowledge the many sacrifices and kindnesses of my research committee. In particular, I am profoundly and gratefully indebted to Drs. Daniel Boudreaux, James McPherson, Jennette Bick, Robert Herold, Kenneth Erley, Thomas Johnson, and Douglas Lancaster for their enduring support and encouragement of this research. I am especially appreciative of Dr. Boudreaux for his boundless curiosity, infectious enthusiasm, and inspired desire to push this project beyond its initial objectives in search of the unknown. In addition, I would like to express my sincere gratitude to Dr. Rodney Phoenix and Dr. Jennette Bick for their indefatigable efforts in mediating the many bureaucratic intricacies of this project.

DEDICATION

To my endearing wife, Shauna, who has not only tolerated my absence throughout my engagement in research and residency, but has, in the process, loved and encouraged, supported and endured, languished and laughed, and, through it all, inspired all that is good and whole within me. Certainly, any attempt to fully convey my gratitude would be, if but sincerely conceived, forever inadequately articulated.

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ABSTRACT

“Effect of prebiotic inulin and initial probiotic optical density variations on *Lactobacillus reuteri* and *Bifidobacterium animalis* subsp. *lactis* sustainability in an *in vitro* biofilm model”

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Thesis directed by: Dr. Daniel M. Boudreaux

Objective: The present research sought to establish a viable *in vitro* model by which to evaluate methods of enhancing the sustainability of two probiotic species, *Lactobacillus reuteri* and *Bifidobacterium animalis* subsp. *lactis*, within the oral microbiota.

Additionally, this pilot study evaluated the longitudinal microbiological effects of those probiotics on the composition of a cultured plaque biofilm.

Materials and methods: Hydroxyapatite disks were utilized as a substrate on which to culture a subgingival plaque biofilm in a tryptic soy broth/hemin/menadione medium, with or without supplementation by low ($\approx 1 \times 10^8$ CFUs/mL) or high ($\approx 1 \times 10^{10}$ CFUs/mL) doses of either *Lactobacillus reuteri* or *Bifidobacterium animalis* subsp. *lactis* via single (initial) or continuous inoculation, in an anaerobic chamber. Biofilms were also cultured in the presence of varying optical densities of *Bifidobacterium animalis* subsp. *lactis* supplemented with prebiotic inulin in concentrations ranging from 0-1,250 μ M. The relative abundances of the probiotics, and of the microbial constituents of the

associated biofilm, were then evaluated at various timepoints via 16S rRNA Next Generation Sequencing technology.

Results: The results of this pilot study demonstrated the capacity of the proposed *in vitro* model to successfully culture plaque biofilms supplemented with probiotic *L. reuteri* and *B. animalis* subsp. *lactis*. Furthermore, *B. animalis* was shown to maintain high relative abundance within the *in vitro* biofilm after five weeks of low-dose continuous application. However, *L. reuteri* demonstrated significantly less viability within the biofilm, even with continuous application, and diminishing, albeit dose-dependent, relative abundance after single-dose initial inoculation. *B. animalis* substantially diminished the relative abundance of *F. nucleatum* and increased the relative abundance of *Veillonella* species within the biofilm over a five-week period, and both probiotics demonstrated an ability to increase the beta diversity of the cultured biofilm over time. At concentrations $\geq 125 \mu\text{M}$, prebiotic inulin significantly enhanced the viability of *B. animalis* subsp. *lactis* within the cultured biofilm, and at a concentration of 1,250 μM , inulin promoted a more favorable plaque microbial composition, even in the absence of *B. animalis*.

Conclusions:

The *in vitro* model utilized herein provides a suitable means of investigating strategies for enhancing probiotic viability within oral biofilms. The results of this study suggest that probiotics may increase the diversity of the oral microbiota over time, *B. animalis* subsp. *lactis* may promote a more healthful oral ecology, and at concentrations $\geq 125 \mu\text{M}$, inulin could enhance *B. animalis* subsp. *lactis* viability within the cultured biofilm.

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LIST OF ABBREVIATIONS

16S rRNA:	16S ribosomal RNA
<i>B. animalis</i> subsp. <i>lactis</i>	<i>Bifidobacterium animalis</i> subspecies <i>lactis</i>
BLAST:	Basic Local Alignment Search Tool
BOP:	Bleeding on probing
GBI:	Gingival bleeding index
GCF:	Gingival crevicular fluid
GI:	Gingival index
HA:	Hydroxyapatite
<i>L. reuteri</i> :	<i>Lactobacillus reuteri</i>
MMP:	Matrix metalloproteinase
NCBI:	National Center for Biotechnology Information
NIH:	National Institutes for Health
OD:	Optical density
PD:	Probing depth
PI:	Plaque index
RNA:	Ribonucleic acid
Spp.:	<i>Species pluralis</i> (Latin for “multiple species”)
SRP:	Scaling and root planing
Subsp.:	Subspecies
TIMP:	Tissue inhibitor of matrix metalloproteinase
TSB-hm:	Tryptic soy broth/hemin/menadione

CHAPTER 1: INTRODUCTION

STATEMENT OF THE PROBLEM

“Probiotics” have been defined by the World Health Organization as live microorganisms that, when administered in adequate amounts, confer a benefit to the host (FAO/WHO, 2001). Among the panoply of novel therapeutics to recently emerge in the field of medicine, probiotic utilization offers promise not only in the maintenance of general systemic health and homeostasis, but also in the enhancement of clinical outcomes afforded by traditional periodontal therapy. The detailed characterization of these benefits, as well as the systemic effects of the indigenous human microbiota, is an area of vigorous current research that has generated a wealth of publications related to the microbiome in the past decade (Day, 2019). However, most of this research has focused on the influence of probiotics on the intestinal microflora, while relatively little is known about probiotic effects on the oral microbiome (Dassi, 2018). Furthermore, although adjunctive probiotic administration has demonstrated great promise in significantly enhancing the clinical outcomes afforded by traditional periodontal therapy and in promoting a more health-conducive oral microbiota (Vivekananda, 2010), numerous unresolved problems preclude recommendations for its widespread utilization (Teughels, 2011). Perhaps chief among these is the inability of administered probiotics to establish permanent residence within the indigenous oral ecology, which, in turn, permits only transient clinical effects (Tekce, 2015). Therefore, there is significant need to elucidate the mechanisms by which beneficial microorganisms can be selectively encouraged to flourish within the established oral biofilms once introduced into the oral cavity.

SIGNIFICANCE

It is widely acknowledged that plaque biofilms and the ensuing host response constitute the primary etiology of periodontal disease (Murakami, 2018; Meyle, 2015). However, pathogenic recolonization of treated sites within a relatively short period of time is a frustrating complication of mechanical debridement, particularly in the presence of poor patient compliance (Magnusson, 1984). In this respect, probiotics have been suggested as a means of mitigating this vexing obstacle to clinical success by enhancing the population of beneficial bacteria, which, in turn, may retard the recolonization of pathogens and mollify the exaggerated immune response against them (Teughels, 2011). As probiotics have been shown to both modify the oral microbial ecology in such a way as preclude or combat dysbiosis (Vivekananda, 2010; Romani Vestman, 2015; Tekce, 2015) and simultaneously modulate the host immune response to favor a controlled inflammatory response (Twetman, 2009; Ince, 2015; Kuru, 2017), efforts to assess the variables influencing probiotic viability within dental biofilms, and which probiotics confer the greatest benefit, are of great import to the advancement of oral health.

Another, equally significant, consideration underscoring the significance of probiotics research concerns the ubiquitous problem of antibiotic resistance. Fifty years of antibiotics notwithstanding, infectious diseases endure as a dominant threat to public health. Moreover, nosocomial infection rates show no promise of decline, and drug-resistant bacteria continue to arise as the limited reservoir of antibiotics is depleted (Teughels, 2011). In particular, Rams and colleagues evaluated the resistance of periodontal pathogens to a spectrum of commonly prescribed antibiotics at therapeutic concentrations and found that 74% of chronic periodontitis patients harbor pathogens that

are resistant at least one such antibiotic. Indeed, periodontal pathogens exhibiting resistance to both amoxicillin and metronidazole were found in 15% of these patients (Rams, 2014). Furthermore, short-term local and systemic antibiotic or antiseptic utilization has afforded limited long-term improvement in clinical outcomes (Quirynen, 2002). Probiotics are particularly promising in the wake of the declining efficacy of some traditional therapeutic approaches and harbor the potential to obviate the concern for deleterious pathogen mutations, as any countervailing mutations of the beneficial species could favor the selective survival of those beneficial species and, in turn, benefit the host. Therefore, as concerned health professionals and researchers forge on in search of novel approaches to human health and maintenance, the encouraging results of probiotic application within the general medical community have resulted in a surge of interest in probiotics within the oral healthcare arena (Teughels, 2011).

Finally, efforts to investigate methods of optimizing probiotic efficacy and oral sustainability are of particular importance in the context of poor patient plaque control and the frequent disregard for strict adherence to maintenance regimens among periodontal patients (Wilson, 1987). A probiotic that could sustain an oral biofilm with reduced relative abundance of periodontal pathogens and promote a regulated immune homeostasis would necessarily mitigate the untoward effects of poor patient compliance. By translation, probiotic therapeutics would bear great significance to military readiness, as deployment conditions frequently offer fewer opportunities for meticulous, high-quality plaque control. In deployment theaters where professional periodontal maintenance and oral hygiene regimens are frequently inadequate, a method of maintaining optimal

periodontal parameters over a six- to fifteen-month period would maximize dental readiness for deployed soldiers in particular.

REVIEW OF THE LITERATURE

INTRODUCTION AND EVOLUTIONARY SIGNIFICANCE

The last decade has witnessed a robust interest in the vast, largely undiscovered realm of the human microbiome and, in particular, its relation to human health and disease. By definition, the microbiome refers to the sum of all microbes living within an organism, their genetic information, and the environment in which they interact. In contradistinction, the *microbiota* of an organism strictly refers to the living organisms constituting the microbiome without reference to their constituent genomes and environment (Kilian, 2016). The human resident microbiota have, over the course of hominid evolution, co-existed and co-evolved to establish a predominantly harmonious symbiotic existence within us. Indeed, we cannot be divorced from our microbiome, but rather should be understood as a “superorganism,” or holobiont, wherein the microbiome confers a significant role in our health and physiology (Kilian, 2016). In fact, estimates indicate that microorganisms comprise 50-90% of all cells within the human body, which implies that we are predominantly microbial in constitution (Savage, 1977; Sender, 2016). The co-evolution of animals and bacteria over an 800-million-year history is evidenced by the existence of an indigenous microbiota found in all animals with at least the complexity of an earthworm (Blaser, 2006), and conservative estimates indicate that resident microbes have been performing metabolic functions within the animal kingdom for at least 500 million years (Cho, 2012).

These facts alone should, at the very least, arouse our intuitions that perhaps microorganisms are fundamental to our physiology. In fact, it would appear that humans invest a great deal of capital in ensuring that certain bacteria, such as those of the genera *Bifidobacteria* and *Lactobacillus*, are present in the developing infant. The oligosaccharides in human breast milk nurture a neonatal microbiota with proportionally higher abundances of *Lactobacillus* and *Bifidobacteria* species, which are considered to be critical to the development of the infant immune system and in the defense against pathogens (Rinne, 2005; Macfarlane, 2006). These discoveries, in turn, have actuated enormous research interest in the precise role of the indigenous microbiome in human health and homeostasis, and have stimulated an academic foray into the unforeseen world of microbial ecology.

THE MICROBIOME AND THE INNER SANCTUM OF HUMAN HEALTH

An extensive body of recent literature has been devoted to examining the role of the human microbiome in human health and the particular effects of microbial dysbiosis on a wide range of developmental and chronic inflammatory diseases. The gut microbiota, for example, is critical to maintaining normal intestinal physiology, and derangements of this microflora, or the dysregulation of the host response to it, are fundamental to the pathogenesis of inflammatory bowel disease (IBD) (Hart, 2006). The microbiome is essential for activation of host immune responses (for example, polysaccharide A produced by *Bacteroides fragilis* mediates conversion of CD4⁺ T cells into regulatory T cells), and IBD is widely acknowledged to result from interactions between intestinal microbes and the host (Cho, 2012). Interestingly, controlled clinical studies have demonstrated the

efficacy of probiotics in treating Crohn's disease and in maintaining remission of ulcerative colitis, and it has been reported that probiotics may enhance gut mucosal barrier function and preclude pathogen invasion (Hart, 2006). Furthermore, preliminary evidence indicates that the human microbiome is involved in a diverse spectrum of additional physiological disturbances, including dermatological disorders, colorectal cancer (wherein short chain fatty acids produced by the intestinal microflora may influence gene expression involved in cell cycle regulation and apoptosis), non-alcoholic fatty liver disease (Cho, 2012), and Alzheimer's disease (Shoemark, 2015). A growing body of research also implicates gut microbial dysbiosis in both types of diabetes via TLR-4-mediated inflammatory pathways (Vallianou, 2018). Regarding obesity, Ley and colleagues reported that relative to lean individuals, obese individuals harbor a gut microbiome with an imbalance of Firmicutes to Bacteroidetes bacteria (Ley, 2006), and in animal models, higher proportions of Firmicutes phylum bacteria corresponds to an increased capacity for dietary energy harvesting (Turnbaugh, 2006). The gut microbiome may also influence the pathogenesis of coronary artery disease, with evidence suggesting that intestinal microbiota can mediate cholesterol metabolism (Ettinger, 2014), influence cholesterol synthesis via HMG-CoA reductase regulation (Wong, 2012), and produce diet-related proatherogenic compounds, as in the case of metabolism of L-carnitine, abundant in red meat, to trimethylamine-*N*-oxide (Koeth, 2013).

Of greater relevance to the domain of periodontal health in particular is the role of microorganisms in immunomodulation. From the very inception of the human organism, the resident microbiota—and, in particular, bacteria of the *Bifidobacterium* genus—is fundamental to the development of the immune system (Macfarlane, 2006; Tuddenham,

2015), and bifidobacteria are known to protect the host against enteric pathogens (Kolida, 2002). Research on the systemic health effects of probiotics has further evidenced the influence of microorganisms on immune regulation. Breastfed infants supplemented with *Lactobacillus rhamnosus* GG exhibited higher total numbers of IgM-, IgA-, and IgG-secreting cells at 12 months versus breastfed infants not receiving the probiotic, suggesting that probiotics can stimulate positive immunomodulatory effects (Rinne, 2005). Moreover, probiotics can influence the expression of neutrophilic phagocyte receptors, stimulate natural killer cell activity, and produce a broad spectrum of antimicrobial compounds, such as lactic acid, hydrogen peroxide, and bacteriocins (Teughels, 2011).

In vitro studies have also demonstrated the capacity of probiotic species to mediate immunomodulation. Ma and colleagues showed that live *Lactobacillus reuteri* was able to exert potent anti-inflammatory effects on cultured human epithelial cells by inhibiting both TNF- α -induced secretion of IL-8 and NF- κ B nuclear translocation (Ma, 2004). Schultz et al. likewise showed that lactobacilli can induce an anti-inflammatory CD4 T-cell response, with upregulation of IL-10 and decreased expression of TNF- α (Schultz, 2000). Similarly, cultured peripheral blood mononuclear cells obtained from individuals receiving probiotic *Lactobacillus rhamnosus* GG exhibited diminished TNF- α production when exposed to Gram-negative bacteria, and peripheral blood mononuclear cells cultured from patients receiving *Bifidobacterium animalis* subsp. *lactis* Bb12 exhibited decreased IL-2 production when stimulated by influenza virus (Kekkonen, 2008). These immunomodulatory effects suggest that probiotics may be particularly well suited to the treatment of periodontal disease, wherein periodontal destruction is mediated by an exaggerated immune response to a dysbiotic plaque biofilm (Meyle, 2015). Indeed, the application of probiotics to

traditional periodontal therapy soon followed its initial development within general medicine.

PROBIOTICS AS THERAPEUTIC AGENTS IN PERIODONTAL DISEASE

INTRODUCTION

Over the past decade, numerous experimental studies and clinical trials have explored the utilization of probiotics in optimizing oral health. A wealth of research now suggests that probiotics are conducive to host-biofilm symbiosis, and investigators are actively seeking a means of utilizing probiotics to combat oral diseases, such as caries and periodontal disease (Allaker, 2017). The aggregate evidence from these *in vitro* and *in vivo* studies indicates that probiotics promote periodontal health and stability by reducing gingival inflammation, mediating salutary shifts in periodontal microbial ecology, and regulating host inflammatory responses (Kuru, 2017).

Numerous modes of probiotic supplementation have been utilized in human studies, and these include lozenges, chewing gums, capsules, as well as alimentary media, such as chocolates and yogurts (Kuru, 2017). Although there is no general consensus on the most favorable probiotics to administer, strains of the genera *Lactobacillus*, *Bifidobacterium*, and *Streptococcus* that are indigenous to the gastrointestinal flora have been most frequently investigated (Allaker, 2017). Strains of each of these genera are also present in the oral cavity, with Gram-positive, predominantly facultative anaerobic, streptococci—such as *S. mitis*, *S. oralis*, and *S. sanguinis*—constituting 60-90% of the initial colonizers within dental plaque biofilms (Kolenbrander, 2006). Indeed, low abundances of *Streptococcus* and *Actinomyces* species—such as *S. mitis*, *S. sanguinis*, *A.*

viscosus, and *A. naeslundii*—have been associated with healthy periodontal tissues (Socransky, 1977). Lactobacilli, on the other hand, are Gram-positive rods that constitute approximately 1% of the cultivable oral microbiota, with *L. casei*, *L. rhamnosus*, *L. salivarius*, and *L. acidophilus* being among the predominant strains (Mayanagi, 2009; Wasfi, 2018). However, lactobacilli are not commonly found in the subgingival microenvironment and are rarely detected in subgingival samples (Stamatova, 2009). Finally, bifidobacteria are Gram-positive anaerobic bacilli that are predominantly found in the gastrointestinal tract (Manome, 2019), although *Bifidobacterium* species—primarily *B. dentium* and *B. longum*—have also been identified in the oral cavity (Beighton, 2008).

It is typically acknowledged that a healthy microbiota characterized by predominantly saccharolytic microorganisms enriched in the genera *Bifidobacteria* and *Lactobacillus*, which do not contain any known periodontal pathogens, is considered protective against invasive microorganisms in the digestive tract and is constituted primarily by carbohydrate-fermenting bacteria (Macfarlane, 2006; Hojo, 2007). With regards to the oral cavity in particular, Hojo et al. examined the presence of salivary *Bifidobacterium* and *Lactobacillus* species among patients with periodontal health and periodontitis, and among well-maintained patients who had completed active periodontal therapy (Hojo, 2007). Although none of the eleven isolated *Lactobacillus* species were specifically correlated with periodontal health, bifidobacterial counts *did* exhibit an association with periodontal health. Interestingly, the two commonly utilized probiotics species *L. reuteri* and *B. animalis* were not identified in the indigenous oral microbiota of any patient.

The conception of microbiological specificity in relation to periodontal health and disease was advanced decades ago in a classic study by Socransky et al., who characterized microbial complexes in subgingival plaque (Socransky, 1998). In a landmark study, it was found that certain bacteria—namely, those of the so-called “red complex” (*Porphyromonas gingivalis*, *Tannerella forsythia*, and *Treponema denticola*)—were strongly associated with periodontal disease. A decade later, Hajishengallis described the “keystone pathogen hypothesis,” whereby certain critical pathogens, such as *Porphyromonas gingivalis*, could remodel a benign, symbiotic microbiota into a dysbiotic, disease-promoting biofilm by subverting the host immune response and modifying the microenvironment in such a way as to allow other periodontopathogens to flourish (Hajishengallis, 2012). It seemed, therefore, that clinical periodontal health required a health-conducting biofilm within which a symbiotic relationship existed between the resident microbiota and the host immune response (Meyle, 2015). Any derangement in this symbiotic relationship that altered the relative abundances of these microbes in favor of periodontal pathogens, then, would disrupt the host-microbe equilibrium and precipitate a dysregulated, excessive, and destructive immune response (Meyle, 2015). These fundamental concepts, in addition to the reports of successful probiotic utilization in the medical literature, prompted a series of clinical and *in vitro* studies that examined the selective utilization of so-called beneficial bacteria as an adjunct to standard periodontal therapy.

ANIMAL STUDIES

One of the first animal studies to assess the utility of probiotics in periodontal therapy evaluated the effect of selective *Streptococcus* species administration as adjunct to

SRP on pathogenic recolonization of periodontal pockets (Teughels, 2007). In this canine model, surgically created osseous defects were superimposed on experimental periodontitis, and then, in each beagle, one pocket in each quadrant received one of four treatments: no treatment, SRP, SRP and a single local probiotic application, or SRP and multiple probiotic applications at predetermined intervals. Twelve weeks after SRP alone was performed, periodontal pathogen counts returned to baseline levels, whereas probiotic application resulted in diminished recurrence of pathogens, with pathogen counts maintained at reduced levels throughout the study when multiple probiotic applications were employed. Furthermore, there was a tendency toward greater reductions in probing depth and BOP with probiotic application.

Another study evaluated the histomorphometric and immunohistochemical outcomes of adjunctive *Bifidobacterium lactis* HN019 administration in rats receiving SRP after induction of experimental periodontitis (Ricoldi, 2017). Reduced alveolar bone loss, a less extensive zone of inflammation, fewer TRAP-positive multinucleated osteoclasts, decreased IL-1 β levels, and increased anti-inflammatory TGF- β and IL-10 levels were observed in rats that had received SRP and the probiotic versus those that had received SRP alone. Moreover, *Bifidobacterium lactis* was observed to modify the composition of the oral microbiota by increasing the ratio of aerobic bacteria to anaerobic bacteria, indicative of reduced pathogen levels.

HUMAN STUDIES: THE EFFECT OF PROBIOTICS ON CLINICAL PERIODONTAL PARAMETERS

Numerous human clinical trials have assessed the effects of probiotics on oral health, with great heterogeneity reported across studies in terms of the particular species administered, delivery mechanism, and dosage. Among the most commonly utilized probiotic species are *Lactobacillus reuteri* (Twetman, 2009; Vivekananda, 2010; Teughels, 2013; Szkaradkiewicz, 2014; Ince, 2015; Romani Vestman, 2015; Tekce, 2015), *Lactobacillus rhamnosus* (Morales, 2016), *Lactobacillus salivarius* (Mayanagi, 2009), and *Bifidobacterium animalis* subsp. *lactis* (Toivainen, 2015; Kuru, 2017). Although current evidence is insufficient to support the use of probiotics for caries risk reduction (Gruner, 2016), numerous authors have reported that adjunctive probiotic application to traditional non-surgical periodontal therapies can minimize pathogenic recolonization of periodontal pockets and afford improved clinical parameters, such plaque index, gingival index, and BOP (Kuru, 2017).

Vivekananda et al. published the first study to clinically evaluate the effects of probiotics as an adjunct to SRP in terms of clinical parameters and microbiological profiles of subgingival plaque (Vivekananda, 2010). In their randomized, placebo-controlled, double-blind, split-mouth study, thirty systemically healthy patients with chronic periodontitis received SRP in two quadrants (left or right), while the other two quadrants remained untreated. Twenty-one days after SRP, patients received either placebo lozenges or lozenges with two strains of *Lactobacillus reuteri* (1×10^8 CFUs each) taken twice a day for three weeks. Baseline clinical parameters and microbial analyses of plaque samples from sites with 5- to 7-mm pockets were evaluated at day 0, day 21 (before probiotic administration), and day 42. The greatest reductions (all statistically significant) in PI, GI, and GBI (%) were achieved according to the hierarchy SRP + probiotic > probiotic alone

> SRP + placebo > placebo. Notably, probiotic administration *alone* demonstrated a significantly greater (15%) reduction in GBI compared to SRP alone. Remarkably, the maximum PD reduction and clinical attachment gains were observed in the SRP + probiotic group, and these values (1.31 mm and 1.09 mm, respectively) were more than double the sum of the corresponding PD reductions and clinical attachment gains in the SRP alone and probiotics alone groups (0.49 mm and 0.46 mm), indicating a positive synergistic effect. Furthermore, the only treatment modalities that demonstrated a significant reduction in microbial counts of the three evaluated periodontal pathogens (*A. actinomycetemcomitans*, *P. gingivalis*, and *P. intermedia*) were those that included the probiotics, which afforded a 90% reduction in all three pathogens.

Similarly, Tekce et al. reported that, relative to those receiving SRP alone, patients who ingested *L. reuteri*-containing lozenges for three weeks in conjunction with SRP exhibited significantly lower mean PD, PI, GI, and BOP; significantly greater clinical attachment gain; significantly fewer sites with residual deep pockets requiring surgical therapy; and a significantly greater reduction in anaerobic bacteria for up to 180 days (Tekce, 2015). Indeed, these favorable outcomes are by no means isolated reports. Numerous additional studies evaluating *Lactobacillus*, *Streptococcus*, and *Bifidobacterium* species as adjunctive and independent therapies for the treatment of periodontal disease have demonstrated similar promising results with respect to improved clinical parameters (Teughels, 2013; Kuru, 2017), reduction in periodontal pathogens (Mayanagi, 2009; Teughels, 2013; Laleman, 2015; Romani Vestman, 2015), reduction in need for surgical therapy (Teughels, 2013; Morales, 2016), decreased collagenolytic MMP-8 activity (Ince, 2015), and modulation of inflammatory cytokine responses, including reductions in GCF

levels of TNF- α , IL-1 β , IL-8, and IL-17 (Twetman, 2009; Szaradkiewicz, 2014; Kuru, 2017). These favorable outcomes notwithstanding, the importance of mechanical debridement in the efficacy of probiotic administration cannot be understated. It is widely acknowledged, for example, that the complex extracellular polymer matrices of biofilms inhibit penetration of therapeutic agents in the absence of prior mechanical disruption (Socransky, 2002; Teughels, 2008; Ince, 2015), and it is reasonable to infer that mechanical biofilm disruption may be a prerequisite for optimal probiotic efficacy.

PROBIOTIC MECHANISMS OF ACTION

Although a particular resident microbe or probiotic species is likely to exert its salutary effects via any number of mechanisms simultaneously, along with myriad others that have yet to be fully elucidated, a broad spectrum of potential mechanisms of action have been reported in the literature. Among the most commonly enumerated include growth and competitive inhibition of pathogens via nutrient utilization and occupancy of shared receptor binding sites (Jones, 2009; Teanpasisan, 2011; Teughels, 2011); suppression of virulence factor expression among pathogens (Jones, 2009); augmentation of mucosal barrier function mediated by SCFA metabolites (Macfarlane, 2006; Teughels, 2011; Kilian, 2016); essential vitamin synthesis (Kolida, 2002); detoxification of environmental chemicals (Kilian, 2016); development and maturation of the immune system (Kilian, 2016); host immunomodulation (Hart, 2003; Rinne, 2005; Kekkonen, 2008; Twetman, 2009; Teughels, 2011, Ricoldi, 2017); and bactericidal effects on pathogens by synthesis of antimicrobial peptides, such as bacteriocins (Talarico, 1989; Teughels, 2011). One antimicrobial peptide of *L. reuteri*, in particular, has been well

characterized. This compound, reuterin, has been shown to exhibit bactericidal and growth-inhibitory effects against a wide spectrum of pathogenic bacteria, yeasts, fungi, protozoa, and viruses (Talarico, 1989)—likely via induction of oxidative stress (Schaefer, 2010)—and its persistence in the GCF up to 70 days after cessation of probiotic administration has been documented (Tekce, 2015). In addition to the aforementioned mechanisms, downregulation of MMP-8 and upregulation of tissue inhibitors of matrix metalloproteinases (TIMPs) are yet another means by which probiotics have been documented to promote periodontal health (Ince, 2015). The collagenolytic activity of MMP-8 has been shown to be a significant mediator of periodontal breakdown in patients with periodontitis, and disease severity appears to correlate with MMP-8 activity (Kinane, 2003). Insofar as MMPs are critical to extracellular matrix degradation and remodeling, with the relative balance of remodeling being influenced by the proportional activity of MMPs and TIMPs (Ince, 2015), it is evident that regulation of these enzymes is integral to periodontal health.

THE PROBLEM REVISITED

Despite the efficacy of probiotics in effecting immunomodulatory responses and improving clinical periodontal parameters, a particularly inconvenient problem has become evident across numerous clinical studies that have evaluated their therapeutic value: The presence of probiotics in the oral microbiome, and therefore their effects, have generally proven to be short lived. While research indicates that probiotics may be capable of modifying the periodontal microbial ecology toward a more health-promoting composition, this effect appears to be unsustainable and may reflect the inability of these

probiotic strains to persist in the oral cavity (Allaker, 2017). Indeed, cessation of probiotic therapy and resumption of a normal, unsupplemented diet invariably leads to reversion to the baseline microbial ecology (Dassi, 2018).

The duration of a probiotic's influence on the periodontium after discontinuation varies, but to the author's knowledge, few reports indicate residual effects beyond 180 days. One study, for example, demonstrated that a three-week regimen of *L. reuteri* lozenges after SRP reduced GCF levels of MMP-8 and increased TIMP-1 for up to 180 days, after which GCF levels of both returned to baseline; however, relative to controls, approximately 1 mm greater PD reduction and clinical attachment gain remained in those who consumed the probiotic 360 days after cessation (Ince, 2015). Romani Vestman reported that although twice-a-day consumption of *L. reuteri* lozenges for twelve weeks induced a favorable shift in the oral microbiota toward more beneficial, less pathogenic bacteria, the oral ecology normalized and reverted to baseline composition one month after probiotic supplementation ended (Romani Vestman, 2015). Similarly, another study demonstrated that although treatment with *L. reuteri* lozenges could significantly reduce the levels of *A. actinomycetemcomitans* and *P. gingivalis* in subgingival plaque, after termination of probiotic therapy, the relative abundance of *P. gingivalis* rebounded after four weeks (Iniesta, 2012). Likewise, Tekce reported that twice-a-day administration of *L. reuteri*-containing lozenges for three weeks resulted in loss of the probiotic from the oral microbiota after 90 days, despite a one-year sustainment of significantly improved clinical periodontal parameters relative to placebo and reduction of anaerobes through day 180 (Tekce, 2015). These reports accord with findings in the medical literature that probiotics generally fail to become permanent residents of the gastrointestinal microbiome (Teughels,

2011) and are indicative of the need for further research to elaborate the principles upon which probiotic sustainability are predicated.

PREBIOTICS, DOSING, AND A WAY FORWARD

In light of the demonstrated therapeutic benefits of probiotic therapy in the treatment of periodontitis, it is critical to seek a means of prolonging probiotic viability within the oral cavity. More specifically, given the significant heterogeneity in probiotic dosing (from 2×10^7 – 2×10^9 CFUs/day) and method of administration reported in the literature (Mayanagi, 2009; Twetman, 2009; Vivekananda, 2010; Teughels, 2011; Teughels, 2013; Toivainen, 2015; Romani Vestman, 2015; Morales, 2016; Kuru, 2017; Ricoldi, 2017), it is worth evaluating the effects of initial probiotic dosing variations on probiotic sustainability within the oral ecology. Furthermore, the literature would suggest it worthwhile to explore the utilization of *prebiotics* in conjunction with oral *probiotic* supplementation, where the term “prebiotic” refers to a “non-digestible food ingredient that selectively stimulates growth and/or activity of one or a limited number of bacteria in the colon, thereby improving host health” (Gibson, 2004). This intuition is governed by the fact that human studies have clearly demonstrated that the prebiotics inulin, fructo-oligosaccharides, trans-galacto-oligosaccharides, and lactulose—even when consumed in small amounts (5-20 g/day)—can selectively allow health-promoting species of the *Bifidobacterium* and *Lactobacillus* genera to flourish (Kolida, 2002; Macfarlane, 2006; Bosscher, 2009). In fact, this targeted species enhancement occurs despite that fact that neither of the aforementioned genera are particularly abundant in the gut, except in breast-fed neonates. (Macfarlane, 2006). Furthermore, a recent report demonstrated a dose-

dependent effect of *N*-acetyl-*D*-mannosamine in selectively shifting an *in vitro* biofilm toward a composition consisting of 97% beneficial species (Slomka, 2018). This latter report, however, did not examine the effects of prebiotics on introduced probiotic species. Nevertheless, these findings indicate the potential of prebiotic supplementation to augment probiotic viability within the oral microbiome. Therefore, an endeavor to evaluate the effects of initial probiotic dosing and prebiotic supplementation could provide an evidence-based foundation for improving the sustainability of therapeutic probiotics for use in adjunctive periodontal therapy. The recent report of an *in vitro* biofilm model that can be sustained for at least 80 days (Velsko, 2018) provides a means of studying such effects and forms the basis of the research proposed herein.

SUMMARY

A number of clinical and *in vitro* studies have demonstrated that probiotics promote favorable effects on periodontal health and stability by reducing gingival inflammation, mediating salutary shifts in periodontal microbial ecology, and modulating host inflammatory responses. However, these effects are transient in nature, given that probiotics have yet to demonstrate permanent, or even long-term, colonization within the indigenous oral microbiota. Nevertheless, probiotics have been shown to target the primary elements of *both* the etiology and pathogenesis of periodontal disease, namely plaque biofilm dysbiosis and the exaggerated host immune response, and, as such, offer a promising and powerful adjunct to current traditional periodontal therapies. Furthermore, given the limitations of current periodontal therapy—including the threat of pathogenic rebound after active therapy and the marginal long-term improvements offered by

antibiotic utilization—the unpredictability of patient compliance with maintenance and personal plaque control, and the perennial emergence of antibiotic-resistant microorganisms, viable therapeutic alternatives such as probiotic therapy are of obvious value and are worthy of vigorous research.

PURPOSE

The aim of the present investigation was to propose and assess a model to evaluate the effects of probiotic optical density variations (simulating patient dosing) and prebiotic inulin on the sustainability of *Lactobacillus reuteri* and *Bifidobacterium animalis* subsp. *lactis* within an *in vitro* biofilm model. This model, in turn, would serve to simulate the ability of increased probiotic dosing and simultaneous prebiotic supplementation to enhance or prolong *Lactobacillus reuteri* or *Bifidobacterium animalis* subsp. *lactis* colonization within the indigenous oral microbiota and, therefore, prolong their health-promoting effects. This study utilized saliva-coated hydroxyapatite disks (as a surrogate for tooth enamel and the associated glycoprotein pellicle) on which plaque biofilms, obtained from the same source as the saliva and supplemented with varying doses of the probiotic with or without inulin, were developed and perpetuated in a tryptic soy broth/hemin/menadione medium. At predetermined intervals, DNA was extracted from the cultured biofilm and the associated 16S rRNA V3-V4 hypervariable regions analyzed via Next Generation Sequencing to determine the relative abundances of the biofilm microbiota and, thus, viability of the probiotics over time.

HYPOTHESES

HYPOTHESIS #1

Increased initial optical densities (i.e. “dosing”) of *L. reuteri* and *B. animalis* subsp. *lactis* will favor prolonged viability within the cultured biofilm.

HYPOTHESIS #2

L. reuteri and *B. animalis* subsp. *lactis* will, over time, modify the composition of the plaque biofilm in favor of decreased pathogens counts and greater relative abundances of beneficial commensal bacteria.

HYPOTHESIS #3

Similar to the effect of oligosaccharides in breast milk on the enhancement of neonate intestinal *Bifidobacteria* and *Lactobacillus* species, prebiotic inulin will afford a selective advantage on the colonization of *L. reuteri* and *B. animalis* subsp. *lactis* within the *in vitro* plaque biofilm.

SPECIFIC AIMS

AIM #1

Demonstrate the ability of the proposed *in vitro* model utilizing saliva-coated hydroxyapatite disks and tryptic soy broth/hemin/menadione medium to develop plaque biofilms supplemented with probiotics and prebiotics and thereby simulate *in vivo* patient dosing with those supplements. The development of the biofilms on the associated disks over time were validated by visual inspection and microbial characterization via 16S rRNA genomic sequencing.

AIM #2

Elucidate methods by which colonization and viability of *L. reuteri* and *B. animalis* subsp. *lactis* can be enhanced and prolonged within the native oral microbiome. This was accomplished by quantitatively varying the initial exposure of the biofilm to *L. reuteri* and *B. animalis* subsp. *lactis* with or without supplemental prebiotic inulin. The presence and quantification of the aforementioned probiotics were measured by Next Generation Sequencing of DNA extracted from the cultured biofilm at varying timepoints.

AIM #3

Demonstrate the effects of *L. reuteri* and *B. animalis* subsp. *lactis* on the composition of indigenous oral microbiota as it exists in the cultured plaque biofilm. Temporal variations in the microbial constitution of the biofilm were evaluated by longitudinally assessing the relative microbial abundances determined by 16S rRNA hypervariable V3-V4 characterization.

CHAPTER 2: METHODS AND MATERIALS

OVERVIEW

The overarching goal of this investigation was to serve as a pilot study for a larger project that will elucidate methods of enhancing the long-term sustainability of the probiotics *Lactobacillus reuteri* and *Bifidobacterium animalis* subsp. *lactis* within an *in vitro* biofilm. More specifically, it was the aim of this study to establish a model by which to longitudinally evaluate whether incremental increases in quantitative probiotic exposure, with or without supplemental prebiotic inulin, to the biofilm can improve the viability, and likelihood of colonization, of those probiotics within the cultured microbiota. This, in turn, would simulate the clinical effect of increasing the dose of a probiotic, supplemented with a substrate selectively fermented by that probiotic, with the goal of affording the probiotic a competitive advantage for survival within a patient's oral microbiome.

The specific aims of the pilot study completed in the present investigation in preparation for the future full-scale project were twofold: (1) to assess the ability of the proposed *in vitro* model to culture *Lactobacillus reuteri* and *Bifidobacterium animalis* subsp. *lactis* within a plaque biofilm in sufficient quantities to evaluate relative species abundances via Next Generation Sequencing techniques and (2) to longitudinally assess the composition of the biofilm over multiple time points in the presence or absence of various quantities of the aforementioned probiotics.

DETAILED METHODOLOGY FOR THE PROPOSED FULL-SCALE PROJECT

The proposed full-scale project described herein was determined to be exempt from IRB review, as identified in 32 CFR 219.101(b), by the U.S. Army Medical Department

Center and School, U.S. Army Health Readiness Center of Excellence (AMEDDC&S HRCoE) Human Research Protections Office.

The detailed protocol for this project was adapted from the *in vitro* biofilm models reported elsewhere in the literature by multiple authors (Walker, 2007; Shaddox, 2010; Slomka, 2018; Velsko, 2018). Note that the methodology detailed below is the model proposed for a future investigation to answer the proposed aims of this study in full. The protocols for the pilot studies completed in the present investigation are subsequently described in this chapter. The general methodology and experimental sequence for the proposed full-scale project and completed pilot study is illustrated in Figure 1 below:

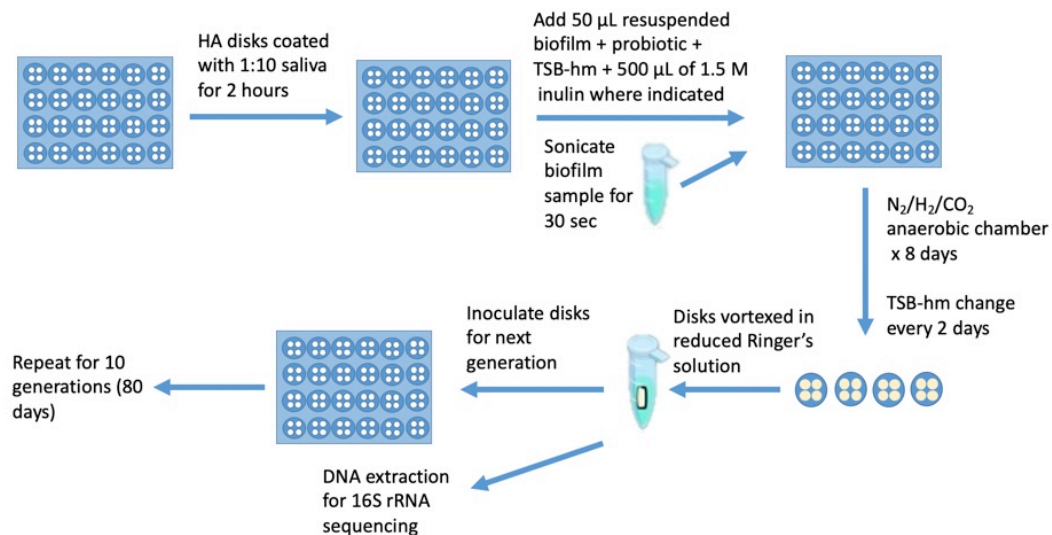


Figure 1. General experimental sequence. Four saliva-coated hydroxyapatite disks per well plate were utilized to culture biofilms from saliva-matched donors. Varying optical densities of either *Lactobacillus reuteri* or *Bifidobacterium animalis* subsp. *lactis* with or without prebiotic inulin were then added to the prescribed well plates and cultured for 8-day periods, after which the disks were vortexed, and 50 µL of the suspension was used to inoculate new disks for biofilm perpetuation. DNA was extracted for biofilm analysis at four time points: the initial HA disk inoculation (time T₀), after 16 days (2 generations) of culture, after 40 days (5 generations) of culture, and after 80 days (10 generations) of culture.

Sample collection

Subgingival plaque and saliva samples from three donors were obtained from Lee BioSolutions (Maryland Heights, MO). Subgingival plaque was collected by inserting a sterile absorbent paper point to the gingival sulcus depth of one mandibular 1st molar and moving it laterally within the sulcus for approximately 10 seconds. The paper point samples were immediately placed into 1-ml aliquots of Amies transport medium supplemented with 0.5% gelatin and 0.1% sodium thioglycollate at 4°C for storage and overnight shipping. Unstimulated saliva (50 ml) was collected from the same donor as the plaque sample. Diluted saliva samples were centrifuged at $1200\text{ g} \times 10\text{ min}$ to remove large particulate matter, then filter-sterilized through a 0.2- μm membrane, divided into 2- mL aliquots, and stored at -20°C.

Biofilm development

Subgingival plaque samples were gently sonicated in a water bath for 30 seconds to break up the biofilm and stored at 4°C while hydroxyapatite (HA) disks were prepared for biofilm inoculation. Four 5 mm x 2 mm HA disks were placed into 24-well cell culture plates, with each well corresponding to a unique probiotic/prebiotic/initial optical density (OD) configuration for each of the three plaque and saliva donors, as outlined below (and illustrated for one of three donors in Figure 2):

1. Donor plaque only
2. Donor plaque only + 1.5 M inulin
3. Donor plaque + *L. reuteri* (OD 0.14), no inulin
4. Donor plaque + *L. reuteri* (OD 0.14) + 1.5 M inulin
5. Donor plaque + *L. reuteri* (OD 0.7), no inulin
6. Donor plaque + *L. reuteri* (OD 0.7) + 1.5 M inulin
7. Donor plaque + *L. reuteri* (OD 1.4), no inulin
8. Donor plaque + *L. reuteri* (OD 1.4) + 1.5 M inulin
9. Donor plaque + *B. animalis* subsp. *lactis* (OD 0.14), no inulin
10. Donor plaque + *B. animalis* subsp. *lactis* (OD 0.14) +1.5 M inulin
11. Donor plaque + *B. animalis* subsp. *lactis* (OD 0.7), no inulin
12. Donor plaque + *B. animalis* subsp. *lactis* (OD 0.7) +1.5 M inulin
13. Donor plaque + *B. animalis* subsp. *lactis* (OD 1.4), no inulin
14. Donor plaque + *B. animalis* subsp. *lactis* (OD 1.4) +1.5 M inulin

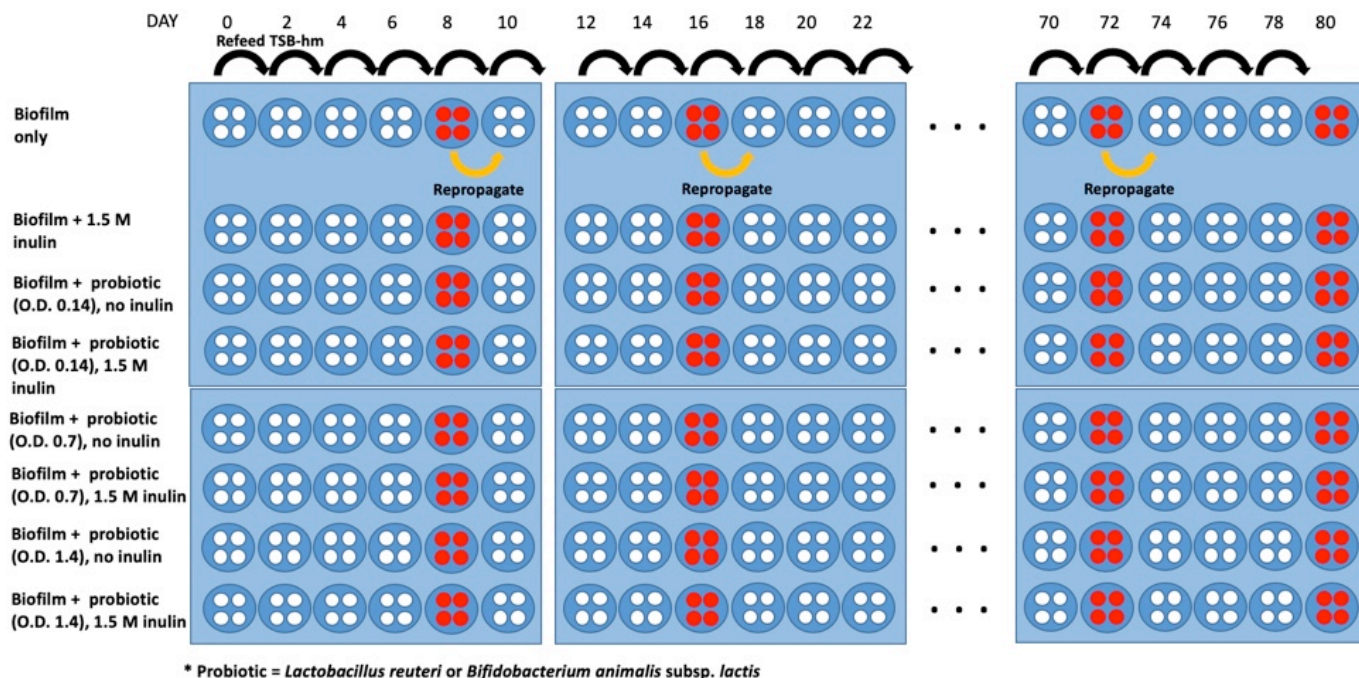


Figure 2. General schematic of biofilm development for a single patient over an 80-day period. Biofilms were developed on hydroxyapatite disks (depicted as red or white circles) coated with saliva obtained from the same patient as the plaque biofilm. Varying optical densities (ODs) of *L. reuteri* or *B. animalis* subsp. *lactis*, with or without inulin, were added to the wells according to a predetermined configuration. The tryptic soy broth/hemin/menadione (TSB-hm) media were replenished every two days. Every eighth day, DNA was extracted from the biofilm on one disk and submitted for analysis, and the biofilms from the remaining disks were reinoculated onto new hydroxyapatite disks (designated in red).

The HA disks were coated for 2 hours at room temperature with sterile 1:10 saliva from the donor whose plaque sample was later added to the well. The saliva was removed, and 2 mL sterile tryptic soy broth supplemented with 5 µg/mL hemin and 1 µg/mL menadione (TSB-hm) was gently added to each well. Additionally, 500 µL of 1.5 M of prebiotic inulin was added to the appropriate wells according to the schematic above. Plaque samples were resuspended by briefly vortexing, and the wells were inoculated with 50 µL of plaque sample from the same donor as the saliva used to coat the wells. Aliquots

of probiotic (either *L. reuteri* or *B. animalis*) in TSB-hm of appropriate volume to achieve the designated optical density were also added to their respective wells. Plates were then placed in an anaerobic incubator at 37°C-75%N₂/10% CO₂/10% H₂ for static growth.

Biofilms were grown for 8 days before dispersal and re-inoculation. Every 48 hours, growth media were gently removed from the wells and replaced by slowly adding a fresh 2 mL of reduced TSB-hm. On the eighth day, new HA disks were added to new wells and coated with respective 1:10 sterile saliva for 2 hours in the anaerobic chamber, then the saliva was removed and replaced with 2 mL fresh reduced TSB-hm. A single disk with mature biofilm (8th day of growth) from each of the 14 wells corresponding to each of the three donors was removed from the well and placed into a tube with 1 mL reduced Ringer's solution. The tubes were sonicated in a water bath for 30 seconds, vortexed briefly to disperse any remaining clumps, and 50 µL of the suspension was used to inoculate the appropriate well for the second generation. This procedure was repeated for 10 generations (80 days) for all three biofilm sources. The biofilm suspensions were further used for viable cell count estimations and DNA extraction.

Viable cell count estimations

The biofilm suspensions were additionally plated to estimate viable cell counts on the HA disk at four time points: the initial HA disk inoculation (time T₀), after 16 days (2 generations) of culture, after 40 days (5 generations) of culture, and after 80 days (10 generations) of culture.

DNA extraction and differential species abundance determination (human oral microbiome identification)

The remaining biofilm suspensions from each of four time points (day 1, day 16, day 40, and day 80) corresponding to generations 1, 2, 5, and 10, respectively, were spun at 10,000 rpm for 2 minutes to pellet the cells, the supernatants were removed, and the pellets were stored at -20°C for DNA extraction. DNA was extracted from the pellet of each biofilm sample according to the protocol provided by The Forsyth Institute (detailed below) for human oral microbe identification using Next Generation Sequencing and submitted to QIAGEN for human oral microbe identification analyses via Illumina MiSeq16S rRNA sequencing.

These analyses provided relative abundance measures of the bacteria present in the biofilm samples at each of the aforementioned time points and, thus, indicated the presence and viability of *Lactobacillus reuteri* and *Bifidobacterium animalis* subsp. *lactis*, as well as any longitudinal effects of those probiotics on the cultured biofilm composition, at those time points.

DNA ISOLATION PROTOCOL*

Lysis

To achieve bacterial cell lysis, the biofilm samples were suspended in 150 µL of TE Buffer. One microliter of Ready-Lyse Lysozyme was then added to each sample, which was subsequently incubated overnight at 37°C. To each sample, 150 µL of 2X T & C Lysis Solution, followed by 1 µL of Proteinase K, was added, followed by sample incubation for

30 minutes at 65°C while briefly vortexing every five minutes. After cooling for 37°C, the samples were placed on ice for 3-5 minutes before proceeding to DNA purification.

DNA Purification

To 300 µL of lysed sample, 175 µL of MPC Protein Precipitation Reagent was added and then vigorously vortexed for 10 seconds. The sample was then pelleted by centrifugation at 4°C for 10 minutes at $\geq 10,000 \times g$ in microcentrifuge and subsequently placed immediately on ice. The supernatant was transferred to a clean microcentrifuge tube and the pellet discarded. To the recovered supernatant, 500 µL of isopropanol was added, and the tube was inverted 30-40 times and then placed on ice for 10 minutes. The DNA was pelleted by centrifugation at 4°C for 10 minutes at $\geq 10,000 \times g$ in a microcentrifuge. After carefully pouring off the isopropanol, a pipette tip was used to remove the remaining isopropanol without dislodging the DNA pellet. The pellet was then rinsed with 500 µL of 75% ethanol and centrifuged briefly if the pellet was dislodged. This rinse was then repeated with an additional 500 µL of 75% ethanol. After removal of the residual ethanol, the DNA was resuspended in 25 µL of TE Buffer in preparation for shipment to QIAGEN for Next Generation Sequencing.

*All reagents can be procured in the Epicentre MasterPure DNA Purification Kit and Epicentre MasterPure Gram Positive DNA Purification Kit.

DETAILED METHODOLOGY FOR THE PILOT STUDY

The protocol for the completed pilot study followed the methodology for the proposed full-scale project detailed above with the exceptions elaborated in the following subsections. Three experimental components were executed as part of this pilot study and are detailed in the remainder of this chapter.

EXPERIMENT #1: Effect of continual high- and low-dose probiotic inoculation

Sample preparation

All paper point plaque samples were stored in 1-ml aliquots of Amies transport medium supplemented with 0.5% gelatin and 0.1% sodium thioglycollate at 4°C. Diluted saliva samples were centrifuged at $1200\text{ g} \times 10\text{ min}$ to remove large particulate matter then filter-sterilized through a 0.2- μm membrane, divided into 2-mL aliquots, and stored at -20°C.

Biofilm development

The subgingival plaque sample was gently sonicated in a water bath for 30 seconds to break up the biofilm and stored at 4°C while HA disks were prepared for biofilm inoculation according to the schematic depicted in Figure 3 below:

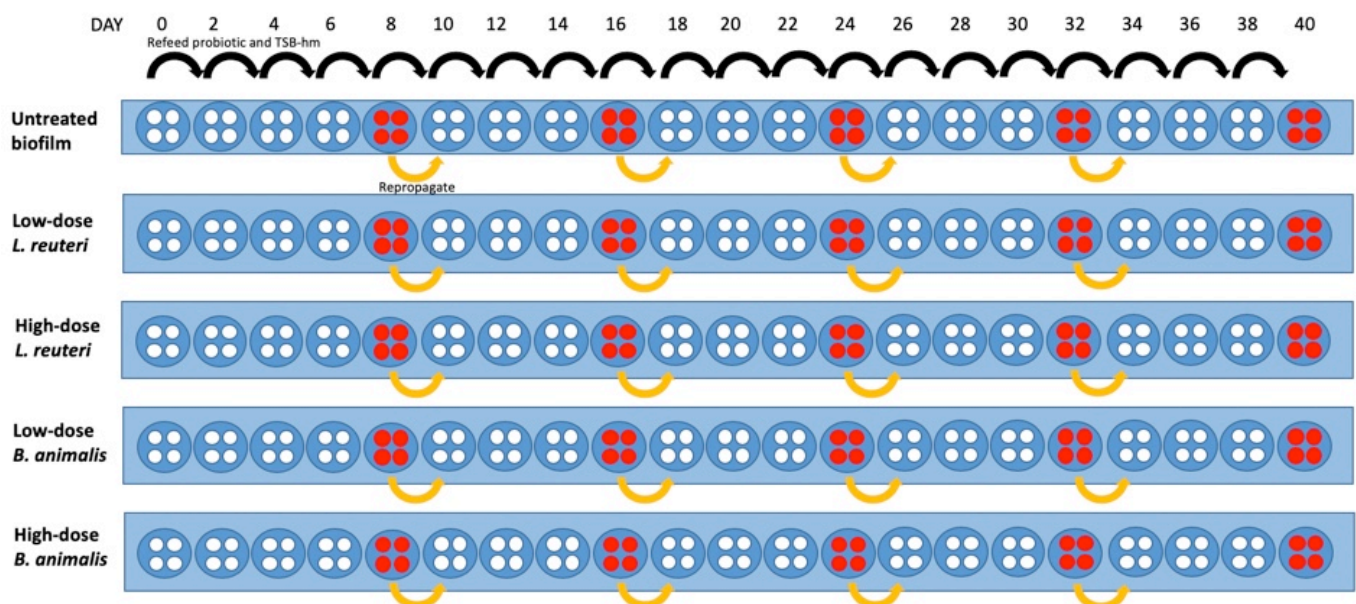


Figure 3. Development of an *in vitro* plaque biofilm with repeated exposure to either high- or low-dose *Lactobacillus reuteri* or *Bifidobacterium animalis* subsp. *lactis* over a 40-day period. Biofilms were developed on hydroxyapatite disks (depicted as red or white circles) coated with saliva obtained from the same patient as the plaque biofilm. Fresh tryptic soy broth/hemin/menadione medium and additional probiotic of the designated dose was added every two days. Every eighth day, DNA was extracted from the biofilm on one disk for analysis, and the biofilms on the remaining disks were reinoculated onto new hydroxyapatite disks (designated in red). Low- and high-dose probiotic indicates $\approx 1 \times 10^8$ CFUs/mL and 1×10^{10} CFUs/mL, respectively.

Four 5mm x 2 mm hydroxyapatite disks were placed into 24-well cell culture plates and coated for 2 hours at room temperature with sterile 1:10 saliva from the donor whose plaque sample was later added to the well. The saliva was removed and 2,970 μ L sterile tryptic soy broth supplemented with 5 μ g/mL hemin and 1 μ g/mL menadione (TSB-hm) was gently added to the “untreated biofilm” well (refer to Figure 3). To the wells corresponding to low-dose probiotic (either *L. reuteri* or *B. animalis* subsp. *lactis*), 2,967 μ L of TSB-hm was added, along with 3 μ L of the designated probiotic suspended in TSB-hm medium at a probiotic concentration of 1×10^8 CFUs/mL, as confirmed by optical density spectrophotometric standardization and viable cell counting. Similarly, to the wells corresponding to high-dose probiotic, 2,670 μ L TSB-hm was added, along with 300 μ L of the designated probiotic suspended in TSB-hm at a probiotic concentration of 1×10^{10} CFUs/mL, as, again, confirmed spectrophotometrically. The plaque sample was resuspended by briefly vortexing, and each well was inoculated with 30 μ L of that plaque sample.

Plates were placed in an anaerobic incubator at 37°C-75%N₂/10% CO₂/10% H₂ for static growth. Biofilms were cultured for 8 days before dispersal and re-inoculation. Every 48 hours, growth media were gently removed from wells and replaced by slowly adding a fresh 2 mL of reduced TSB-hm, in addition to re-inoculation with either high- or low-dose

of the predetermined probiotic, in accordance with the initial pre-determined configuration (refer to Figure 3). On the 8th day, new HA disks were added to new wells and coated with respective 1:10 sterile saliva for 2 hours in the anaerobic chamber, then the saliva was removed and replaced with fresh reduced TSB-hm according to the aforementioned volumes. A single disk with mature biofilm (8th day of growth) from each of the six wells was removed and placed into a tube with 1 mL reduced Ringer's solution. The tubes were sonicated in a water bath for 30 seconds, vortexed briefly to disperse any remaining clumps, and 30 μ L of the suspension was used to inoculate the appropriate well, to which additional probiotic of the designated dose (volume) and species were added for the second generation. This procedure was repeated for five generations (40 days). Due to monetary limitations, however, only samples corresponding to the "untreated biofilm," "low-dose *L. reuteri*," and "low-dose *B. animalis*" cultures were subsequently sequenced for DNA analysis. In brief, DNA extraction was completed for the three aforementioned biofilm cultures at days 8, 24, and 40, roughly corresponding to weeks 1, 3, and 5 of culture, respectively. DNA extractions were performed according to the Forsyth Institute protocol described previously in this chapter and submitted to QIAGEN for Next Generation Sequencing and relative species abundance determination. The sequenced 16S rRNA V3-V4 hypervariable regions for the twenty most abundant bacterial species within each sample were then compared to known sequences within the N.I.H. N.C.B.I. BLAST database to identify the specific species present, along with their relative abundances.

EXPERIMENT #2: Effect of single-dose *L. reuteri* inoculation

In order to evaluate probiotic sustainability in the *in vitro* model, a second experiment was performed with high- and low-dose *Lactobacillus reuteri* exactly as described above, with the exception that the biofilm was not re-inoculated with the probiotic every two days at the time of TSB-hm medium change (and, thus, the volume of TSB-hm was increased by 3 μ L or 300 μ L, as appropriate), and the duration of the experiment was shortened to two generations, as depicted in the schematic in Figure 4 below:

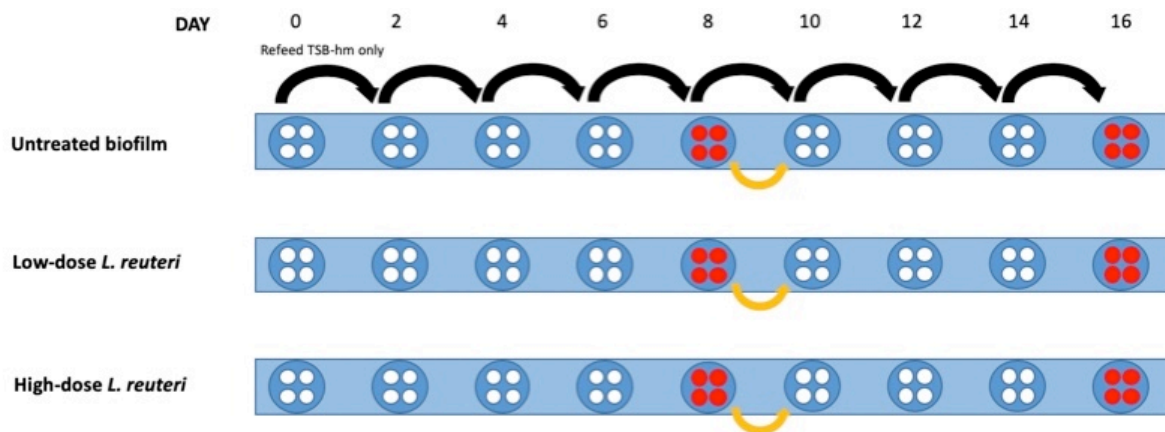


Figure 4. Development of a biofilm in the presence of a single (initial) exposure to high- or low-dose *Lactobacillus reuteri* over a 16-day period. Biofilms were developed on hydroxyapatite disks (depicted as red or white circles) coated with saliva obtained from the same patient as the plaque biofilm. Fresh tryptic soy broth/hemin/menadione (TSB-hm) medium was added every two days. Every eighth day, DNA was extracted from the biofilm on one disk for analysis, and the biofilms on the remaining disks were reinoculated onto new hydroxyapatite disks (designated in red). Low- and high-dose probiotic indicates $\approx 1 \times 10^8$ CFUs/mL and 1×10^{10} CFUs/mL, respectively.

DNA was extracted from the “untreated biofilm,” “low-dose *L. reuteri*,” and “high-dose *L. reuteri*” cultures at days 8 and 16—roughly corresponding to one and two weeks of culture, respectively—and submitted to QIAGEN for analysis, as detailed above.

EXPERIMENT #3: Effect of inulin on *B. animalis* subsp. *lactis* viability

In order to evaluate the effect of prebiotic inulin supplementation on the viability of *B. animalis* subsp. *lactis* within a dental biofilm, *in vitro* biofilms were cultured in a TSB-hm medium inoculated with varying quantities of *B. animalis* subsp. *lactis* and then supplemented with inulin at differing concentrations. A solution of *B. animalis* subsp. *lactis* suspended in TSB-hm medium was prepared to an optical density of one, as determined by spectrophotometric standardization. Four stock solutions of this *B. animalis* preparation were then prepared as an O.D. 1 stock solution (i.e. no dilution) and three serial dilutions of 1:10, 1:100, and 1:1,000 in TSB-hm medium. Dental plaque biofilms were cultured alone in TSB-hm for three generations, each of eight days in duration, on single 9.5 mm x 2 mm HA disks. Expended TSB-hm media was exchanged for fresh TSB-hm media every 48 hours. After these 24 days of biofilm culture, 10 μ L of the cultured plaque biofilm and 1 mL of TSB-hm were added to single 9.5 mm x 2 mm HA disks in each of 25 wells. To each well, 10 μ L of one of the prepared *B. animalis* subsp. *lactis* solutions (no dilution, 1:10, 1:100, or 1:1,000) were added such that five wells received the undiluted probiotic, five received the 1:10 dilution, five received the 1:100 dilution, and five received the 1:1,000 dilution. No additional probiotic was administered at any future timepoint. An additional five biofilms were cultured without *B. animalis* to serve as a control. To one of the five biofilm cultures within each of the five probiotic-inoculated biofilm cultures, one milliliter of either 0 μ M, 0.5 μ M, 5 μ M, 50 μ M, or 500 μ M inulin solution—prepared by dissolution at room temperature in TSB-hm, followed by sterile filtration with a Pall filtration membrane of 0.22 μ M pore size—was

then added. Each of these biofilms was then cultured for an additional three generations, each of eight days in duration. Every 48 hours, growth media was gently removed from wells and replaced by slowly adding a fresh 1 mL of reduced TSB-hm supplemented by 1 mL of the appropriate 0 μ M, 0.5 μ M, 5 μ M, 50 μ M, or 500 μ M inulin solution. Every eighth day, DNA was extracted from a portion of the cultured biofilm for analysis, and the remaining vortexed biofilm resuspended in TSB-hm was reinoculated onto new hydroxyapatite disks, along with the appropriate inulin solution. After 8, 16, and 24 days of biofilm culture with probiotic and prebiotic, sample DNA extractions were performed as described above and submitted to GENEWIZ for Next Generation Sequencing and relative species abundance determination. Figure 5 illustrates the general protocol for this experiment.

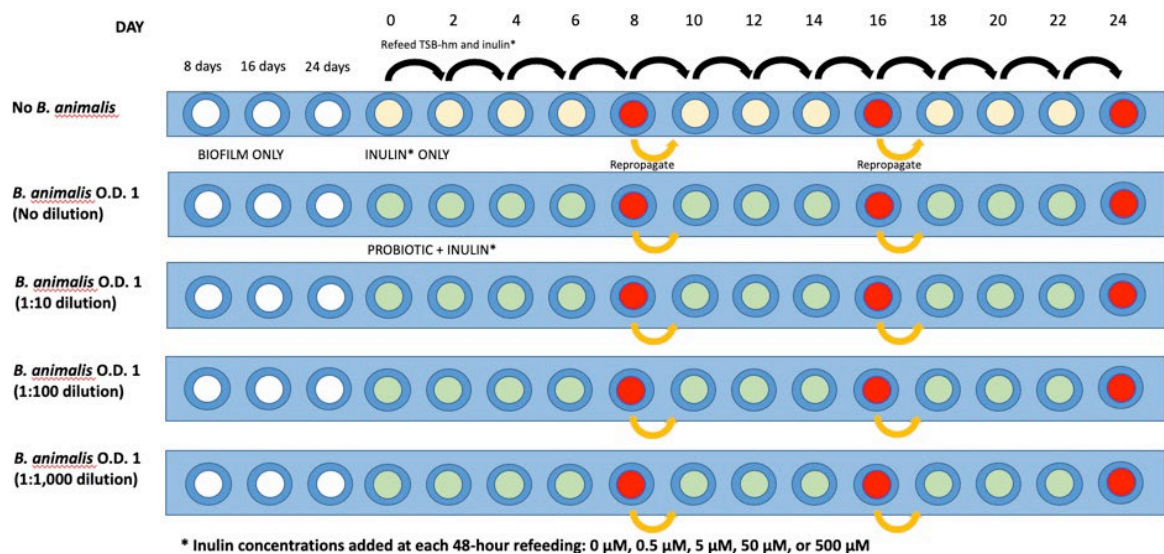


Figure 5. Effect of prebiotic inulin on *B. animalis* subsp. *lactis* viability within a cultured plaque biofilm. Biofilms alone (without probiotic or prebiotic) were cultured in TSB-hm for three 8-day generations on single hydroxyapatite disks (depicted as white circles) coated with saliva obtained from the same patient as the plaque biofilm. After the three initial generations, five groups of five biofilm lines (indicated by the five depicted rows) were inoculated with varying quantities of *B. animalis* subsp. *lactis* by addition of 10 μ L of either no probiotic (i.e. TSB-hm alone) or of probiotic from one of four prepared stock solutions of differing degrees of dilution. To each of the five biofilms within each of these five biofilm lines, 1 mL of inulin of one of five different concentrations (0 μ M, 0.5 μ M, 5 μ M, 50 μ M, or 500 μ M) was added. Expended media were replaced by fresh TSB-hm media with the appropriate inulin solution every 48 hours for three additional 8-day generations. Every eighth day, DNA was extracted from a portion of the biofilm, and the remaining biofilm was reinoculated onto a new hydroxyapatite disk (designated in red). Note that cream-colored disks denote biofilms to which inulin (of varying concentrations) alone was added, and green-colored disks denote biofilms exposed to the various permutations of *B. animalis* dose and inulin concentration.

INDEPENDENT VARIABLES

The independent variables in this pilot study were optical density/dosing of probiotic, probiotic species, prebiotic inulin concentration, and time.

DEPENDENT VARIABLES

The dependent variables in both the pilot study and full-scale investigation were relative abundances of probiotic (*Lactobacillus reuteri* or *Bifidobacterium animalis* subsp. *lactis*) and composition of the biofilm (in terms of relative microbe abundance) over time.

DATA ANALYSIS

All extracted DNA samples were submitted to either QIAGEN Corporation or GENEWIZ for 16S rRNA hypervariable region V3-V4 analysis and species identification via Next Generation Sequencing technology. This analysis provided relative microbial species

abundances for each biofilm sample, which, in turn, permitted the determination of longitudinal probiotic viability, as well as the influence of the respective probiotics on resident microbial composition over time.

CHAPTER 3: RESULTS

THE *IN VITRO* BIOFILM MODEL

Inasmuch as the primary aim of this research was to propose and evaluate methods of enhancing probiotic sustainability within the oral microbiota, an *in vitro* model by which to assess variables that may influence probiotic viability within a complex plaque biofilm was developed. As the ultimate intent of this research is to better inform the clinical application of probiotics in periodontal therapy, a flexible model capable of assessing a spectrum of variables that may influence probiotic colonization within a biofilm is needed. The model utilized in this investigation was adapted from *in vitro* plaque biofilm models proposed elsewhere in the literature (Walker, 2007; Shaddox, 2010; Slomka, 2018; Velsko, 2018) and, as detailed below, demonstrated the capacity to longitudinally assess *Lactobacillus reuteri* and *Bifidobacterium animalis* subsp. *lactis* viability within a simulacrum of the indigenous oral microbiota.

STABILITY OF *IN VITRO* BIOFILM MICROBIOTA IN THE ABSENCE OF PROBIOTICS

Prior to evaluating both the viability of *L. reuteri* and *B. animalis* subsp. *lactis* within an *in vitro* biofilm cultured from a single donor and the influence of those probiotics on the composition of that biofilm, the sustainability and constitution of the biofilm, itself, was first evaluated. This biofilm cultured in the absence of probiotics, then, served as a control for both components of the pilot study, namely the evaluations of probiotic sustainability with continual supplementation of the probiotic every two days at the time

of TSB-hm medium change (Experiment #1) and after single (initial) exposure of the biofilm to the probiotic in question without subsequent probiotic re-inoculation (Experiment #2). Table 1 and Table 2 illustrate the control biofilm microbial composition over time for each of these two aforementioned experiments, respectively.

CONTROL: Continual repopulation No probiotic Week 1	CONTROL: Continual repopulation No probiotic Week 3	CONTROL: Continual repopulation No probiotic Week 5
Streptococcus anginosus	Streptococcus anginosus	Parvimonas, peptostreptococcus
Fusobacterium nucleatum	Parvimonas, peptostreptococcus	Streptococcus anginosus
Streptococcus peiodonticum or mitis	Streptococcus anginosus	Streptococcus anginosus
Streptococcus, unknown	Clostridium sp., parvimonas sp. (uncult.)	Clostridium sp., parvimonas sp. (uncult.)
Streptococcus chosunense	Moryella sp., uncultured	Fusobacterium spp.
Streptococcus chosunense	Apotobium parvulum	Streptococcus anginosus
Granulicatella adiacens	Solobacterium moorei or Bulleidia spp.	Solobacterium moorei or Bulleidia spp.
Apotobium parvulum	Stomatobaculum longum	Apotobium parvulum
Actinomyces spp.	Streptococcus anginosus	Fusobacterium spp.
Fusobacterium nucleatum	Slackia spp.	Selenomonas 920
Veillonella parvula	Veillonella, uncultured bacterium	Moryella sp., uncultured
Veillonella tobetsuensis	Selenomonas spp.	Slackia spp.
Fusobacterium spp.	Granulicatella adiacens	Fusobacterium nucleatum
Veillonella spp., uncultured	Solobacterium moorei or Bulleidia spp.	Veillonella tobetsuensis
Granulicatella adiacens	Veillonella spp., uncultured	Fusobacterium ChDC F332
Veillonella spp.	Fusobacterium spp.	Selenomonas 920
Streptococcus gordonii	Veillonella parvula	Streptococcus anginosus 4188
Streptococcus parasanguinis	Streptococcus anginosus 4188	Micromonas micros/Parvimonas
Veillonella parvula	Fusobacterium spp.	Stomatobaculum longum
Veillonella spp., uncultured	Selenomonas, uncultured bacterium	Fusobacterium nucleatum GS17A

Table 1. Longitudinal biofilm microbial composition in the absence of probiotic exposure (Experiment #1, control). Listed are the twenty most abundant microbes, ranked from most to least abundant, as assessed at three different time points over the course of biofilm culture. Note that duplicate entries indicate different strains within the same species.

CONTROL: Initial probiotic only No probiotic Week 1	CONTROL: Initial probiotic only No probiotic Week 2
Streptococcus anginosus	Streptococcus anginosus
Parvimonas, peptostreptococcus	Parvimonas, peptostreptococcus
Streptococcus anginosus	Clostridium sp., parvimonas sp. (uncult.)
Clostridium sp., parvimonas sp. (uncult.)	Streptococcus anginosus
Fusobacterium nucleatum	Moryella sp., uncultured
Streptococcus anginosus	Peptostreptococcus, uncultured sp.
Apotobium parvulum	Fusobacterium nucleatum
Peptostreptococcus, uncultured sp.	Streptococcus anginosus
Fusobacterium nucleatum	Fusobacterium nucleatum
Selenomonas spp., uncultured	Eubacterium nodatum, uncultured bacterium
Granulicatella adiacens	Granulicatella adiacens
Moryella sp., uncultured	Peptostreptococcus, uncultured sp.
Dialister pneumosintes	Dialister spp.
Oribacterium spp., uncultured	Fusobacterium spp.
Lachnospiraceae spp., Streptococcus spp.	Fusobacterium nucleatum
Streptococcus intermedius	Dialister spp.
Uncultured clone 071072	Catonella morbi, Cantonella spp.
Veillonella tobetsuensis	Veillonella tobetsuensis
Fusobacterium nucleatum	Uncultured clone 071072
Mogibacterium sp., uncultured	Dialister sp., uncultured clone

Table 2. Longitudinal biofilm microbial composition in the absence of probiotic exposure (Experiment #2, control). Listed are the twenty most abundant microbes, ranked from most to least abundant, as assessed at two different time points of biofilm culture. Note that duplicate entries indicate different strains within the same species.

The assessment of physical biofilm development and adherence to the hydroxyapatite disks *in vitro*, in conjunction with the species identification via 16S rRNA hypervariable V3-V4 analysis depicted in Table 1 and 2, not only indicates that the experimental model utilized in this study is capable of developing and perpetuating a plaque biofilm, but also that the DNA extracted from the biofilm cultured from one 5 mm x 2 mm HA disk is of sufficient quantity for analysis by Next Generation Sequencing.

With regards to the microbial composition of the control biofilm, unexposed to probiotics, it can readily be seen that in both experiments, streptococci and species of the *Parvimonas* and *Clostridium* genera are the most abundant, and that this composition remains consistent over time. Likewise, *Fusobacterium* species are abundant and remain, on the whole, consistently distributed over time. In the control biofilm of the first

experiment, *Apotobium parvulum* remains in nearly identical relative abundance for the duration of the experiment, as do *Granulicatella adiacens* and *Dialister* species in the second experiment. It is obvious, however, that significant temporal variability exists with regards to both relative abundances of individual species and overall microbial composition. Therefore, it cannot be concluded that the constitution of the biofilm microbiota remains stable over time in a strict sense. Nevertheless, general trends, such as those specified above, can be observed, as can the fact that Gram-positive species predominate throughout the course of both experiments.

***LACTOBACILLUS REUTERI* SUSTAINABILITY AND EFFECT ON MICROBIAL COMPOSITION**

Of primary interest in this investigation were (1) the ability to culture *Lactobacillus reuteri* and *Bifidobacterium animalis* subsp. *lactis* within an *in vitro* plaque biofilm in TSB-hm medium and (2) the evaluation of both their sustainability within that model and their effect(s) on the resident biofilm microbiota. The results of continual low-dose exposure of *Lactobacillus reuteri*, in terms of probiotic viability and influence on biofilm composition, are shown in Table 3, which is shown in juxtaposition to Table 1 for ease of comparison with the control biofilm, below:

CONTROL: Continual repopulation No probiotic Week 1	CONTROL: Continual repopulation No probiotic Week 3	CONTROL: Continual repopulation No probiotic Week 5
Streptococcus anginosus	Streptococcus anginosus	Parvimonas, peptostreptococcus
Fusobacterium nucleatum	Parvimonas, peptostreptococcus	Streptococcus anginosus
Streptococcus peiodonticum or mitis	Streptococcus anginosus	Streptococcus anginosus
Streptococcus, unknown	Clostridium sp., parvimonas sp. (uncult.)	Clostridium sp., parvimonas sp. (uncult.)
Streptococcus chosunense	Moryella sp., uncultured	Fusobacterium spp.
Streptococcus chosunense	Apotobium parvulum	Streptococcus anginosus
Granulicatella adiacens	Solobacterium moorei or Bulleidia spp.	Solobacterium moorei or Bulleidia spp.
Apotobium parvulum	Stomatobaculum longum	Apotobium parvulum
Actinomyces spp.	Streptococcus anginosus	Fusobacterium spp.
Fusobacterium nucleatum	Slackia spp.	Selenomonas 920
Veillonella parvula	Veillonella, uncultured bacterium	Moryella sp., uncultured
Veillonella tobetsuensis	Selenomonas spp.	Slackia spp.
Fusobacterium spp.	Granulicatella adiacens	Fusobacterium nucleatum
Veillonella spp., uncultured	Solobacterium moorei or Bulleidia spp.	Veillonella tobetsuensis
Granulicatella adiacens	Veillonella spp., uncultured	Fusobacterium ChDC F332
Veillonella spp.	Fusobacterium spp.	Selenomonas 920
Streptococcus gordonii	Veillonella parvula	Streptococcus anginosus 4188
Streptococcus parasanguinis	Streptococcus anginosus 4188	Micromonas micros/Parvimonas
Veillonella parvula	Fusobacterium spp.	Stomatobaculum longum
Veillonella spp., uncultured	Selenomonas, uncultured bacterium	Fusobacterium nucleatum GS17A

Table 1. Longitudinal biofilm microbial composition in the absence of probiotic exposure (Experiment #1, control). Listed are the twenty most abundant microbes, ranked from most to least abundant, as assessed at three different time points of biofilm culture. Note that duplicate entries indicate different strains within the same species.

TEST: Continual probiotic repopulation LOW-DOSE <i>L. reuteri</i> Week 1	TEST: Continual probiotic repopulation LOW-DOSE <i>L. reuteri</i> Week 3	TEST: Continual probiotic repopulation LOW-DOSE <i>L. reuteri</i> Week 5
Streptococcus anginosus	Streptococcus anginosus	Parvimonas, peptostreptococcus
Streptococcus peiodonticum or mitis	Parvimonas, peptostreptococcus	Streptococcus anginosus
Fusobacterium nucleatum	Streptococcus anginosus	Streptococcus anginosus
Streptococcus peiodonticum or mitis	Clostridium sp., parvimonas sp. (uncult.)	Clostridium sp., parvimonas sp. (uncult.)
Streptococcus, unknown	Moryella sp., uncultured	Moryella sp., uncultured
Streptococcus peiodonticum or mitis	Solobacterium moorei or Bulleidia spp.	Solobacterium moorei or Bulleidia spp.
Granulicatella adiacens	Fusobacterium nucleatum	Fusobacterium nucleatum
Solobacterium moorei or Bulleidia spp.	Streptococcus anginosus	Apotobium parvulum
Veillonella spp.	Apotobium parvulum	Streptococcus anginosus
Lactobacillus reuteri	Veillonella parvula	Fusobacterium spp.
Fusobacterium nucleatum	Selenomonas spp., uncultured	Slackia spp.
Fusobacterium spp.	Fusobacterium nucleatum	Stomatobaculum longum
Veillonella spp.	Stomatobaculum longum	Veillonella parvula
Actinomyces spp.	Fusobacterium nucleatum	Fusobacterium nucleatum
Veillonella spp.	Veillonella parvula, uncultured variant	Veillonella parvula, uncultured variant
Apotobium parvulum	Veillonella parvula UTDB1-3	Veillonella parvula UTDB1-3
Campylobacter rectus/showae	Veillonella spp.	Bifidobacterium animalis subsp. lactis
Streptococcus gordonii	Campylobacter rectus/showae	Fusobacterium nucleatum
Granulicatella adiacens	Fusobacterium nucleatum	Selenomonas spp., uncultured
Moryella sp., uncultured	Veillonella parvula VP57	Selenomonas spp., uncultured

TABLE 3. Longitudinal effect of continuous low-dose (1×10^8 CFUs/mL) *Lactobacillus reuteri* on biofilm microbial composition (Experiment #1, test, *L. reuteri*). Listed are the twenty most abundant microbes, ranked from most to least abundant, as assessed at three different time points of biofilm culture. Note that duplicate entries indicate different strains within the same species. Note also that *L. reuteri* is denoted in lime green.

It is readily evident from Table 3 that although *L. reuteri* is present at week one of biofilm culture, is it not among the twenty most abundant species at subsequent timepoints, which would seem to indicate that *L. reuteri* is not viable within the proposed model, not competitive for niche occupation within the biofilm, or both. Nevertheless, its reasonably high abundance at week one of biofilm culture is, at the very least, suggestive of potential viability under suitable circumstances.

With regards to an influence on the indigenous microbiota, it would appear that *L. reuteri* has little to no influence at low-dose exposure. Indeed, even when *L. reuteri* is present at relatively high abundance at week one, the microbial composition is hardly variant from that of the biofilm unexposed to probiotic, as evidenced by a comparison of the first column of Table 1 with that of Table 3. Furthermore, with the exception of a greater abundance of *Veillonella* species relative to the control, the overall biofilm composition appears only marginally affected by low-dose *L. reuteri* exposure over time, as indicated by comparison of the last two columns of Table 1 and Table 3. Incidentally, *Fusobacterium* counts are not diminished by the presence of *L. reuteri*. Note also that the presence of *B. animalis* subsp. *lactis* at week five of low-dose *L. reuteri* inoculation is suggestive of sample contamination.

To evaluate the influence of increasing probiotic dose on the ability of *L. reuteri* to colonize the resident microbiota and alter the biofilm constitution over a two-week period, a second experiment (Experiment #2) was performed in which the effect of a single 1×10^8 CFU/mL (“low”) dose of *L. reuteri* was compared to that of a single 1×10^{10} CFU/mL (“high”) dose. Note that a second distinctive feature of this experiment is that the probiotic was only administered at the time of initial biofilm development

without subsequent supplementation at the time of TSB-hm media changes. This was performed to provide a better indication of probiotic viability upon single application. The results of this experiment are shown in Table 4. To facilitate comparison with the control condition of biofilm composition in the absence of *L. reuteri* exposure, Table 2 is shown in juxtaposition.

CONTROL: Initial probiotic only No probiotic Week 1	CONTROL: Initial probiotic only No probiotic Week 2
Streptococcus anginosus	Streptococcus anginosus
Parvimonas, peptostreptococcus	Parvimonas, peptostreptococcus
Streptococcus anginosus	Clostridium sp., parvimonas sp. (uncult.)
Clostridium sp., parvimonas sp. (uncult.)	Streptococcus anginosus
Fusobacterium nucleatum	Moryella sp., uncultured
Streptococcus anginosus	Peptostreptococcus, uncultured sp.
Apotobium parvulum	Fusobacterium nucleatum
Peptostreptococcus, uncultured sp.	Streptococcus anginosus
Fusobacterium nucleatum	Fusobacterium nucleatum
Selenomonas spp., uncultured	Eubacterium nodatum, uncultured bacterium
Granulicatella adiacens	Granulicatella adiacens
Moryella sp., uncultured	Peptostreptococcus, uncultured sp.
Dialister pneumosintes	Dialister spp.
Oribacterium spp., uncultured	Fusobacterium spp.
Lachnospiraceae spp., Streptococcus spp.	Fusobacterium nucleatum
Streptococcus intermedius	Dialister spp.
Uncultured clone 071072	Catonella morbi, Cantonella spp.
Veillonella tobetsuensis	Veillonella tobetsuensis
Fusobacterium nucleatum	Uncultured clone 071072
Mogibacterium sp., uncultured	Dialister sp., uncultured clone

Table 2. Longitudinal biofilm microbial composition in the absence of probiotic exposure (Experiment #2, control). Listed are the twenty most abundant microbes, ranked from most to least abundant, as assessed at two different time points of biofilm culture. Note that duplicate entries indicate different strains within the same species.

TEST: Initial probiotic only LOW-DOSE <i>L. reuteri</i> Week 1	TEST: Initial probiotic only LOW-DOSE <i>L. reuteri</i> Week 2	TEST: Initial probiotic only HIGH-DOSE <i>L. reuteri</i> Week 1	TEST: Initial probiotic only HIGH-DOSE <i>L. reuteri</i> Week 2
Streptococcus anginosus	Parvimonas, peptostreptococcus	Streptococcus anginosus	Streptococcus anginosus
Parvimonas, peptostreptococcus	Streptococcus anginosus	Parvimonas, peptostreptococcus	Parvimonas, peptostreptococcus
Clostridium sp., parvimonas sp. (uncult.)	Clostridium sp., parvimonas sp. (uncult.)	Clostridium sp., parvimonas sp. (uncult.)	Clostridium sp., parvimonas sp. (uncult.)
Streptococcus anginosus	Peptostreptococcus spp.	Moryella sp., uncultured	Streptococcus anginosus
Peptostreptococcus spp.	Streptococcus anginosus	Peptostreptococcus spp.	Peptostreptococcus spp.
Moryella sp., uncultured	Moryella sp., uncultured	Streptococcus anginosus	Moryella sp., uncultured
Streptococcus anginosus	Eubacterium nodatum, uncultured bacterium	Streptococcus anginosus	Streptococcus anginosus
Eubacterium nodatum, uncultured bacterium	Peptostreptococcus spp.	Lactobacillus reuteri	Streptococcus mutans
Anaerogolbus germintus, uncultured	Bifidobacterium animalis subsp. lactis	Fusobacterium nucleatum	Eubacterium nodatum, uncultured bacterium
Dialister pneumosintes	Streptococcus anginosus	Bifidobacterium animalis subsp. lactis	Peptostreptococcus spp.
Fusobacterium nucleatum	Streptococcus mutans	Mogibacterium sp., uncultured	Fusobacterium nucleatum
Mogibacterium sp., uncultured	Catonella morbi, Catonella spp.	Fusobacterium nucleatum	Catonella morbi, Catonella spp.
Peptostreptococcus spp., uncultured	Peptostreptococcus spp.	Dialister pneumosintes	Fusobacterium spp.
Oribacterium spp., uncultured	Shuttleworthia spp.	Solobacterium moorei or Bulleidia spp.	Fusobacterium nucleatum
Fusobacterium nucleatum	Fusobacterium nucleatum	Peptostreptococcus spp.	Dialister spp.
Lactobacillus reuteri	Fusobacterium spp.	Fusobacterium spp.	Bifidobacterium animalis subsp. lactis
Apotobium parvulum	Fusobacterium nucleatum	Oribacterium spp., uncultured	Dialister spp.
Slackia exagla	Veillonella parvula	Apotobium parvulum	Prevotella spp., uncultured/unknown
Uncultured clone 071072	Olsenella/Coriobacteriaceae	Uncultured clone 071072	Veillonella tobetsuensis
Veillonella parvula VP57	Uncultured bacterium	Eubacterium nodatum, uncultured bacterium	Veillonella parvula VP57

Table 4. Longitudinal effect of single low-dose (1×10^8 CFUs/mL) versus single high-dose (1×10^{10} CFU/mL) *Lactobacillus reuteri* on biofilm microbial composition (Experiment #2, test). Listed are the twenty most abundant microbes, ranked from most to least abundant, as assessed at three different time points of biofilm culture. Note that duplicate entries indicate different strains within the same species. Note also that *L. reuteri* is denoted in lime green.

The presence, and dose-dependent relative abundance, of *L. reuteri* at one week of biofilm culture despite a mere single inoculation of the probiotic is perhaps even more suggestive that, provided selective favorable circumstances, *L. reuteri* could be “encouraged” to colonize an *in vitro* biofilm. Nevertheless, by the second week of culture, *L. reuteri* was no longer among the twenty most abundant species, regardless of the initially administered dose. In fact, Next Generation Sequencing analysis revealed that by the second week of culture, *L. reuteri* had diminished from the 16th most abundant species to the 248th most abundant species for the low-dose *L. reuteri* sample, and from the 8th most abundant species to the 229th most abundant species in the high-dose sample. This indicates that *L. reuteri* is not robustly sustainable beyond one week in the proposed *in vitro* model, despite a 100-fold increase in initial dose. Furthermore, in concordance with the findings of the Experiment #1, in which low-dose *L. reuteri* was administered every two days, only

marginal differences in biofilm microbial composition could be discriminated in the samples with probiotic exposure relative to the control sample. Again, an insignificant effect of *L. reuteri* inoculation on *Fusobacterium species* and *Eubacterium nodatum* abundance within the biofilm could be discerned (see Table 2 and Table 4). The presence of *Bifidobacterium animalis* subsp. *lactis* in the test samples is likely due to contamination and, therefore, caution should be exercised in attempting to infer its viability and the effect of its presence, as it is unknown at what point and in what quantities it was introduced into the sample.

***BIFIDOBACTERIUM ANIMALIS* SUBSP. *LACTIS* SUSTAINABILITY AND EFFECT ON MICROBIAL COMPOSITION**

The second probiotic evaluated in this pilot study was *Bifidobacterium animalis* subsp. *lactis*. As with *Lactobacillus reuteri*, the specific intent of the investigation was to assess both the viability of *B. animalis* within the *in vitro* biofilm model, as well as the effect of that probiotic on the resident microbial composition of the cultured biofilm. Furthermore, this investigation provided a comparative analysis of the behavior of two different probiotics—namely, *L. reuteri* and *B. animalis*—within the biofilm model. The results of continuous low-dose *Bifidobacterium animalis* subsp. *lactis* inoculation of the cultured biofilm are summarized in Table 5. For ease of comparison with the control biofilm culture devoid of probiotic exposure, Table 1 is shown in juxtaposition.

CONTROL: Continual repopulation No probiotic Week 1	CONTROL: Continual repopulation No probiotic Week 3	CONTROL: Continual repopulation No probiotic Week 5
Streptococcus anginosus	Streptococcus anginosus	Parvimonas, peptostreptococcus
Fusobacterium nucleatum	Parvimonas, peptostreptococcus	Streptococcus anginosus
Streptococcus peiodonticum or mitis	Streptococcus anginosus	Streptococcus anginosus
Streptococcus, unknown	Clostridium sp., parvimonas sp. (uncult.)	Clostridium sp., parvimonas sp. (uncult.)
Streptococcus chosunense	Moryella sp., uncultured	Fusobacterium spp.
Streptococcus chosunense	Apotobium parvulum	Streptococcus anginosus
Granulicatella adiacens	Solobacterium moorei or Bulleidia spp.	Solobacterium moorei or Bulleidia spp.
Apotobium parvulum	Stomatobaculum longum	Apotobium parvulum
Actinomyces spp.	Streptococcus anginosus	Fusobacterium spp.
Fusobacterium nucleatum	Slackia spp.	Selenomonas 920
Veillonella parvula	Veillonella, uncultured bacterium	Moryella sp., uncultured
Veillonella tobetsuensis	Selenomonas spp.	Slackia spp.
Fusobacterium spp.	Granulicatella adiacens	Fusobacterium nucleatum
Veillonella spp., uncultured	Solobacterium moorei or Bulleidia spp.	Veillonella tobetsuensis
Granulicatella adiacens	Veillonella spp., uncultured	Fusobacterium ChDC F332
Veillonella spp.	Fusobacterium spp.	Selenomonas 920
Streptococcus gordonii	Veillonella parvula	Streptococcus anginosus 4188
Streptococcus parasanguinis	Streptococcus anginosus 4188	Micromonas micros/Parvimonas
Veillonella parvula	Fusobacterium spp.	Stomatobaculum longum
Veillonella spp., uncultured	Selenomonas, uncultured bacterium	Fusobacterium nucleatum GS17A

Table 1. Longitudinal biofilm microbial composition in the absence of probiotic exposure (Experiment #1, control). Listed are the twenty most abundant microbes, ranked from most to least abundant, as assessed at three different time points of biofilm culture. Note that duplicate entries indicate different strains within the same species.

TEST: Continual probiotic repopulation LOW-DOSE <i>B. animalis</i> Week 1	TEST: Continual probiotic repopulation LOW-DOSE <i>B. animalis</i> Week 3	TEST: Continual probiotic repopulation LOW-DOSE <i>B. animalis</i> Week 5
Streptococcus anginosus	Streptococcus anginosus	Streptococcus anginosus
Parvimonas, peptostreptococcus	Parvimonas, peptostreptococcus	Moryella sp., uncultured
Solobacterium moorei or Bulleidia spp.	Clostridium sp., parvimonas sp. (uncult.)	Solobacterium moorei or Bulleidia spp.
Fusobacterium nucleatum	Streptococcus anginosus	Parvimonas, peptostreptococcus
Clostridium sp., parvimonas sp. (uncult.)	Solobacterium moorei or Bulleidia spp.	Bifidobacterium animalis subsp. lactis
Veillonella parvula	Veillonella tobetsuensis	Clostridium sp., parvimonas sp. (uncult.)
Bifidobacterium animalis subsp. lactis	Peptostreptococcus spp.	Streptococcus anginosus
Moryella sp., uncultured	Streptococcus anginosus	Stomatobaculum longum
Lactobacillus reuteri	Slackia spp.	Apotobium parvulum
Veillonella tobetsuensis	Apotobium parvulum	Veillonella parvula
Streptococcus anginosus	Veillonella spp., uncultured/unknown	Streptococcus anginosus
Fusobacterium nucleatum	Veillonella parvula	Catonella morbi, Catonella spp.
Streptococcus anginosus	Granulicatella adiacens	Slackia spp.
Streptococcus peiodonticum or mitis	Fusobacterium nucleatum	Veillonella tobetsuensis
Streptococcus peiodonticum or mitis	Veillonella sp., uncultured variant	Selenomonas spp., uncultured
Streptococcus, unknown	Actinomyces sp. A3	Solobacterium moorei or Bulleidia spp.
Streptococcus peiodonticum or mitis	Solobacterium moorei or Bulleidia spp.	Veillonella parvula, uncultured variant
Granulicatella adiacens	Lachnospiraceae spp., Streptococcus spp.	Veillonella parvula UTDB1-3
Veillonella spp.	Dialister sp., uncultured	Veillonella spp., uncultured/unknown
Fusobacterium spp.	Veillonella parvula, uncultured variant	Lachnospiraceae spp., Streptococcus spp.

Table 5. Longitudinal effect of continuous low-dose (1×10^8 CFUs/mL) *Bifidobacterium animalis* subsp. *lactis* on biofilm microbial composition (Experiment #1, test, *B. animalis*). Listed are the twenty most abundant microbes, ranked from most to least abundant, as assessed at three different time points of biofilm culture. Note that duplicate entries indicate different strains within the same species. Note also that *B. animalis* subsp. *lactis* is denoted in light blue.

The relative species abundances depicted in Table 5 attest to two significant differences with regards to probiotic viability and biofilm composition when compared to the results obtained with *Lactobacillus reuteri*. The first important observation is that unlike *L. reuteri*, *B. animalis* subsp. *lactis* was sustainable over the entire five-week duration of biofilm culture. Although *B. animalis* appears to exhibit significantly diminished relative abundance by the third week, as testified by its absence from the second column in Table 5, it was the fifth most abundant species by the fifth week. Moreover, according to the QIAGEN 16S rRNA sequencing analysis, *B. animalis*, being the 27th most abundant species, was still reasonably abundant at week three. This is in stark contradistinction to *L. reuteri*, whose relative abundance at the third and fifth week of biofilm after comparable low-dose continuous administration was 49th and 23rd, respectively.

The second significant observation pertains to the longitudinal effect of *B. animalis* on the “indigenous” plaque biofilm composition over the five-week study period. Indeed, a comparison of the resident microbiota in the absence of probiotic exposure with the microbiota in coexistence with *B. animalis* reveals a substantial difference in composition. Notably, one week after biofilm culture, there is a reversal in relative abundance of *Streptococcus* and *Veillonella* species after exposure to low-dose *B. animalis* relative to control. Thereafter, there is a greater representation of *Veillonella* species with time after continual inoculation with the probiotic. Perhaps most significantly, and most reflective of a health-promoting microbiological effect of *B. animalis*, is the dramatic decrease in *Fusobacterium nucleatum* with time, such that by the fifth week of biofilm culture with

the probiotic, *F. nucleatum* is no longer among the twenty most abundant microbial species. Incidentally, the presence of *L. reuteri* at one week of culture with *B. animalis* is almost certainly reflective of contamination.

PROBIOTIC EFFECTS ON ALPHA AND BETA DIVERSITY

Another parameter that can be assessed by 16S rRNA sequencing data is that of overall species richness, as reflected in the alpha diversity and beta diversity of the evaluated samples. Alpha diversity reflects the microbial diversity, in terms of species number (i.e. “richness”), within a particular microbial ecosystem. Beta diversity, on the other hand, evaluates the *change* in species diversity between ecosystems. The alpha and beta diversity plots for this experiment are shown in Figure 6 and Figure 7.

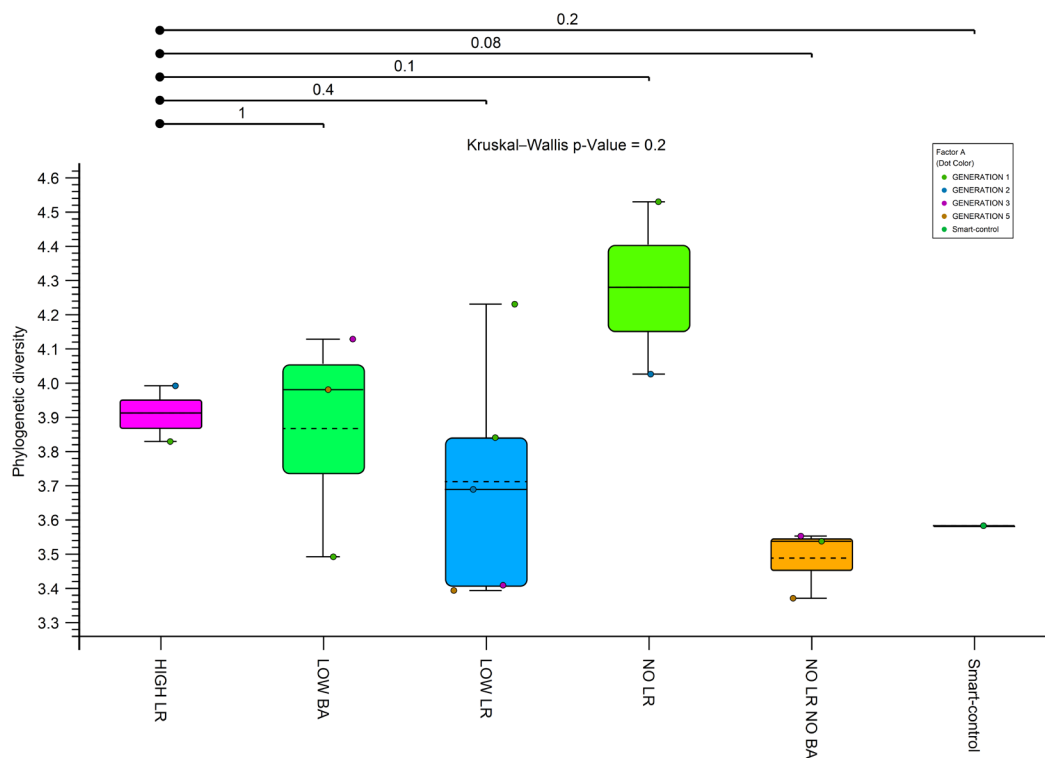


Figure 6. Influence of probiotics on alpha diversity. Boxplot reflects species diversity under the conditions of low- and high-dose *Lactobacillus reuteri* (LOW LR and HIGH LR), low-dose *Bifidobacterium animalis* subsp. *lactis* (LOW BA), and the absence of probiotic (NO LR, NO LR, NO BA). Note that the LOW LR reflects the combined data of the samples exposed to single (initial) and continuous low-dose *Lactobacillus reuteri* exposure.

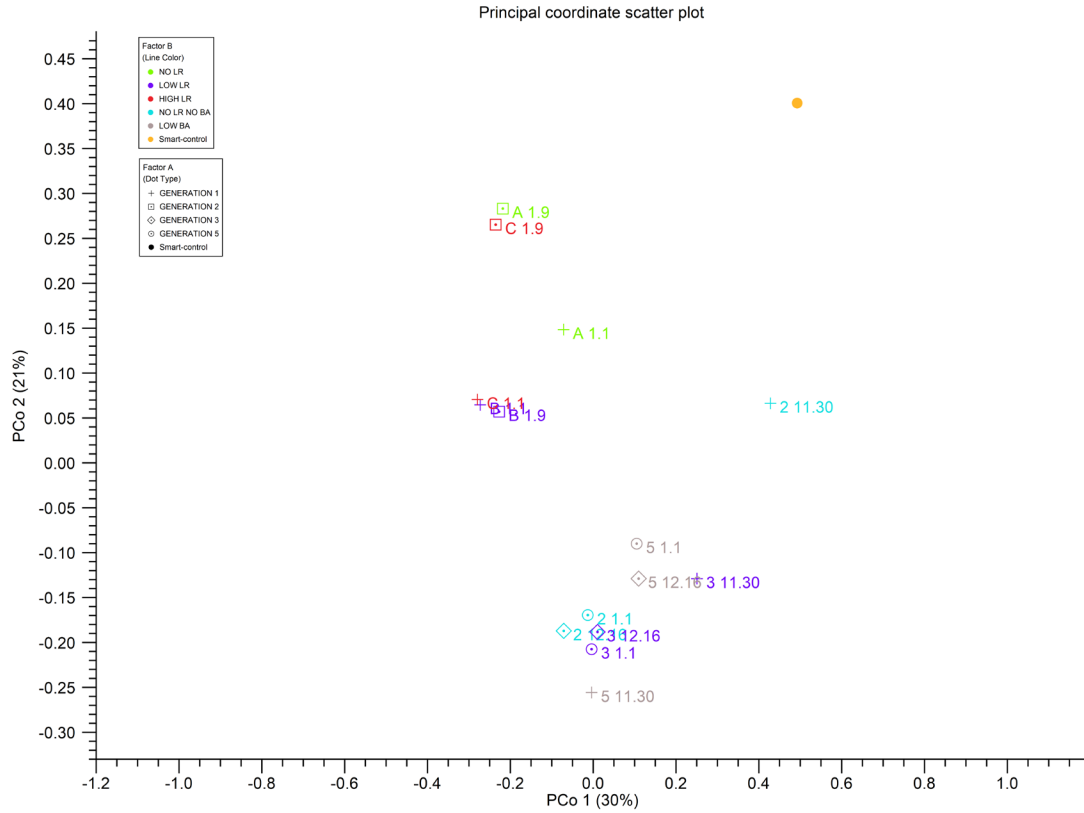


Figure 7. Influence of probiotics on beta diversity. Scatter plot reflects species diversity under the conditions of low- and high-dose *Lactobacillus reuteri* (LOW LR and HIGH LR), low-dose *Bifidobacterium animalis* subsp. *lactis* (LOW BA), and the absence of probiotic (NO LR, NO LR, NO BA). The numbers within the scatter plot reflect the date of DNA extraction within that particular sample (i.e. the gray “5 1.1” reflects the low-dose *Bifidobacterium animalis* subsp. *lactis* culture—internally labelled the fifth sample for tracking purposes—from which the DNA was extracted on 01 January 2020; similarly, the gray “5 11.30” would indicate DNA extraction of that sample on 30 November 2019, and so on). Note that all DNA extractions occurred between 30 November 2019 and 09 January 2020.

From the alpha diversity data, it can be concluded that the greatest median species diversity was found in the collective *control* samples for Experiment #2 (designated NO

LR), wherein the plaque biofilm was cultured in the absence of any probiotic. The second highest phylogenetic diversity was found in the collective samples (designated LOW BA) corresponding to continuous low-dose *Bifidobacterium animalis* subsp. *lactis* administration; however, there is considerable overlap with the maximum range of the HIGH LR samples and with third quartile range of LOW LR samples, which correspond to the single administrations of high-dose *Lactobacillus reuteri* and low-dose *Lactobacillus reuteri*, respectively, of Experiment #2. The lowest median species diversity was found in the collective samples (designated NO LR NO BA) corresponding to the control cultures of Experiment #1, in which the plaque biofilm was developed over five weeks in the absence of either *Lactobacillus reuteri* or *Bifidobacterium animalis* subsp. *lactis*.

The beta diversity scatter plot depicted in Figure 7 illustrates the *change* in species richness (phylogenetic diversity) over time within the individual cultured samples of Experiment #1 and Experiment #2. From the beta diversity data, a general trend toward increased species diversity with time is evident in all samples *except* in the control sample of Experiment #1 (designated in light blue as NO LR NO BA), in which the plaque biofilm was cultured without exposure to *either* probiotic, and in the samples corresponding to continual low-dose exposure to *Lactobacillus reuteri* (designated by the purple data points that begin with “2”). Indeed, in these culture lines, the overall species richness sharply *declined* over time, although there was an insignificant increase in diversity between 16 December 2019 and 01 January 2020 in the samples cultured without either probiotic. However, it is paradoxical to find a much higher species diversity in the samples (designated by the two purple data points beginning with the letter “B”) corresponding to single low-dose exposure to *L. reuteri* in Experiment #2 relative to that found with

continuous low-dose *L. reuteri* exposure. Nevertheless, biofilms cultured with a single high-dose *L. reuteri* administration (indicated by red data points beginning with the letter “C”) or continuous low-dose *B. animalis* subsp. *lactis* (indicated by gray data points beginning with the number “5”), demonstrated *increases* in species richness within the biofilm over time.

EFFECT OF INULIN ON *B. ANIMALIS* SUBSP. *LACTIS* VIABILITY AND BIOFILM MICROBIAL COMPOSITION

As demonstrated in the first experiment of this pilot study, *B. animalis* subsp. *lactis* appears to exhibit greater viability within the cultured biofilm three weeks after low-dose (1×10^8 CFUs/mL) inoculation than does *L. reuteri*. Nevertheless, it is of interest to elucidate mechanisms by which *B. animalis* subsp. *lactis* sustainability might be further enhanced within a dental biofilm. The third experiment of this pilot study assessed the effect of prebiotic inulin on both *B. animalis* subsp. *lactis* viability within the cultured biofilm and on the microbial composition of the resident microflora within that biofilm over a three-week period. The results of this experiment demonstrated that inulin supplementation increased the viability of *B. animalis* subsp. *lactis*, as elucidated by the total number of *B. animalis* 16S rRNA sequences identified within each sample by DNA analysis, at inulin concentrations of at least 50 μ M (see Table 6 and Table 7 below). Below this concentration, inulin only negligibly influenced viability of the probiotic relative to no inulin supplementation. However, at that highest inulin concentration (500 μ M), *B. animalis* subsp. *lactis* growth increased 28- to 600-fold relative to control (no inulin). Note that, as shown in Table 6, some *B. animalis* was present even in the initial three generations

of biofilm culture, to which neither probiotic nor prebiotic was added. The reason for this is unknown, but could conceivably reflect the presence of an unidentified *B. animalis* subspecies other than *lactis* that naturally exists in the oral cavity.

<i>B. animalis</i> O.D. 1 (No dilution)					
Inulin concentration (μM)	Biofilm only Generation 1	Biofilm only Generation 3	Probiotic + inulin Generation 1	Probiotic + inulin Generation 2	Probiotic + inulin Generation 3
0	4	28	81	38	4
0.5	4	28	63	18	7
5	4	28	99	22	3
50	4	28	371	157	63
500	4	28	2336	1627	1056

<i>B. animalis</i> O.D. 1 (1:10)					
Inulin concentration (μM)	Biofilm only Generation 1	Biofilm only Generation 3	Probiotic + inulin Generation 1	Probiotic + inulin Generation 2	Probiotic + inulin Generation 3
0	4	28	41	10	4
0.5	4	28	21	0	3
5	4	28	54	37	2
50	4	28	454	118	79
500	4	28	1894	1338	1250

<i>B. animalis</i> O.D. 1 (1:100)					
Inulin concentration (μM)	Biofilm only Generation 1	Biofilm only Generation 3	Probiotic + inulin Generation 1	Probiotic + inulin Generation 2	Probiotic + inulin Generation 3
0	4	28	28	8	3
0.5	4	28	12	1	2
5	4	28	41	4	2
50	4	28	493	188	117
500	4	28	1626	973	1799

<i>B. animalis</i> O.D. 1 (1:1,000)					
Inulin concentration (μM)	Biofilm only Generation 1	Biofilm only Generation 3	Probiotic + inulin Generation 1	Probiotic + inulin Generation 2	Probiotic + inulin Generation 3
0	4	28	28	4	0
0.5	4	28	23	5	2
5	4	28	35	14	1
50	4	28	465	223	3
500	4	28	1544	1563	1092

Table 6. Effect of inulin *B. animalis* subsp. *lactis* viability within a cultured plaque biofilm. The data tables provide the total *B. animalis* 16S rRNA sequence counts evaluated for each plaque biofilm as a function of both the initial quantity of inoculated *B. animalis* subsp. *lactis* and the concentration of supplemental inulin added to the culture media. Note that the quantities of administered probiotic were based upon dilutions (none, 1:10, 1:100, 1:1,000) of an initial stock solution prepared to an optical density of one. Note also that the “biofilm only” columns denote the initial biofilm development in the absence of probiotic or prebiotic.

Mean <i>B. animalis</i> Counts (All probiotic quantities)						
Inulin concentration (μM)	Biofilm only (1st Generation)	Biofilm only (3rd Generation)	Biofilm with <i>B. animalis</i> 1st Generation	Biofilm with <i>B. animalis</i> 2nd Generation	Biofilm with <i>B. animalis</i> 3rd Generation	Mean (All probiotic generations)
0	4	28	45	15	3	21
0.5	4	28	30	6	4	13
5	4	28	57	19	2	26
50	4	28	446	172	66	228
500	4	28	1850	1375	1299	1508

Table 7. Summary of the effect of inulin on *B. animalis* subsp. *lactis* viability within a cultured plaque biofilm. The data table provides the total *B. animalis* 16S rRNA sequence counts over time when averaged across all cultured biofilms (i.e. samples of all quantities of administered *B. animalis* probiotic) as a function of the concentration of supplemental inulin added to the culture media. Note that the “biofilm only” columns denote the initial biofilm development in the absence of probiotic or prebiotic.

The effect of inulin supplementation on the resident biofilm microbial composition was also assessed by 16S rRNA Next Generation Sequencing, and those results for several key periodontal genera are shown in Table 8 and summarized in Table 9. Interestingly, inulin appears to produce an unremarkable effect on resident microbial composition of the five analyzed genera, except at the highest concentration of 500 μM, wherein *Fusobacterium* species all but disappear. Indeed, at 500 μM, there is a greater than a 400-fold decrease *Fusobacterium* after three generations (24 days) of culture with inulin and *B. animalis*. Furthermore, at 500 μM of inulin, *Prevotella* counts are elevated in excess of twelve-fold relative to baseline after three generations of biofilm culture with pro- and prebiotic. However, inulin appears to exert a less pronounced effect on the levels of early

dental biofilm colonizers of the genera *Streptococcus*, *Actinomyces*, and *Veillonella*. Nevertheless, after three generations of biofilm culture with probiotic and prebiotic, there was a three-fold increase in *Actinomyces* counts and a nearly two-fold increase in *Veillonella*. It is of interest to note that these general trends are even observed when the *in vitro* biofilms are cultured with inulin in the *absence* of *B. animalis* subsp. *lactis*. For example, inulin dramatically and independently reduced the levels of *Fusobacterium*, as shown in Table 10. Another interesting finding was that when biofilms were cultured with the highest concentration of inulin (500 μ M), a drastically reduced biofilm mass was observed on the HA disks at each generation, and this, in turn, corresponded to substantially decreased quantities of extracted biofilm DNA, as shown in Table 11. However, this correlation between extracted DNA quantity and inulin concentration did not exist at inulin concentrations less than 500 μ M.

Mean <i>Fusobacterium</i> Counts (All <i>B. animalis</i> quantities)						
Inulin concentration (μM)	Biofilm only Generation 1	Biofilm only Generation 3	Biofilm with <i>B. animalis</i> 1st Generation	Biofilm with <i>B. animalis</i> 2nd Generation	Biofilm with <i>B. animalis</i> 3rd Generation	Mean (All probiotic generations)
0	4	28	859	657	1740	1086
0.5	4	28	1063	862	1568	1164
5	4	28	974	1006	1891	1290
50	4	28	777	1031	1834	1214
500	4	28	57	72	4	44

Mean <i>Prevotella</i> Counts (All <i>B. animalis</i> quantities)						
Inulin concentration (μM)	Biofilm only Generation 1	Biofilm only Generation 3	Biofilm with <i>B. animalis</i> 1st Generation	Biofilm with <i>B. animalis</i> 2nd Generation	Biofilm with <i>B. animalis</i> 3rd Generation	Mean (All probiotic generations)
0	18	35	10	5	24	13
0.5	18	35	7	20	38	21
5	18	35	8	6	43	19
50	18	35	24	17	78	40
500	18	35	147	111	294	184

Mean <i>Actinomyces</i> Counts (All <i>B. animalis</i> quantities)						
Inulin concentration (μM)	Biofilm only Generation 1	Biofilm only Generation 3	Biofilm with <i>B. animalis</i> 1st Generation	Biofilm with <i>B. animalis</i> 2nd Generation	Biofilm with <i>B. animalis</i> 3rd Generation	Mean (All probiotic generations)
0	52	55	79	71	27	59
0.5	52	55	83	91	29	68
5	52	55	86	62	21	56
50	52	55	99	68	18	62
500	52	55	186	114	94	131

Mean <i>Veillonella</i> Counts (All <i>B. animalis</i> quantities)						
Inulin concentration (μM)	Biofilm only Generation 1	Biofilm only Generation 3	Biofilm with <i>B. animalis</i> 1st Generation	Biofilm with <i>B. animalis</i> 2nd Generation	Biofilm with <i>B. animalis</i> 3rd Generation	Mean (All probiotic generations)
0	905	685	578	1043	1238	953
0.5	905	685	685	1456	1289	1144
5	905	685	446	1265	1297	1003
50	905	685	509	2102	1230	1280
500	905	685	566	1153	2200	1306

Mean <i>Streptococcus</i> Counts (All <i>B. animalis</i> quantities)						
Inulin concentration (μM)	Biofilm only Generation 1	Biofilm only Generation 3	Biofilm with <i>B. animalis</i> 1st Generation	Biofilm with <i>B. animalis</i> 2nd Generation	Biofilm with <i>B. animalis</i> 3rd Generation	Mean (All probiotic generations)
0	1760	2531	2128	2431	1287	1949
0.5	1760	2531	2307	1941	1248	1832
5	1760	2531	2246	2267	1158	1890
50	1760	2531	2211	2045	1215	1824
500	1760	2531	1624	1485	974	1361

Table 8. Effect of inulin on resident biofilm microbial composition in the presence of *B. animalis* subsp. *lactis*. The data table provides the mean 16S rRNA sequence counts over time for five bacterial genera within biofilms cultured for three generations with varying concentrations of inulin supplementation when averaged across samples of all initial *B. animalis* subsp. *lactis* inoculation quantities. Note that the “biofilm only” columns denote the initial biofilm development in the absence of probiotic or prebiotic.

Mean Taxon Counts by Inulin Concentration (All <i>B. animalis</i> Quantities and Generations)					
Taxon	Inulin concentration (μM)				
	0	0.5	5	50	500
<i>B. animalis</i>	21	13	26	228	1508
<i>Streptococcus</i>	1949	1832	1890	1824	1361
<i>Actinomyces</i>	59	68	56	62	131
<i>Veillonella</i>	953	1144	1003	1280	1306
<i>Fusobacterium</i>	1086	1164	1290	1213	44
<i>Prevotella</i>	13	21	19	40	184

Table 9. Summary of the effect of inulin on resident biofilm microbial composition in the presence of *B. animalis* subsp. *lactis*. The data table provides the mean 16S rRNA sequence counts, averaged across all samples of all three generations of culture, for five bacterial genera within biofilms cultured with varying concentrations of inulin supplementation.

Mean <i>Fusobacterium</i> Counts (No <i>B. animalis</i> subsp. <i>lactis</i>)					
Inulin concentration (μM)	Biofilm only Generation 1	Biofilm only Generation 3	Biofilm with <i>B. animalis</i> 1st Generation	Biofilm with <i>B. animalis</i> 2nd Generation	Biofilm with <i>B. animalis</i> 3rd Generation
0	694	226	859	692	2170
0.5	694	226	1138	856	1458
5	694	226	714	1096	2024
50	694	226	192	729	1882
500	694	226	108	2	8

Mean <i>Prevotella</i> Counts (No <i>B. animalis</i> subsp. <i>lactis</i>)					
Inulin concentration (μM)	Biofilm only Generation 1	Biofilm only Generation 3	Biofilm with <i>B. animalis</i> 1st Generation	Biofilm with <i>B. animalis</i> 2nd Generation	Biofilm with <i>B. animalis</i> 3rd Generation
0	18	35	6	0	7
0.5	18	35	5	13	1
5	18	35	3	3	34
50	18	35	3	36	118
500	18	35	241	193	119

Mean <i>Actinomyces</i> Counts (No <i>B. animalis</i> subsp. <i>lactis</i>)					
Inulin concentration (μM)	Biofilm only Generation 1	Biofilm only Generation 3	Biofilm with <i>B. animalis</i> 1st Generation	Biofilm with <i>B. animalis</i> 2nd Generation	Biofilm with <i>B. animalis</i> 3rd Generation
0	52	55	84	50	35
0.5	52	55	77	86	29
5	52	55	73	63	26
50	52	55	61	51	12
500	52	55	247	26	21

Mean <i>Veillella</i> Counts (No <i>B. animalis</i> subsp. <i>lactis</i>)					
Inulin concentration (μM)	Biofilm only Generation 1	Biofilm only Generation 3	Biofilm with <i>B. animalis</i> 1st Generation	Biofilm with <i>B. animalis</i> 2nd Generation	Biofilm with <i>B. animalis</i> 3rd Generation
0	905	685	340	768	730
0.5	905	685	1024	1644	1032
5	905	685	842	995	1019
50	905	685	278	2465	938
500	905	685	391	1733	2946

Mean <i>Streptococcus</i> Counts (No <i>B. animalis</i> subsp. <i>lactis</i>)					
Inulin concentration (μM)	Biofilm only Generation 1	Biofilm only Generation 3	Biofilm with <i>B. animalis</i> 1st Generation	Biofilm with <i>B. animalis</i> 2nd Generation	Biofilm with <i>B. animalis</i> 3rd Generation
0	1760	2531	2328	2442	1651
0.5	1760	2531	2293	1585	1492
5	1760	2531	2246	2344	1102
50	1760	2531	2390	1905	1225
500	1760	2531	1868	1553	1207

Table 10. Effect of inulin on resident biofilm microbial composition in the absence of *B. animalis* subsp. *lactis*. The data table provides the mean 16S rRNA sequence counts for five bacterial genera within biofilms cultured for three generations with varying concentrations of inulin supplementation in the absence of *B. animalis* subsp. *lactis*. Note that the “biofilm only” columns denote the initial biofilm development in the absence of probiotic or prebiotic.

Mean Extracted Biofilm DNA Concentration Relative to Inulin Concentration (All probiotic quantities)	
Inulin concentration (μM)	DNA concentration (ng/μL)
0	170
0.5	179
5	172
50	186
500	52

Table 11. Mean extracted biofilm DNA concentration relative to inulin concentration. The data table reveals that at supplemental inulin concentrations of 500 μM, dramatically decreased quantities of DNA were extracted from the developing biofilms. Note that these values reflect mean DNA concentrations calculated for all biofilms across all generations and all initial *B. animalis* subsp. *lactis* inoculation quantities.

CHAPTER 4: DISCUSSION

Over the past two decades, the medical literature has provided robust and encouraging evidence that the microorganisms existing on and within the human body constitute a functional organ *in toto* that is fundamental to the preservation of health and homeostasis (Mayanagi, 2009). This fact, in conjunction with evidence suggesting that humans are 50-90% microbial in cellular constitution (Savage, 1977; Sender, 2016), has challenged our intuitions about the very nature of human physiology. The oral cavity alone harbors over 700 species of bacteria that exist in an established homeostatic ecosystem wherein perturbations can yield significant deleterious consequences to systemic and oral health (Kilian, 2016). More specifically, disruptions in the relative abundances of microorganisms within this native ecosystem signify a dysbiosis that ultimately manifests as caries, gingivitis, or periodontal disease. (Kilian, 2016; Meyle, 2015)

The elucidation of the fundamental role that the indigenous oral and intestinal microbiome plays in the provision, security, and maintenance of systemic homeostasis and oral health has fostered vigorous interest in the utility of probiotic therapy in virtually all domains of medicine. In periodontics, specifically, the aggregate evidence indicates that probiotic applications may afford not only improved clinical periodontal parameters, but also exert a favorable influence on the oral microbiota (Mayanagi, 2009). Furthermore, there is an unequivocal need for advancements in therapeutic techniques and concepts in order to overcome several limitations that currently hobble the periodontal surgeon's attempts to achieve optimal clinical outcomes. A frustrating complication of mechanical tooth debridement, for example, is the reality of pathogenic recolonization of treated sites

within a relatively short period of time, particularly in the presence of poor patient compliance (Magnusson, 1984). In this respect, probiotics have been suggested as a means of mitigating this vexing obstacle to clinical success by enhancing the population of beneficial bacteria, which, in turn, may retard the recolonization of pathogens and mollify the exaggerated immune response against them (Teughels, 2011). Furthermore, with the perpetual emergence of antibiotic-resistant microorganisms and the desire among public consumers for more “natural” remedies, there is ample impetus for the development of novel therapeutics that avoid traditional antibiotic and antimicrobial mechanisms. Therefore, preventive approaches that seek to restore host-microbe homeostasis have been investigated, and these include utilization of probiotics to promote a health-promoting microbial ecology with physiologic benefit to the host. If effective toward this end, it is reasonable to assume that probiotics would offer a treatment modality that diminishes the threat and burden of antibiotic resistance (Allaker, 2017).

Although a number of studies utilizing probiotics as an adjunct to standard periodontal therapy report encouraging results (Twetman, 2009; Vivekananda, 2010; Teughels, 2013; Ince, 2015; Tekce, 2015; Morales, 2016; Kuru, 2017), the implementation of probiotic therapy has also been beset by challenges. Just as many gastrointestinal probiotics fail to colonize and disappear as soon as ingestion ceases (Teughels, 2011), so, too, have probiotics failed to demonstrate prolonged colonization within the oral cavity. Tekce reported that twice-a-day administration of *L. reuteri*-containing lozenges for three weeks resulted in loss of the probiotic from the oral microbiota after 90 days and that, moreover, *Lactobacillus reuteri* could not be detected in all patients who consumed the probiotic, which may indicate that environmental factors and/or the existing microbiota are

critical in establishing viability and colonization (Tekce, 2015). Similarly, Szkaradkiewicz et al. reported that although 75% of individuals administered *L. reuteri* tablets twice a day for two weeks exhibited improved clinical periodontal parameters and reduction in GCF cytokine levels, 25% demonstrated no significant benefit of probiotic therapy (Szkaradkiewicz, 2014). Thus, it is conceivable that only a subset of those consuming probiotics may demonstrate diminished probiotic colonization, or that biofilms may require altered environmental conditions and/or prolonged probiotic administration to achieve permanent colonization and enduring beneficial effects.

Given these challenges associated with adjunctive probiotic administration in periodontal therapy, the present research sought to establish an *in vitro* model that could be utilized to evaluate a broad spectrum of environmental and technical factors that might affect probiotic colonization and sustainability within plaque biofilms. A secondary benefit of such a model, if successful, would be the capacity to evaluate the microbiological effects of probiotics on the resident oral microbiota with the ultimate aim of manipulating the indigenous microbial flora toward a more health-promoting oral ecology. Indeed, Velsko and Shaddox recently demonstrated that plaque biofilms could be developed *in vitro* and perpetuated for 80 days with great stability in species preservation and relative abundance (Velsko, 2018). Walker and Sedlacek likewise demonstrated the ability of *in vitro* biofilm models developed on hydroxyapatite disks to preserve the integrity of the native oral microflora (Walker and Sedlacek, 2007). Their study showed that subgingival plaque biofilms obtained from periodontally healthy subjects could be developed in an *in vitro* model on hydroxyapatite disks in an anaerobic chamber with, relative to the initial plaque samples, 81% and 70% fidelity in species preservation and relative abundance,

respectively, after 10 days of culture. Biofilms obtained from subjects with periodontal disease, however, were maintained over 10 days of culture with less fidelity in species preservation and abundance (69% and 57%, respectively) and, thus, were less reflective of the native oral microbiota.

In addition to the need to successfully culture a biofilm, and any concomitant probiotic to which they might be exposed *in vitro*, it is necessary to secure a means by which to assess the presence and relative abundance of the constituent microorganisms. The 16S ribosomal RNA molecule is an integral part of the small ribosomal subunit of prokaryotes and contains both highly conserved regions common to all prokaryotes and hypervariable regions that are unique to particular species. As the 16S rRNA gene is present in all prokaryotes, and the variable regions within this gene are unique among microbes, it can be utilized as a means of bacterial identification (Kilian, 2016). The advent of high-throughput genomic sequencing technologies, such as Next-Generation Sequencing, has afforded a powerful tool by which to investigate the constitution of the human microbiome across a spectrum of health and disease and, thus, elucidate its relationship to health (Kilian, 2016). Genomic sequencing technologies have permitted DNA analyses which, in turn, have generated vast data sets that can inform an ever-expanding knowledge of the compositional and functional characteristics of microbial communities (Cho, 2012). The NIH Human Microbiome Project, for instance, has amassed a 2.3-terabyte 16S rRNA metagenomic dataset acquired from 690 samples from 300 U.S. subjects at 15 body sites (Cho, 2012). The emergence of Next Generation Sequencing techniques, such as Illumina MiSeq, have enabled an enormous increase in sample throughput capacity, with up to 27 million sequences being generated in a single run.

(Kilian, 2016). This technology, in turn, provided the means of longitudinally assessing biofilm microbial constitution and relative species abundance in this investigation, and, thus, both the sustainability and effects of probiotics cultured within the biofilm.

The present study adapted the model proposed by Velsko and Shaddox to investigate the viability of two probiotics commonly utilized in periodontics, *Lactobacillus reuteri* and *Bifidobacterium animalis* subsp. *lactis*, within an *in vitro* plaque biofilm. The results of this research suggest substantially greater viability of *B. animalis* within the *in vitro* plaque biofilm relative to that of *L. reuteri*. Indeed, after one week of continuous low-dose exposure of the biofilm to *L. reuteri*, the probiotic was no longer present among the twenty most abundant species. This is somewhat surprising, giving that *L. reuteri* was replenished, as it were, within the culture biofilm every two days and, thus, would be expected to be present in reasonably high abundance throughout the duration of the experiment. Nevertheless, at weeks three and five of culturing, *L. reuteri* was the 49th and 23rd most abundant microorganism, respectively, which is still quite significant, given that a total of 7,723 microorganisms were detected in total. Even after a single administration of low- and high-dose *L. reuteri* (i.e. without further supplementation at future time points), the probiotic was present as the 16th most abundant microbe after one week and the 248th most abundant microbe two weeks after low-dose application. After a single high-dose administration, *L. reuteri* was the 8th most abundant microbe after one week and, after two weeks of biofilm culture, the 229th most abundant microbe. These data suggest that *L. reuteri* is, in fact, sustainable within an *in vitro* plaque biofilm model and that, furthermore, there is a dose-dependent relationship to its relative viability within the biofilm. Nevertheless, the fact that its abundance diminishes with time coheres with the finding of

a clinical trial demonstrating that *L. reuteri* disappears from the oral microbiota after discontinuation of administration (Tekce, 2015).

In contrast to *L. reuteri*, *B. animalis* subsp. *lactis* appeared to be significantly more viable within the cultured *in vitro* biofilm. Low-dose continual administration of *B. animalis* subsp. *lactis* conferred substantial sustainability within the biofilm; after five weeks of culture, *B. animalis* was the fifth most abundant microorganism present within the biofilm. Although the sustainability of *B. animalis* after single administration was not evaluated in this pilot study, the limited data are suggestive that this probiotic may, under specific (as-yet undetermined) environmental conditions, be a preferable candidate to *L. reuteri* for long-term colonization within the resident oral microbiota.

Perhaps more significant is the fact that *B. animalis* subsp. *lactis* appeared to confer more favorable microbiological effects on the indigenous *in vitro* biofilm composition over time. Although no appreciable microbiological effects on resident biofilm composition were observed upon inoculation with *L. reuteri*, *B. animalis* subsp. *lactis* appeared to effect a significant alteration in the indigenous *in vitro* microbiota. Specifically, *B. animalis* increased the relative proportions of *Veillonella* species and dramatically decreased *Fusobacterium nucleatum* counts over the course of a five-week period.

A health-promoting oral microbiota is characterized by high proportions of Gram-positive aerobic species (Roberts, 2002). More specifically, species of the *Streptococcus* and *Actinomyces* genera, and occasionally of the *Veillonella* genus, are characteristic of the minimal plaque accumulations compatible with healthy periodontal tissues (Socransky, 1977; Kolenbrander, 2006). Furthermore, the initial colonizers within dental plaque biofilms are predominantly streptococci, along with minor representatives of other genera,

such as *Actinomyces*, *Gemella*, *Granulicatella*, *Neisseria*, *Prevotella*, *Rothia*, *Clostridia*, and *Veillonella* (Kolenbrander, 2006). However, the initial communities of *Veillonella parvula* may serve as a “bridging species” leading to the orange complex, of which *Fusobacterium nucleatum* is representative (Socransky, 1977). This is significant insofar as *F. nucleatum*, as a quintessential coaggregator, is fundamental to the transition toward a more pathogenic biofilm characterized by the presence of pathogenic “red complex” bacteria (Socransky, 1977; Kolenbrander, 2006). Although species of the red complex—namely, *Porphyromonas gingivalis*, *Tannerella forsythia*, and *Treponema denticola*—demonstrate a particularly strong association with deep periodontal pockets, the “orange complex” bacteria—exemplified by *F. nucleatum*—are also associated with increasing pocket depths (Socransky, 1998). Moreover, *Eubacterium nodatum* is known to co-aggregate with *F. nucleatum* and was found to occasionally cluster with the “red complex” pathogens (Socransky, 1998). Therefore, the presence of *E. nodatum* and the persistence of *F. nucleatum* within the cultured biofilm after exposure to *L. reuteri*, in contrast to the ability of *B. animalis* subsp. *lactis* to maintain high proportions of Gram-positive early colonizers while drastically reducing *F. nucleatum*, in the present study suggests that *B. animalis* may be preferable for clinical use as an adjunct to standard periodontal therapy.

Nevertheless, the apparent inefficacy of *L. reuteri* to induce appreciable changes in the composition of the cultured biofilm is surprising in light of reports in the literature of the opposite effect. Indeed, numerous authors have found that clinical administration of *Lactobacillus reuteri* favorably, albeit transiently, alters the oral microbiota by reducing colony counts of Gram-negative anaerobes and common periodontal pathogens, such as *P. gingivalis*, *A. actinomycetemcomitans*, *P. intermedia* (Vivekananda, 2010; Iniesta, 2012;

Teughels, 2013; Romani Vestman, 2015). This apparent contradiction may be explained by differences in the periodontal health of the patients treated in the latter studies relative to that of the single subgingival plaque donor evaluated in the present study. Moreover, one should hazard extreme caution in extrapolating the results obtained from a sample size of one, as in the case of this pilot study.

The microbiological effects of probiotics, however, are not merely confined to their influence on *individual* microorganisms within the biofilm; rather, probiotics also affect the overall diversity of the biofilm microbiota *as a whole*, and this, too, is of great interest to the researcher and clinician. It has been reported in the medical literature that a healthy gut microbiota exhibits great diversity and likely contains over 1000 species (Tuddenham, 2015). Greater diversity within the gut microbiota has been associated with improved health, and those with decreased species richness have been found to exhibit greater adiposity, insulin resistance, and dyslipidemia when compared to those with greater bacteria diversity (Tuddenham, 2015). The importance of alpha diversity and species richness has also been demonstrated in the context of periodontal health. In a study that utilized 16S rRNA sequencing to assess plaque samples obtained from patient-matched healthy and diseased periodontal sites, it was demonstrated that diseased sites were significantly less diverse and exhibited less species richness than health-associated microbial populations (Jorth, 2014). In the present study, the lowest median alpha diversity was found in the control samples for the continuous-probiotic-application cultures of Experiment #2—that is to say, the samples in which *no probiotic* was introduced. The second- and third-highest alpha diversity populations were those corresponding to the addition of continuous low-dose *B. animalis* subsp. *lactis* and high-dose single

administration of *L. reuteri*, which would seem to indicate that the addition of probiotics favorably increases species richness within the biofilm microbiota. Paradoxically, however, the highest alpha diversity was found in the control sample of the single-dose *L. reuteri* cultures (Experiment #2). It is difficult to posit an explanation for this observation, especially in light of the fact that the same control for Experiment #1 demonstrated the *least* alpha diversity. Nevertheless, the beneficial effect of probiotics upon the resident microbial composition is further underscored by the results of the beta diversity analysis, which showed that single high-dose inoculation with *L. reuteri* and low-dose continuous administration of *B. animalis* increased species diversity over time. These limited observations are suggestive of the beneficial effects of probiotics on the resident biofilm microbiota and, furthermore, may even support their co-administration in those clinical scenarios that necessitate antibiotic therapy. It has been demonstrated, for example, that antibiotics significantly alter the gut microbiota in the short term, and a reduction in species diversity occurs within 3-4 days of antibiotic administration (Tuddenham, 2015). Even antibiotic regimens of short duration may have long-term effects on the intestinal microbiota that may last up 18 months (Tuddenham, 2015). Furthermore, antibiotic disruption of the gastrointestinal microbiota may result in diminished resistance to pathogens such as *Clostridium difficile* (Goldenberg, 2017). Among cited prophylactic therapies, probiotic administration is substantiated by the highest-quality evidence with regards to efficacy in the prevention of *C. difficile*-associated diarrhea, with mean 60% reductions in this adverse antibiotic-associated outcome (Goldenberg, 2017). Therefore, the effect of simultaneous probiotic therapy on mitigating detrimental effects of antibiotics certainly warrants further investigation.

Despite the promising effects of probiotic therapy highlighted in both clinical trials and this investigation, the problem of achieving prolonged probiotic colonization within any indigenous microbiota remains. Although the limited evidence from this pilot study is suggestive that merely increasing the exposure (i.e. the “dose”) of *L. reuteri* may increase its relative abundance within an oral biofilm and, therefore, potentially encourage a more enduring colonization, it is worth investigating the means by which altering environmental conditions may be leveraged to favor probiotic colonization. One way in which this may be done is via simultaneous *prebiotic* application, wherein specific fermentable substrates (complex carbohydrates) may be synchronously administered with probiotics in an attempt to selectively allow those species to flourish. The genesis of this idea follows from an understanding of the influence of breast milk on neonatal microbial development. Indeed, the microbiota of exclusively breast-fed infants exhibit proportionally higher abundances of bifidobacteria, which are considered to be critical to the development of the neonate immune system and in the defense against pathogens. Insofar as the oligosaccharides in breast milk nurture this primordial microbiota, they may, therefore, be considered the original prebiotics (Macfarlane, 2006). Furthermore, the preponderance of *in vivo* and *in vitro* studies has reported selective fermentation of inulin by health-promoting microorganisms such as bifidobacteria and, to a lesser degree, lactobacilli (Kolida, 2002). Inulin is a generic term that encompasses compounds with linear chains of either α -D-glucopyranosyl- $[\beta$ -D-fructofuranosyl]_{n-1}- β -D-fructofuranoside or β -D-fructopyranosyl- $[\beta$ -D-fructofuranosyl]_{n-1}- β -D-fructofuranoside with $\beta(2\rightarrow1)$ fructose-glucose linkages (Gibson, 2004). Partial enzymatic hydrolysis of inulin produces oligofructose, and insofar as inulin and oligofructose evade digestion in the upper gastrointestinal tract, and thus serve

as intact substrates for colonic microbial fermentation, inulin fulfils the criteria of a prebiotic (Gibson, 2004).

In the present study, inulin supplementation was shown to increase the viability of *B. animalis* subsp. *lactis* within the cultured biofilm when administered at concentrations $\geq 50 \mu\text{M}$. Below this concentration, negligible effects on *B. animalis* subsp. *lactis* quantities were observed, whereas at the highest inulin concentration of $500 \mu\text{M}$, a mean 433-fold increase in *B. animalis* 16S rRNA count was observed after three generations of biofilm culture. These results cohere with the favorable effects of breast milk oligosaccharide fermentation on bifidobacteria development within the neonate gut microbiota; however, the present study indicates that a dose-dependent relationship may exist.

As observed in second experiment, in which *B. animalis* subsp. *lactis* was shown to confer a favorable shift toward a more health-promoting microbial composition within the cultured plaque biofilm, at concentrations of $500 \mu\text{M}$, supplemental inulin demonstrated similar favorable microbiological effects in the presence *or absence* of *B. animalis* subsp. *lactis*. In other words, at high concentrations, inulin may independently induce a favorable biofilm microbial profile characteristic of reduced pathogen counts and higher relative proportions of commensal early colonizers. More specifically, at a concentration of $500 \mu\text{M}$, inulin supplementation resulted in a 414-fold decrease in *Fusobacterium* counts, a 3-fold increase in *Actinomyces*, a roughly 2-fold increase in *Veillonella*, and a relatively negligible influence on *Streptococcus* levels. As detailed above, these shifts correspond to a dental biofilm microbial composition consistent with enhanced periodontal health. Furthermore, these results are in accordance with those from

a recent study by Slomka and colleagues, who utilized an *in vitro* model of 14 representative oral bacterial species, including a spectrum of periodontal pathogens and beneficial *Streptococcus* and *Veillonella* species, that were cultured for 24 hours and then treated with three different prebiotic substrates (N-acetyl-D-mannosamine, succinic acid, and Met-Pro). After treatment with these prebiotic compounds three times a day for three days, the microbial composition exhibited a significant shift from a baseline 43% beneficial species constitution toward a > 95% relative abundance of beneficial species, with N-acetyl-D-mannosamine demonstrating the most potent effect in a dose-dependent manner (Slomka, 2018). Although this study did not specifically evaluate prebiotic effects on probiotic growth and colonization, it is nevertheless indicative that prebiotics can potentially modify the oral microflora. Both the Slomka study and the present pilot study, along with the aforementioned evidence of prebiotic influence on *Bifidobacterium* species development, suggest that co-administration of inulin with prebiotic *L. reuteri* and *B. animalis* may be a viable means of favoring the colonization of those probiotics within the oral microbiome.

Despite the salutary effects of inulin observed in this study, an unexpected finding was the fact that at the highest administered concentration (500 μ M), supplemental inulin appeared to dramatically reduce the biofilm mass visibly growing on the HA disks at each generation of culture. As shown in Table 11, this reduction, in turn, corresponded to a substantial decrease in the total quantity of DNA extracted from each biofilm. Although the precise cause of this reduction is unknown, it is reasonable to speculate that the severe decrease in *Fusobacteria* induced by 500 μ M of inulin supplementation may be contributory. As described above, *Fusobacterium nucleatum* functions as the

“quintessential coaggregator” for biofilm development, given its capacity to bind to, or coaggregate with, numerous other bacterial species. If *Fusobacterium nucleatum* is not present within the biofilm to promote this coaggregation, then the overall result might conceivably be a disruption in biofilm formation and, hence, a reduction in biofilm mass. Whether such a reduction results in a decrease in biofilm diversity or otherwise induces deleterious effects on the resident microbial composition is unknown.

Before departing the concept of prebiotics, it should be acknowledged that if, in fact, prebiotic supplementation of probiotic therapy is successful in promoting probiotic viability within the native oral flora, it would necessarily have potential implications regarding the influence of diet on the viability of probiotics. Diet is a significant influential factor in the gut microbial composition, with dietary nutrients favoring the growth of particular species (Ettinger, 2014). Those with diets rich in fiber and complex carbohydrates and low in animal protein demonstrate greater microbial diversity and greater *Prevotella*:*Bacteroides* ratios, whereas diets rich in cholesterol, saturated fats, and refined carbohydrates correlate with a gut microbiota enriched in bacteria belonging to the Firmicutes phylum (Wong, 2012; Tuddenham, 2015). In fact, one study demonstrated that short-term consumption of an exclusively animal- or plant-based diet rapidly alters the gut microbiota within a single day of the diet reaching the gut microbiota in such a way as to mirror the metabolic activity of carnivorous or herbivorous animals (David, 2014). Perhaps, then, one factor contributing to the difficulty in establishing a healthful oral and gut microbiome supplemented by probiotics is merely reflective of a diet devoid of the appropriate fermentable substrates. The modern Western diet is characterized by high lipid and fructose consumption and low fermentable fiber consumption, which is mismatched to

the metabolic requirements of our “ancestral” microbiota (Wong, 2012). A return to unprocessed foods rich in plant-based fibers, seasonal raw foods, and low in red meat might allow the indigenous beneficial microorganisms to reestablish preponderance within our resident microbiota, which likely evolved in parallel with the diet of our evolutionary ancestors (Wong, 2012). This observation, too, may have significant bearing on the success of probiotic supplementation in clinical trials.

A final, and equally significant, concern related to probiotic therapy is that of their safety. Indeed, a considerable spectrum of probiotics is available in over-the-counter preparations, but the manufacture and microbial validation of these marketed strains have not been tightly regulated. This, in turn, has translated to numerous commercial preparations failing to provide the species specified on the label in the concentrations advertised or in the purities implied (Hart, 2003). Nevertheless, probiotics derived from the normal host flora should afford at least an intuitive level of safety, and such bacteria utilized in alimentary vehicles—such as fermented milk, vegetables, and cereals—have afforded an excellent safety record with rare disease manifestations through translocation (Hart, 2003). In fact, a particularly appealing aspect of probiotics, relative to their pharmacological counterparts, is their relatively few side effects (Ettinger, 2014).

Although a rare occurrence, isolated reports of probiotic-related adverse effects, such as bacteremia, have been documented in the literature (Snydman, 2008). However, risk for such adverse effects appears to be limited to a small subset of the population, such as the immunocompromised, premature infants, and patients with cardiac valvulopathies (Doron, 2015). Rautio, for example, reported a liver abscess related to *L. rhamnosus* consumption in a 74-year-old diabetic (Rautio, 1999). Moreover, lactobacillemias have

also been documented in immunocompromised patients (Hart, 2003; Horwitch, 1995). This suggests the capability of probiotics to serve as opportunistic pathogens in the context of immunocompromised states. Nevertheless, the estimated risk for bacteremia secondary to probiotic lactobacilli or bifidobacteria ingestion is less than one case per one million individuals (Boriello, 2003), thus attesting to the safety of probiotic therapy.

SUMMARY: EXPERIMENTAL HYPOTHESES REVISITED

HYPOTHESIS #1

The first hypothesis proposed in this study was that increased exposures (i.e. “dose”) of *L. reuteri* and *B. animalis* subsp. *lactis* would favor prolonged viability within the cultured biofilm. Although variation in *B. animalis* dosing was not evaluated, it was found that increasing the dose of *L. reuteri* from 1×10^8 CFUs/mL (“low dose”) to 1×10^{10} CFUs/mL (“high dose”) afforded, as intuition would suggest, a greater relative abundance of the probiotic within the cultured biofilm over time. Nevertheless, after a single initial inoculation of the biofilm *L. reuteri*, Next Generation Sequencing analysis revealed that by the second week of culture, *L. reuteri* had diminished from the 16th most abundant species to the 248th most abundant for the low-dose *L. reuteri* sample, and from the 8th most abundant species to the 229th most abundant species in the high-dose sample. This indicates that *L. reuteri* is not robustly sustainable beyond one week in the proposed *in vitro* model, despite a 100-fold increase in initial dose. However, when comparing the viability of *L. reuteri* to that of *B. animalis* subsp. *lactis* after low-dose continuous inoculation (i.e. every two days), it was discovered that *B. animalis* was significantly more abundant at week five of biofilm culture than was *L. reuteri*.

(fifth most abundant microorganism within the biofilm vs. 23rd most abundant microorganisms, respectively). This observation suggests that at comparable doses, *B. animalis* is more viable within an *in vitro* plaque biofilm than is *L. reuteri*.

HYPOTHESIS #2

The second hypothesis proposed for this investigation was that *L. reuteri* and *B. animalis* subsp. *lactis* would, over time, modify the composition of the plaque biofilm in favor of decreased pathogen counts and greater relative abundances of beneficial commensal bacteria. The limited evidence afforded by this investigation suggests that although *L. reuteri* exerts minimal influence on the longitudinal composition of the cultured plaque biofilm, *B. animalis* subsp. *lactis* confers a more substantial microbiological effect in terms of altering the constitution of the biofilm microbiota. Specifically, *B. animalis* increased the relative proportions of *Veillonella* species and dramatically decreased *Fusobacterium nucleatum* counts over the course of a five-week period. However, both single high-dose *L. reuteri* and continuous low-dose *B. animalis* subsp. *lactis* increased the species diversity (richness) within the indigenous cultured biofilm, suggesting an additional salubrious effect of the probiotics on the biofilm microflora.

HYPOTHESIS #3

The third hypothesis proposed in this investigation was that, similar to the effect of oligosaccharides in breast milk on the enhancement of neonate intestinal *Bifidobacteria* and *Lactobacillus* species, prebiotic inulin would afford a selective advantage on the

colonization of *L. reuteri* and *B. animalis* subsp. *lactis* within the *in vitro* plaque biofilm. Although this hypothesis was investigated in the present study with respect to *B. animalis* subsp. *lactis* viability only, the results of our experiment indicated that inulin supplementation could increase the sustainability of *B. animalis* subsp. *lactis* within the cultured biofilm at inulin concentrations of at least 50 μ M. Below this concentration, only negligible influence on probiotic viability relative to control (i.e. no inulin supplementation) was observed. However, at that highest inulin concentration (500 μ M), *B. animalis* subsp. *lactis* growth increased up to 600-fold in comparison to no inulin supplementation, and the ensuing increased relative abundance of the probiotic, in turn, corresponded to favorable effects on biofilm composition. Indeed, the addition of 500 μ M of inulin, with or *without* probiotic administration, resulted in greater biofilm colony counts of beneficial commensal early colonizers and a decreased relative abundance of bacteria traditionally characterized as pathogenic. These observations suggest that appropriate oral pro- and prebiotic administration may, indeed, prove beneficial in establishing a health-promoting oral microbiome and, in turn, periodontal health.

STUDY LIMITATIONS

The limitations of this pilot study include an exceedingly small sample size of one and an associated lack of diversity in the constitution of the baseline biofilm (i.e. lack of assessment of biofilms derived from sites corresponding to a spectrum of periodontal health and disease), the potential for unreliable results secondary to sample contaminations,

and the inability to replicate the *in vivo* host biofilm constitution with 100% fidelity in an *in vitro* model sustained in an anaerobic chamber.

RECOMMENDATIONS FOR FUTURE STUDY

Despite evidence that is generally supportive of probiotic administration in the management of periodontal disease, a number of variables require further elucidation prior to widespread recommendation. Among these are duration and mode of administration to prevent reversion to a dysbiotic ecology subsequent to probiotic discontinuation, evaluation of potential cariogenicity of particular probiotic strains, and the characterization of systemic risks of probiotic consumption in particular high-risk populations (Allaker, 2017). Certainly, the considerable heterogeneity among probiotics studies—particularly with regard to probiotic dosing ($2 \times 10^7 - 2 \times 10^9$ CFU/day), treatment duration (1 day-12 weeks), strains utilized, models (human vs. animal), and delivery vehicle (chewing gum, mouthrinse, lozenge, sachet, etc.)—complicates definitive conclusions on probiotic efficacy and the establishment of therapeutic recommendations (Teughels, 2011). Therefore, future research efforts should focus on discriminating among these variables to establish the parameters for optimal clinical outcomes with probiotic therapy. In addition, an assessment of systemic conditions or habits, such as smoking and diet, in relation to variability of inter-individual probiotic efficacy should be pursued. Furthermore, the possible synergistic effects of multiple probiotic strains delivered synchronously should be explored as well. For example, the effect of combining *L. reuteri* and *B. animalis*, and various combinations of strains within those species, should be evaluated. Finally, and perhaps most importantly, considerable effort should be made toward discovering

environmental factors that encourage or preclude probiotic colonization and long-term viability within the oral microbiome. Currently, probiotics should not be viewed as a treatment modality for permanently modifying the oral microbiota, and the literature suggests that the favorable clinical effects of probiotic application are transient and likely to dissipate as soon as the probiotic is discontinued. Therefore, it currently appears that long-term, sustainable transitions to a stable, non-pathogenic microbiota are unlikely (Teughels, 2011). Nevertheless, simultaneous prebiotic supplementation that would provide substrates for selective probiotic metabolism may provide a means for encouraging probiotic growth and colonization and should be the subject of future research. However, not all species within the *Bifidobacterium* and *Lactobacillus* genera are equally capable of fermenting prebiotics or have validated health-promoting effects. Therefore, until the particular species within these genera capable of prebiotic stimulation are characterized, the health benefits of prebiotic consumption will remain poorly defined (Macfarlane, 2006). However, as with all scientific inquiry, any present stalemate is merely reflective of inadequate information, intellectual myopia, and/or an inability to frame the question correctly. One should be encouraged, therefore, toward further investigation into the promising and dynamic, yet still largely undiscovered realm, of probiotic therapy.

CHAPTER 5: CONCLUSIONS

The relationship of the resident microbiome to, and of exogenous microbial influences on, human health and homeostasis represents a burgeoning field of active research in periodontics and general medicine. Nevertheless, given the great heterogeneity among studies implementing probiotic therapy and an inchoate knowledge of the detailed physiological influences inherent to microbe-host dynamics, innumerable unresolved questions persist. The present research establishes a viable *in vitro* model capable of exploring the nuances of probiotic dynamics within a plaque biofilm. More specifically, this model provides a means of not only culturing specific probiotics, such as *L. reuteri* and *B. animalis* subsp. *lactis*, within a biofilm, but of also investigating environmental and extrinsic variables on their viability within that biofilm.

The very limited evidence concerning probiotic viability and microbiological effects on biofilm composition provided by this pilot study is suggestive, but is by no means generalizable or immediately translatable to a clinical setting. However, within the limitations of this study, cautious inference may be made that increasing the probiotic dose could enhance viability within a resident microbiome, and probiotics and high-concentration prebiotic inulin appear to be influential in modifying the constitution of the biofilm microflora. In particular, *B. animalis* subsp. *lactis* and 500 μ M inulin (alone or in the presence of *B. animalis*) substantially diminished the presence of *Fusobacterium nucleatum* and increased the relative proportions of early commensal colonizers within the cultured biofilm. Furthermore, single high-dose inoculation with *L. reuteri* and continuous

low-dose inoculation with *B. animalis* subsp. *lactis* increased the beta diversity (species richness) within the plaque biofilm over a two- and five-week period, respectively.

In summary, the results of this research are coherent with the aggregate published periodontics literature implicating the favorable microbiological effects of probiotics and prebiotics. Nevertheless, the decrease in relative abundance of *L. reuteri* within the cultured biofilm over time also accords with findings that probiotics generally fail to become permanent residents of the gastrointestinal and oral microbiomes. This failure to attain permanent probiotic colonization, in turn, suggests that any promising clinical outcomes will be transient in nature and is indicative of the need for further research to elaborate the principles upon which probiotic sustainability are predicated.

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