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The Role of Streptococcus salivarius as a Modulator of Homeostasis in the Oral Cavity

Kyle W. MacDonald

The University of Western Ontario

Supervisor
Dr. Gregor Reid
The University of Western Ontario

Graduate Program in Microbiology and Immunology

A thesis submitted in partial fulfillment of the requirements for the degree in Master of Science

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THE ROLE OF *STREPTOCOCCUS SALIVARIUS* AS A MODULATOR OF
HOMEOSTASIS IN THE ORAL CAVITY

(Thesis format: Monograph)

by

Kyle William MacDonald

Graduate Program in Microbiology and Immunology

A thesis submitted in partial fulfillment
of the requirements for the degree of
Masters of Science

The School of Graduate and Postdoctoral Studies
The University of Western Ontario
London, Ontario, Canada

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Abstract

The oral cavity contains many different surfaces all colonized by prokaryotes, of which over 700 have been identified. While almost all people have some degree of plaque formation, the more concerning diseases of caries, candidiasis and periodontal disease afflict many patients and represent a major public health concern. As these are all diseases which have a component attributable to parts of the microbiota, efforts to manipulate the microbes has until recently involved use of antimicrobial agents. However, due to side effects, resistance and failure to restore homeostasis, this approach is limited. As an alternative, the administration of beneficial microbes (probiotics) has been considered. In this thesis, probiotic *Streptococcus salivarius* K12 and M18 and their by-products were shown to interfere with adhesion and coaggregation of pathogenic bacteria and yeast, and lower inflammatory factors. A human trial of healthy subjects showed the probiotics to be safe and not induce inflammation or disrupt the indigenous microbiota.

**Keywords:** oral cavity, probiotic, *Streptococcus salivarius*, adhesion, inflammation, coaggregation, humans
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Chapter 1

1 Introduction

1.1 Focus of Thesis

The purpose of this study was to investigate the ability of the human commensal bacterial species *Streptococcus salivarius* to maintain homeostasis in the oral cavity. This section will cover information on the human oral microbiome, how diseases develop as this bacterial community shifts in composition, and how probiotics may offer promise in preventing and treating these highly prevalent oral diseases, with specific emphasis placed on the oral probiotic strains *S. salivarius* K12 and M18.

1.2 The Human Microbiome

The human body plays host to numerous bacterial populations which colonize and persist at various sites including the skin, gastrointestinal tract, vagina, urogenital tract, and oral cavity (1). These complex and diverse communities are established and influenced based on a variety of factors such as the host's genetics, behaviours, environment, and diet (2). The human microbiome, defined as the collective genomes of our microbial constituents, provide additional metabolic functions above and beyond what the human genome alone is capable of driving (3). It has been increasingly recognized that these microorganisms dynamically interact with the host in ways that can have profound effects on health and disease (1, 2). Perhaps nowhere is the association of commensal microbes with our well-being more apparent than in the oral cavity.
1.3 Healthy Oral Microbiome

The oral cavity contains many different surfaces including the teeth, tongue, cheeks, hard and soft palates, gingival sulcus (area of space between a tooth and the surrounding gingival tissue), and tonsils, which are colonized by distinct and intricate microbial communities (4). Early research focused on culturing the vast number of bacterial species inhabiting this environment, with many aerobes as well as facultative and obligate anaerobes isolated and identified. These organisms demonstrated a wide range of metabolic processes, including the ability to degrade and utilize the complex sugars and proteins consumed by their human host in their diet (5). However, it is well established that a significant percentage of bacteria cannot be grown in the laboratory using traditional culture methods (6). The advancement of non-culture based methods of detection and classification in recent years, including high-throughput next generation sequencing technologies, has shed light on the truly immense range of bacterial species that colonize and persist in the human oral cavity (4, 7, 8). Approximately 700 oral prokaryotic species have currently been identified, of which 34% are presently uncultivated (4). The existence of such microorganisms is not unique to the oral cavity, as uncultivated species have been shown to represent around 40-50% of the total taxa colonizing the human skin, vagina, and stomach (9). Studies have identified a healthy "core microbiome" consisting predominantly of taxa belonging to Firmicutes, Proteobacteria, Actinobacteria, Bacteroidetes, and Fusobacteria phyla (10). These commensals preserve homeostasis in the oral cavity by helping to produce nutrients, maintain pH, modulate saliva production, and generate inhibitory substances, all of which act to prevent colonisation and growth of exogenous or pathogenic species (11).
1.4 Diseases caused by the Disruption of the Oral Microbiome

While the oral microbiome of an individual remains fairly stable over time (12), problems arise when bacterial populations shift, allowing different metabolic processes to occur, often resulting in a drastically altered local environment. The resulting oral diseases can have a significant negative impact on the quality of life of the patient, and often result in chronic conditions, particularly if access to dental professionals is limited. Some of the most prevalent oral microbial associated diseases are summarized below.

1.4.1 Dental Caries

Dental caries (otherwise known as tooth decay or cavities) is one of the most prevalent chronic diseases of people worldwide, with patients susceptible to developing caries throughout their lifetime (13). This process is mediated by an increase in acidogenic and/or acid-tolerating bacteria found in dental plaques (biofilms) on the teeth, which ferment sugars into organic acids (14). The consequent reduction in pH catalyzes the demineralization of enamel, dentin and cementum in teeth, resulting in caries lesions (13). Production of short-chain carboxylic acids by Streptococci and Lactobacilli – chiefly lactic acid production by *Streptococcus mutans* – is the primary etiology of caries, however other bacteria with similar properties such as *Actinomyces*, *Bifidobacteria*, *Atopobium*, *Propionibacterium*, and *Veillonella* can contribute (15).

Management of caries in dental practice has traditionally and mainly been carried out through surgical methods (16). The demineralized/diseased tooth structure is removed and restorative materials are applied to promote reformation. This, however, does not remove the causative infectious agent. Broad-spectrum antibiotics and antimicrobials are
occasionally used post-surgery to suppress further infection, however any success will be temporary (16).

1.4.2 Oral Candidiasis

Oral candidiasis is a fungal infection of the oral cavity caused by various species of yeast from the genus *Candida*. *Candida* species are frequently found in the oral cavity of healthy individuals, with a recent study detecting the genera in 75% of participants (17). The most common isolate found in humans is *Candida albicans*, which colonizes the posterior dorsum of the tongue (18). While typically carried asymptptomatically, *C. albicans* can act as an opportunistic pathogen in patients with compromised oral health or immune deficiencies, such as the elderly or transplant recipients (19). While oral lesions caused by *C. albicans* infection can cause discomfort, in more serious cases the yeast can gain access to the bloodstream and systemically spread (20). Proper oral hygiene practices are important for controlling natural oral yeast populations, with antifungal agents used as treatment once oral candidiasis has been established (19).

1.4.3 Periodontal Disease

Periodontal disease is characterized by inflammation of the tissues that surround and support the teeth, including the gums and periodontal ligaments. It arises as a consequence of long-term dental plaque build up on the teeth. This plaque is made up of bacteria and food debris, which eventually turns into a hard deposit (tartar) if not removed by regular brushing and flossing. The bacteria trapped in the tartar irritate the gums, causing an overt immune response directed against them, leading to inflammation
Symptoms of periodontal disease include bleeding, swelling, and tenderness of the gums. In more advanced cases, such as periodontitis, alveolar bone that surrounds and supports the teeth is destroyed, which can lead to tooth loss. A recent study indicated that approximately 50% of North American adults suffer from some form of periodontal disease, making it a major public health concern.

During periodontal disease, the oral microbiota shifts from a Gram-positive dominated community to one comprised mainly of Gram-negative bacteria. Bacteria classically considered to be strongly associated with periodontal disease include *Porphyromonas gingivalis*, *Aggregatibacter actinomycetemcomitans*, and *Fusobacterium nucleatum*. These anaerobes trigger the release of pro-inflammatory cytokines, leading to immune cell recruitment, tissue destruction, and eventual bone loss. Cytokines important in this destructive cycle include IL-1β (bone resorption, metalloproteinase production), IL-6 (B-cell activation), IL-8 (attraction and activation of neutrophils), TNF-α (bone resorption), and IFN-γ (modulates local immune responses).

When periodontal disease is diagnosed, plaque and tartar is typically removed by a dental professional in a process known as "scaling". In more advanced cases, surgery may be required to allow deep cleaning and reduce periodontal pocket depth. Newer therapies aimed at periodontal regeneration include the use of soft tissue and bone grafts, guided tissue/bone regeneration, and the delivery of growth factors by implanted biomaterials. Management of periodontal disease typically requires multiple trips per year to the dentist for cleanings, and even with regular home oral care inflammation may return.
1.4.4 Influence at Distant Sites

The effects of a disruption of the oral microbiome are not confined solely to the oral cavity. Members of the oral microbiota can have a bearing upon heart health, *Streptococcus pyogenes* being the classic example, where cross reactive antibodies affect the heart valves and other parts of the body (27). Periodontal disease has been associated with cardiovascular disease through a variety of epidemiological studies, and meta-analyses combining the findings from multiple independent studies conducted since the 1950's (28, 29). The increase in gingival bleeding during periodontal disease offers oral bacteria access to the bloodstream, where they can circulate and interact with atheromatous plaque deposits. Numerous studies have detected oral bacterial DNA in atherosclerotic lesions (30). The bacteria appear capable of invading and activating endothelial cells, increasing Toll-like receptor (TLR) interactions, or inducing the expression of metalloproteinases, all of which contribute to the development of cardiovascular disease (31).

In summary, disruptions of the oral microbiome are not only common, but can result in debilitating and chronic diseases for patients in the oral cavity and at distal body sites as well. Identifying novel ways to help prevent, manage, and treat such diseases remains a focus for researchers and clinicians worldwide, with the use of probiotics offering a promising avenue for tackling these conditions.

1.5 Probiotics

Probiotics are defined as live microorganisms that when administered in adequate amounts confer a health benefit on the host (32). Of the many clinical studies showing the ability of probiotics to prevent and treat local and distant site condition (32, 33), the
effects are mostly strain specific. There are a number of ways in which probiotics interact with the host, including competition with other organisms, epithelial cross talk with the host immune system, improving the integrity of epithelial barriers and function of tight junctions, and production of many metabolites, enzymes, co-factors, and vitamins K, B2, B12, and folate, all of which are utilized by the host (33–38).

1.5.1 Probiotics for Dentistry

The ability of probiotics to help maintain a healthy oral cavity or manage and treat oral diseases has been increasingly examined in recent years. As oral disease is the fourth most expensive disease to treat worldwide (39), regular probiotic usage may offer the opportunity to economically complement visits with trained dental professionals. Dental caries, traditionally associated with the species *Streptococcus mutans*, has been targeted by a variety of health-associated bacteria. Studies have demonstrated that consumption of *Lactobacillus rhamnosus* in cheese (40), *Lactobacillus reuteri* in tablets/straws (41), and *Bifidobacterium* in yogurt (42) are capable of reducing salivary *S. mutans* counts. Interestingly, these *Lactobacillus* and *Bifidobacterium* species are usually recognized as dietary probiotics aimed at improving gastrointestinal health, and have been derived from the intestinal tract. As such, it has been suggested that using bacteria that traditionally colonize the oral cavity may offer a better chance to impact oral health parameters. An interesting strategy along this line has been designed by Hillman and coworkers, who isolated a strain of *S. mutans* from a human subject capable of producing a bacteriocin with potent activity against virtually all other *S. mutans* strains (43). This strain was able to stably colonize the oral cavity of human volunteers, and resulted in an overall decrease
of *S. mutans* levels (44). In an effort to reduce its cariogenic potential, this group successfully deleted the lactate dehydrogenase operon from the bacterial genome, then demonstrated that the resulting mutant strain resulted in a lower incidence of dental caries development in rat infection models compared to the wild type strain (45). As lactic acid production is presumed to be the driving pathogenic mechanism behind the development of cariogenic lesions, this *S. mutans* strain offers intriguing potential for "replacement therapy", where indigenous, potentially pathogenic bacterial species are eliminated from their ecological niche and replaced with a strain designed to prevent oral damage (46).

Future oral probiotic development may be well served by following a similar approach; identifying and isolating potential beneficial species from healthy volunteers, determining colonization potential, and engineering the strain to generate favourable products/eliminate harmful ones.

Another oral health related condition that has been tackled by probiotics is halitosis (bad breath). Driven mainly by the production of volatile sulfur compounds (VSCs) by bacteria (particularly Gram negative anaerobes, including *P. gingivalis* and *Prevotella intermedia*) colonizing the tongue (47), it is believed to affect a large proportion of the population to various degrees. Traditional treatment options have focussed on the non-specific elimination of oral bacteria by various anti-bacterial chemical therapies. However, such efforts are costly and provide short-term relief, as malodour generating bacteria quickly return when treatment is ceased (48). In one probiotic study, healthy individuals who gargled a solution containing a *Weissella cibaria* isolate (selected based on its observed hydrogen peroxide generating ability *in vitro*) had lower VSC levels the following day than those who gargled a solution of distilled water.
Probiotics designed to treat periodontal disease have also been tested with promising results. Teughels et al. recently examined the daily usage of lozenges containing *Lactobacillus reuteri* by patients suffering from chronic periodontitis following standard dental scaling and root planing (50). Probiotic consumption resulted in significantly more pocket depth reduction and attachment gain in deep periodontal pockets, as well as a decrease in *P. gingivalis* levels, compared to those subjects who received a placebo lozenge. A similar study using *Lactobacillus salivarius* WB21-containing tablets demonstrated the ability of this probiotic to reduce the plaque index and periodontal pocket depth in subjects at high risk of periodontal disease (51).

While the above examples clearly demonstrate that a variety of bacterial species and strains have been used successfully as probiotics in dentistry, our research focused specifically on the oral species *Streptococcus salivarius*, and ways that it could be applied to help maintain homeostasis in the oral cavity.

1.6 *Streptococcus salivarius* K12 and M18

*Streptococcus salivarius* is a Gram-positive bacterial commensal which colonizes the human oral cavity throughout the host's life, and is generally associated with health (52). These characteristics made this species attractive to investigate as a potential oral probiotic. *S. salivarius* strains K12 and M18 were selected for further study based on their *in vitro* inhibitory activity against *Streptococcus pyogenes* (53). Both strains encode multiple bacteriocins; bacterially produced substances with the capacity to either inhibit other bacteria attempting to colonize the same niche, or to act as signaling molecules. *S. salivarius* K12 produces the bacteriocins Salivaricin A2 and Salivaricin B (54), while M18 produces Salivaricin A2, Salivaricin 9, and the bacteriocin MPS (55). Studies have
demonstrated that these *S. salivarius* strains are safe for human consumption (56), and can colonize and persist in the human oral cavity (57, 58), particularly on the tongue dorsum and other mucosal membranes. *S. salivarius* K12 has been shown in placebo controlled studies to prevent recurrent Streptococcal induced pharyngitis in adults (20 patients receiving the probiotic for 90 days) (59) and children (45 patients receiving the probiotic for 90 days, then a 6 month follow-up period) (60), as well as reduce halitosis by limiting the production of volatile sulfur compounds from anaerobic bacteria (48). *S. salivarius* M18 consumption was able to reduce dental plaque scores and *S. mutans* numbers in children (61).

Given *S. salivarius* K12 and M18's successful ability in treating other oral diseases, our group was interested if this probiotic species could be used to target periodontal disease. Interestingly, a wide variety of Streptococci species have been shown to exhibit anti-inflammatory effects on human cells. For example, Kaci *et al.* have demonstrated that multiple *S. salivarius* and *S. vestibularis* isolates are capable of inhibiting the inflammatory response of TNF-\(\alpha\) stimulated intestinal epithelial cells (62), and that intragastric administration of a live *S. salivarius* strain significantly inhibited inflammation in severe and moderate colitis mouse models (63). Similarly, groups have shown that *S. salivarius*, *S. mitis*, and *S. sanguinis* can reduce the release of the pro-inflammatory cytokine IL-8 from pathogen stimulated human cells (64, 65). As periodontal disease is primarily inflammation driven, and *S. salivarius* K12 and M18 have a proven record of safety and efficient colonization in the human oral cavity, we set out to determine whether these probiotic strains could potentially play a role in the management of this disease.
1.7 Hypothesis and Objectives

We hypothesize that *Streptococcus salivarius* strains K12 and M18 are capable of modulating inflammation in the oral cavity.

The objectives of this thesis are to:

1. Characterize the ability of *S. salivarius* strains to interact with a variety of oral microbes.
2. Investigate whether *S. salivarius* can modulate inflammatory factors produced by human oral fibroblasts exposed to common dental pathogens.
3. Assess in humans the extent to which *S. salivarius* K12 administration can modulate the oral microbiome and inflammatory mediators over the course of 14 days.
Chapter 2

2 Materials and Methods

2.1 General Materials and Methods

2.1.1 Bacterial Growth Media

All bacteriological media types were prepared according to the manufacturer's instructions, then sterilized by autoclaving at 121°C, 15 psi for 15 minutes.

2.1.2 Bacterial Strains - Origin

*Streptococcus salivarius* K12 and M18, *Candida albicans* TIMM 1768, as well as the nine standard indicator strains used (I₁ to I₉) were kindly provided from the laboratory collection of Dr J. R. Tagg (University of Otago, Dunedin, New Zealand). These were *Micrococcus luteus* (I₁), *S. pyogenes* M-type 52 (I₂), *Streptococcus constellatus* (I₃), *Streptococcus uberis* (I⁴), *S. pyogenes* M-type 4 (I₅), *Lactococcus lactis* ssp. *lactis* (I₆), *S. pyogenes* M-type 28 (I₇), *S. pyogenes* M-type 87 (I₈) and *Streptococcus dysgalactiae* (I₉). *Aggregatibacter actinomycetemcomitans* Y4 (AA), *Porphyromonas gingivalis* 33277 (PG), *Fusobacterium nucleatum* 10593 (FN), and *Streptococcus mutans* 25175 were purchased from ATCC. *Lactobacillus reuteri* RC-14, *Lactobacillus plantarum* Lp-2001, and *Lactobacillus helveticus* Lafti L10 were obtained from the Reid/Burton culture collection.

2.1.3 Bacterial Strains - Storage and General Culture

Stock cultures were stored in 30% (v/v) glycerol in MRS (de Man, Rogosa and Sharpe) broth (*Lactobacilli* species) or Brain Heart Infusion with Yeast Extract (BHYE) broth (all other species) at -80°C, and regularly subcultured every two weeks on the
appropriate solid media. Solid media was prepared in 10cm petri dishes. *S. salivarius* K12 and M18, along with I1 to I9 were maintained on Columbia Blood Agar plates (CBA) (Difco Columbia Agar Base [BD]) supplemented with 0.1% CaCO₃ and 5% (v/v) sheep's blood. *L. reuteri, L. plantarum,* and *L. helveticus* were maintained on MRS plates with 1.5% agar. Unless otherwise noted, these species were routinely grown at 37°C in 5% CO₂ in an anaerobic jar. *C. albicans* was cultured aerobically at 30°C on Sabouraud Dextrose Agar. *A. actinomycetemcomitans, P. gingivalis,* and *F. nucleatum* were grown at 37°C on BYHE with 1.5% agar in an anaerobic chamber containing 85% (v/v) N₂, 10% (v/v) H₂, and 5% (v/v) CO₂.

### 2.1.4 Primary Human Gingival Fibroblasts

Gingival fibroblasts were cultured from explanted tissue obtained from healthy volunteers undergoing periodontal procedures in the Oral Surgery Clinic at UWO in accordance with the guidelines of the University’s Research Ethics Board with informed patient consent. Fibroblasts from four separate patients were used in this thesis, and were routinely cultured in Minimum Essential Medium (MEM) supplemented with 10% FBS and 100 mM L-glutamine, at 5% CO₂ and 37°C. Fibroblast passages between 4-9 were used for the studies. For experiments, fibroblasts were inoculated into the wells of a 24-well plate and allowed to grow for 48 hours to reach confluency (approximately 5 x 10⁵ fibroblasts per mL).
2.2 *S. salivarius* Experiments *in vitro*

2.2.1 Simultaneous Bacterial Antagonism

Simultaneous bacterial antagonism assays were conducted as previously described (53). Briefly, overnight cultures of pathogens of interest were swabbed evenly over the surface of a blood agar plate. K12 and M18 colonies were then stab inoculated into the agar. Plates were incubated for 48 hours at 5% CO$_2$, then examined for zones of inhibition.

2.2.2 Deferred Bacterial Antagonism

K12 or M18 were grown as a 1 cm wide streak on a blood agar plate for 18 hours. The bacterial growth was then removed from the plate, with the surface of the plate then sterilized for 20 minutes with chloroform vapour. After drying, indicator strains and oral pathogens were streaked across the plate perpendicular to the original growth. The plate was then incubated for 48 hours at 5% CO$_2$, and examined for inhibitory activity.

2.2.3 Co-aggregation

To determine whether K12 and M18 could co-aggregate with the oral pathogens, a qualitative visual assay was carried out. Overnight cultures of each bacterial strain were centrifuged (10 minutes at 3000 x g), washed three times in PBS, then resuspended in PBS to an equivalent Optical Density (1.0). Each periodontal pathogen was mixed with an equal volume of the test *S. salivarius* strain. The turbidity of the mixture was then recorded at 8 hours and given a score based on the observed aggregation. Known positive (*Candida albicans*) and negative (*Streptococcus mutans*) controls were carried out as well to act as a reference.
2.2.4 Attachment to Host Cells

K12 and M18 were tested for their ability to adhere to cultured monolayers of human gingival fibroblasts. Overnight bacterial cultures were centrifuged (10 minutes at 3000 x g), washed three times in PBS, then resuspended in fibroblast growth media and added to the monolayers at a Multiplicity of Infection (MOI) of 25:1, and incubated for 8 hours. The monolayers were then washed three times with sterile PBS to remove non-adherent bacteria, then lysed with 0.1% Triton X-100. This concentration of Triton X-100 was tested to ensure it completely lysed all fibroblasts, while not affecting bacterial viability. Remaining bacteria were then dilution plated on blood agar and allowed to grow overnight at 5% CO₂.

2.2.5 Pro-inflammatory Cytokine Release

Potential anti-inflammatory effects of *S. salivarius* K12 and M18 were examined using a gingival fibroblast challenge model. Briefly, fibroblasts were added to the wells of a 24 well plate and allowed to grow for 48 hours until they reached confluency. At this point, cultures of either the probiotic, pathogen, or a combination of strains were added to the fibroblasts at an MOI of 25:1. Bacteria were co-incubated with the fibroblasts for 8 hours. After this time period, the culture supernatant was collected, centrifuged to clear debris, then stored at -20°C. Levels of the pro-inflammatory cytokines IL-6 and IL-8 were then determined using a Luminex multiplex immunoassay kit.

To determine whether *S. salivarius* K12 produced any soluble anti-inflammatory factors, overnight K12 cultures were centrifuged, with the resulting supernatant filter-sterilized, then applied to *F. nucleatum* stimulated fibroblasts for 8 hours. The level of IL-8 secretion was then determined using a commercial IL-8 ELISA kit. K12 supernatant
was also fractionated based on size (using a 10 kDa centrifuge filter), with both the <10 kDa fraction and >10 kDa tested on stimulated fibroblasts. Based on the fact that *S. salivarius* is known to secrete some molecules to a higher degree when grown on a solid surface, a freeze thaw extract from a K12 bacterial lawn was prepared. In this procedure, an agar plate covered in bacterial growth is placed at -80°C for 4 hours, then allowed to thaw with the resulting liquid collected from the degraded matrix and filter sterilized. Finally, this freeze thaw extract was subjected to heat treatment (10 minutes at 80°C) and trypsin digest (10 minutes).

### 2.3 *C. albicans* Experiments *in vitro*

Hyphae formation/*C. albicans* adhesion to 96-well plates was assessed after 3 hours, by fixation of cells in 70% ethanol, followed by crystal violet staining and OD$_{600}$ reading as previously described (66). Sterile-filtered supernatant (SFS) collected from select probiotic strains grown anaerobically for 22 hours in YEPD were mixed 50:50 with YEPD (1% FBS), to maintain a constant concentration of FBS and YEPD between conditions. Pooled, sterile-filtered human saliva was also used as a mixture in some experiments. As well, previously established biofilms were washed carefully three times with PBS, with plates then incubated for a further 3 hours with probiotic culture supernatant mixtures, then assayed for attachment as before.

### 2.4 Pilot Study on the Effects of a Probiotic Gum on the Healthy Oral Cavity

#### 2.4.1 Human Study Objective and Primary Outcome

The major objective of this section was to test our hypothesis that a protective anti-inflammatory effect would be produced in the saliva of individuals consuming a
probiotic gum tablet containing *S. salivarius* K12 bacteria. As such, our primary outcome of interest was observing levels of several pro-inflammatory cytokines important in oral disease conditions at various time points, and comparing these to levels in individuals chewing regular, non-probiotic gum. Additionally, we wanted to determine how effective *S. salivarius* K12 delivered in gum colonizes and persists in the oral cavity, and what, if any, effects this gum would have on the established oral microbiome.

2.4.2 Ethics Statement

Details of the proposed pilot study were reviewed and approved by both the Health Sciences Research Ethics Board at the University of Western Ontario (Appendix 1) and the Clinical Research Impact Committee at the Lawson Health Research Institute (Appendix 2). Each participant received a package explaining the relevant details of the study, were given the opportunity to have any questions of theirs answered, and signed a letter of consent prior to the commencement of the study (Appendix 3).

2.4.3 Study Population and Recruitment

Recruitment of individuals between the ages of 20-60 years with general good oral health took place in London, Ontario. Recruitment posters (Appendix 4) were placed in the Lawson Health Research Institute, and emphasized a need for healthy individuals interested in being part of a short, two week study. Communication between the study coordinator and those interested in participating was carried out by phone and in person. Participants were excluded if they had any oral disease, an oral implanted device, were currently taking antibiotics, or had a dental appointment scheduled during the course of the study.
2.4.4 Study Design and Sample Collection

The study recruited twenty healthy adult volunteers, who were randomly assigned into two groups containing 10 individuals each, matched for sex and age. Participants received either probiotic CulturedCare™ with BLIS K12™ gum tablets (Group 1) or regular gum tablets lacking probiotic bacteria (Group 2). CulturedCare™ gum tablets are commercially available in Canada. Each individual was assigned a unique identifier code, to ensure anonymity and that we would be blinded to which group a sample belonged too. Both gum types were similar in taste, appearance, and texture. Participants were supplied enough gum tablets to last the duration of the study. Over the course of the next 15 days, participants followed the timeline detailed in the "Study Design Flowchart" (Appendix 5). On Day 1 of the study, participants brushed their teeth at 8:00 AM, then collected 3 mL of unstimulated saliva in a supplied 15 mL conical tube at 9:00 AM. This served as a baseline sample for the participant. Individuals then chewed one gum tablet for 10 minutes, before discarding the material. This was the standard chewing procedure used throughout the study. A second piece was chewed following tooth brushing at 8:00 PM. On Days 1-7 participants followed this twice daily pattern of brushing/chewing. On Day 8 a "wash-out" period begun, where participants followed the same tooth brushing pattern, but without gum tablet consumption. Additional saliva samples were collected at 1:00 PM on Day 1, and at 9:00 AM on Days 2, 8, and 15. These time points corresponded to 4 Hours, 24 Hours, 7 Days, and 14 Days post first gum tablet exposure. All saliva samples were delivered by the participants to the Burton lab at the Lawson Health Research Institute where they were immediately aliquoted into 1.5 mL eppendorf tubes and stored at -80°C until use.
2.4.5 Salivary Pro-Inflammatory Cytokine Levels

Levels of pro-inflammatory cytokines (IL-6, IL-8, IL-1β, and IFN-γ) in the saliva of subjects in the probiotic gum group were measured using multiplexed immunoassay kits according to the manufacturers’ instructions (Bio-Rad Laboratories Inc., Hercules, CA). A Bio-Plex 200 readout system was used (Bio-Rad), which utilizes Luminex® xMAP fluorescent bead-based technology (Luminex Corporation, Austin, TX). Cytokine levels (pg/mL) were automatically calculated from standard curves using Bio-Plex Manager software (v. 4.1.1, Bio-Rad).

2.4.6 DNA Extraction and PCR Amplification

DNA was extracted from the saliva samples using the PowerSoil®-htp 96 Well Soil DNA Isolation Kit (MoBio). The extraction was carried out as per the manufacturer’s protocol, with two changes; the addition of a 10-minute incubation step at 65°C in a bead bath before the bead-beating step, and a doubling of the centrifugation times. 500 µL of saliva were used for the extractions. In total, 94 samples were extracted for sequencing.

Samples were PCR amplified for the V4 region of the 16S rRNA gene using barcoded primers as follows: V4L (forward) 5’ GTGCCAGC[CA]GCCGCGGTAA 3’ and V4R (reverse) 5’ GGACTAC[ATC][ACG]GGGT[AT]TCTAAT 3’. Amplification was carried out in a 42 µL reaction with 10 µL of each primer (3.2 pMol/µL stock), 20 µL GoTaq hot start colorless master mix (Promega) and 2 µL extracted DNA. Thermocycling conditions were as follows: initial hot start activation at 95°C for 2 min, then 25 cycles of 1 minute at 95°C for denaturation, 1 minute 55°C for primer annealing, and 1 min at 72°C for extension.
2.4.7 Sequencing and Read Processing

PCR products were quantified with a Qubit 2.0 Fluorometer and high sensitivity dsDNA specific fluorescent probes (Life Technologies). Samples were mixed at equimolar concentrations and purified with the QIAquick PCR Purification kit (QIAGEN). The pooled product was sent to the London Regional Genomics Centre (LRGC) for sequencing on the Illumina MiSeq platform using the 600-cycle kit to produce 2x300 paired-end reads. Using in-house Perl and Shell scripts, reads were retained if sequence matched the primer while allowing 2bp mismatches, and with perfect matches to expected sequence barcodes. Paired reads passing this filter were overlapped using pandaseq (https://github.com/neufeld/pandaseq) (67) to produce full-length V4 sequences assigned by sample. Operational taxonomic units (OTUs) were constructed by clustering V4 reads at 97% sequence identity using USEarch v. 7 (http://www.drive5.com/usearch/) (68). OTUs were retained if they represented at least 0.1% abundance of any one sample. The most abundant sequence in the cluster was used as the reference sequence for taxonomic classification. The reference OTU sequences were compared to the RDP database release 11.2 (https://rdp.cme.msu.edu) using Seqmatch v. 3 (69), and the lowest common taxonomy was retained out of the top 20 hits with an S_ab score ≥ 0.5. OTU sequences from differential taxonomic groups were further validated by BLAST (70) against the Human Oral Microbiome Database (HOMD) v. 13.2 (http://www.homd.org).

2.4.8 Statistical and Exploratory Analyses

The OTU table with assigned taxonomies was imported into QIIME (http://qiime.org) (71) for exploratory analyses including summarizing reads to different
taxonomic levels, generating beta diversity with weighted UniFrac distance (72) based on OTU sequence alignment with MUSCLE (73), and principal coordinate analysis (PCoA). Bar, stripchart, and PCoA plots were generated using R (74). Between-group comparisons for differential microbiota analyses were conducted with ALDEx2 package (http://www.bioconductor.org/packages/release/bioc/html/ALDEx2.html) (75) in R. Taxonomic clusters were considered differential between groups with an adjusted p-value < 0.01 using Welch’s t-test with Benjamini-Hochberg multiple test correction, and with an effect size ≥ 1.5.
Chapter 3

3 Results

3.1 *S. salivarius* Interaction with Oral Microbes

3.1.1 Bacterial Antagonism

The first step in this investigation was to determine whether *S. salivarius* K12 and M18 could inhibit the growth of common oral bacterial species, especially those associated with disease conditions. In a simultaneous antagonism assay, with the probiotic of interest stab inoculated into an agar plate immediately after it had been swabbed with a given indicator strain, both K12 and M18 failed to inhibit the growth of *P. gingivalis*, *F. nucleatum*, *A. actinomycetemcomitans*, and *C. albicans* (Table 1). However, they did demonstrate strong inhibition against indicator strains I₁ to I₉.

Additionally, a deferred antagonism assay was conducted to determine if initial growth of a probiotic would result in the restriction of later indicator growth. Once again, K12 and M18 demonstrated strong inhibition of strains I₁ to I₉, while having no effect on growth of the four oral pathogens tested (Table 2).
<table>
<thead>
<tr>
<th>Indicator Strain</th>
<th>Producer Strain</th>
<th>S. salivarius K12</th>
<th>S. salivarius M18</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>P. gingivalis</strong></td>
<td></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>F. nucleatum</strong></td>
<td></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>A. actinomycetemcomitans</strong></td>
<td></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>S. mutans</strong></td>
<td></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>C. albicans</strong></td>
<td></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><strong>Micrococcus luteus (I₅)</strong></td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td><strong>S. pyogenes M-type 52 (I₂)</strong></td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td><strong>Streptococcus constellatus (I₃)</strong></td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td><strong>Streptococcus uberis (I₄)</strong></td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td><strong>S. pyogenes M-type 4 (I₅)</strong></td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td><strong>Lactococcus lactis ssp. lactis (I₆)</strong></td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td><strong>S. pyogenes M-type 28 (I₇)</strong></td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td><strong>S. pyogenes M-type 87 (I₈)</strong></td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td><strong>Streptococcus dysgalactiae (I₉)</strong></td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
</tbody>
</table>

**Table 1. Simultaneous Bacterial Antagonism.** Inhibition after 48 hours of growth of *S. salivarius* K12/M18 and indicator strain on agar plates. The zone of inhibition of the indicator strain is indicated by: '-' no inhibition, '+' zone of inhibition diameter $\geq$ 3mm, '++' zone of inhibition diameter $\geq$ 5mm. Results were consistent across the three experiments conducted.
<table>
<thead>
<tr>
<th>Indicator Strain</th>
<th>Producer Strain</th>
<th>S. salivarius K12</th>
<th>S. salivarius M18</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>P. gingivalis</em></td>
<td></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>F. nucleatum</em></td>
<td></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>A. actinomycetemcomitans</em></td>
<td></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>S. mutans</em></td>
<td></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>C. albicans</em></td>
<td></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Micrococcus luteus</em> (I1)</td>
<td></td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td><em>S. pyogenes</em> M-type 52 (I2)</td>
<td></td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td><em>Streptococcus constellatus</em> (I3)</td>
<td></td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td><em>Streptococcus uberis</em> (I4)</td>
<td></td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td><em>S. pyogenes</em> M-type 4 (I5)</td>
<td></td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td><em>Lactococcus lactis</em> ssp. lactis* (I6)</td>
<td></td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td><em>S. pyogenes</em> M-type 28 (I7)</td>
<td></td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td><em>S. pyogenes</em> M-type 87 (I8)</td>
<td></td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td><em>Streptococcus dysgalactiae</em> (I9)</td>
<td></td>
<td>++</td>
<td>++</td>
</tr>
</tbody>
</table>

Table 2. Deferred Bacterial Antagonism. Inhibition after 18 hours of *S. salivarius* K12/M18 growth on agar plates, followed by 48 hours of incubation of the indicator streaks. The degree of inhibition of the indicator streaks is noted by: '-' no inhibition, '+' zone of inhibition is the same width as the producer streak, '++' zone of inhibition is at least 1.5 times the width of the producer streak. Results were consistent across the three experiments conducted.
3.1.2 Bacterial Co-aggregation

The ability of the S. salivarius strains to interact with the same oral microbes through co-aggregation was then examined. S. salivarius K12 and M18 displayed identical co-aggregation patterns (Table 3). Both co-aggregated moderately well with P. gingivalis and F. nucleatum, and weakly with A. actinomycetemcomitans. C. albicans co-aggregated extremely strongly with the S. salivarius strains, while S. mutans demonstrated no noticeable co-aggregation.

3.1.3 Bacterial Adherence to Human Oral Cells

Both S. salivarius K12 and M18 adhered to primary human gingival fibroblasts after co-incubation for 8 hours (Figure 1). There was no significant difference in attachment between the strains, with each resulting in approximately 30 bacterial cells adhered per fibroblast at the endpoint. The level of adherence for S. salivarius was significantly higher than demonstrated by L. reuteri, with less than 5 bacterial cells bound per fibroblast. Finally, S. mutans did not adhere at all to the gingival fibroblasts.
Table 3. Bacterial Co-aggregation. Ability of *S. salivarius* K12/M18 to co-aggregate in solution with various pathogens after 8 hours: “-“ no co-aggregation, evenly turbid suspension; “+”weak precipitation with evenly turbid supernatant; “++”moderate precipitation with evenly turbid supernatant and evidence of flocculation; “+++”substantial precipitation with clear supernatant and some flocculation. Results were consistent across the three experiments conducted.

<table>
<thead>
<tr>
<th><em>S. salivarius</em> Strain</th>
<th>Pathogen</th>
<th>PG</th>
<th>FN</th>
<th>AA</th>
<th><em>S. mutans</em></th>
<th><em>C. albicans</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>K12</td>
<td></td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>-</td>
<td>+++</td>
</tr>
<tr>
<td>M18</td>
<td></td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>-</td>
<td>+++</td>
</tr>
</tbody>
</table>

Figure 1. Bacterial Adherence to Human Gingival Fibroblasts. Attachment of various bacterial species to primary human gingival fibroblasts *in vitro* following 8 hours co-incubation. Assay was carried out in triplet on three separate occasions. (*p<0.05 compared to K12 attachment, unpaired t-test).
3.1.4 Prevention of \textit{C. albicans} Adherence

The ability of several oral probiotics to inhibit the binding of yeast to a solid surface was explored. \textit{C. albicans} was grown for three hours in the wells of a 96 well plate in either pure growth media, or with a mixture of probiotic culture supernatants. Microscopy was used to confirm that it was the hyphael form of \textit{C. albicans} adhering to the bottom of the plastic wells. After this period of growth the level of attachment of \textit{C. albicans} to the wells was determined using a crystal violet staining assay. The addition of supernatant from both \textit{S. salivarius} K12 and M18 significantly reduced adherence of \textit{C. albicans} to the wells (Figure 2). This was also observed with \textit{L. plantarum} and \textit{L. helveticus} supernatants, which almost completely abolished \textit{C. albicans} attachment. Decreasing the amount of probiotic supernatant added (from 1/2 to 1/4 of the total growth media) was less effective, but still resulted in significant attachment reduction.
Figure 2. Adherence of *C. albicans* to 96-well Plates. Ability of *C. albicans* to attach to the wells of a 96-well plate after 3 hours of incubation in YEPD (Control) or a mixture of YEPD with either 1/2 or 1/4 bacterial culture supernatant. 'Neg. Ctrl': YEPD alone with no *C. albicans*. (**) *p*<0.01 compared to control, One-way ANOVA with Dunnett's multiple comparison test).
Based on the observation that the collected probiotic culture supernatants had a lower pH than the normal *C. albicans* growth media, we wanted to determine if a lowered pH could be solely responsible for the observed decrease in attachment. *C. albicans* was therefore grown for 3 hours in the plastic wells in YEPD with the pH balanced to varying levels between 4 and 9 (Figure 3). A clear trend was observed, with the lowest pH media significantly reducing attachment. As such, the probiotic supernatants were pH balanced to 7.0, with the same *C. albicans* adherence assay conducted. Balancing the pH of these supernatants resulted in no significant attachment decrease for any of the tested probiotics (Figure 4), suggesting that pH reduction of the supernatants by these probiotic strains is the primary cause of the observed yeast attachment interference.
Figure 3. Effect of pH on *C. albicans* Adherence. Attachment of *C. albicans* to a 96-well plate after 3 hours of incubation in YEPD adjusted to various pH levels. (* p<0.05 compared to control, One-way ANOVA with Dunnett's multiple comparison test).

Figure 4. Effect of pH-Balanced Probiotic Supernatants on *C. albicans* Adherence. Attachment of *C. albicans* to a 96-well plate after 3 hours of incubation in a mixture of YEPD with bacterial culture supernatants adjusted to pH 7.0.
With the success of using non-pH balanced supernatant from individual probiotic strains to decrease *C. albicans* adherence, we wanted to test whether using combinations of these probiotics would result in similar, or perhaps even enhanced reduction. Using combinations of *S. salivarius* M18, *L. plantarum*, and *L. helveticus* supernatants resulted in a significant decrease in *C. albicans* adherence compared to the control (Figure 5). This was the case regardless if the bacteria were grown together to produce a single supernatant, or grown separately with their supernatants combined after the fact. The addition of *L. helveticus* supernatant to a *S. salivarius* M18/*L. plantarum* mix resulted in further, though non-significant, adherence reduction. While these combinations were effective, they did not result in significantly lowered attachment compared to the use of their individual strains alone, although this may be due to the minimum detection limit of this assay. Of particular note, using supernatant from a *C. albicans* culture had no effect on yeast attachment, suggesting that nutrient deprivation in the probiotic supernatants was not an important factor.

Finally, in an effort to create an assay that more closely mimics conditions in the oral cavity, we integrated the use of pooled, filtered human saliva into the *C. albicans* attachment experiment. Adding saliva to either the regular growth media or *C. albicans* culture supernatant resulted in a significant increase in yeast attachment (Figure 6). As before, combinations of *S. salivarius* M18, *L. plantarum*, and *L. helveticus* supernatants were able to significantly reduce *C. albicans* adherence to the wells, with the addition of saliva not impeding this ability.
Figure 5. Impact of Probiotic Combinations on *C. albicans* Adherence. Attachment of *C. albicans* to a 96-well plate after 3 hours of incubation in a mixture of YEPD with multiple, non-pH balanced bacterial supernatants. Growth media was 50% YEPD, 50% supernatant (equal mixture of species). For growth conditions indicated by '+' in the bar label, the probiotic species were grown overnight together in the same culture to produce the resulting supernatant, rather than pooling supernatants from individually grown species. (** p<0.01 compared to control, One-way ANOVA with Dunnett's multiple comparison test).
Figure 6. Effect of Saliva on Supernatant Mediated Reduction in *C. albicans* Adherence. Attachment of *C. albicans* to a 96-well plate after 3 hours of incubation in a mixture of YEPD and pooled, filter sterilized human saliva, combined with multiple, bacterial supernatants. Growth media was 50% YEPD, 50% supernatant (equal mixture of species), with an equivalent volume of saliva where indicated. For growth conditions indicated by ‘+’ in the bar label, the probiotic species were grown overnight together in the same culture to produce the resulting supernatant, rather than pooling supernatants from individually grown species. (* p<0.05, ** p<0.01 compared to control, One-way ANOVA with Dunnett's multiple comparison test).
3.1.5 Disruption of *C. albicans* Biofilms

While oral probiotic supernatants proved capable of preventing *C. albicans* from attaching effectively to plastic wells, we were also interested if they could remove yeast that had already adhered and formed a biofilm. *C. albicans* cultures were grown for 3 hours in regular growth media as before, then had the media carefully aspirated and the wells washed briefly, before the addition of our probiotic supernatants of interest. The presence of a thick yeast biofilm was confirmed by microscopy before the addition of the supernatants. The plate was allowed to incubate for a further three hours, then assayed for *C. albicans* attachment using the same crystal violet staining protocol. When compared to the use of a *C. albicans* culture supernatant, combinations of *S. salivarius* M18, *L. plantarum* and *L. helveticus* supernatant significantly reduced the amount of remaining attached *C. albicans* (Figure 7).
Figure 7. Disruption of *C. albicans* Biofilms by Probiotic Supernatants. Adherence of pre-attached *C. albicans* to 96-well plates following 3 hour exposure to various bacterial culture supernatants. For media conditions indicated by ‘+’ in the bar label, the probiotic species were grown overnight together in the same culture to produce the resulting supernatant, rather than pooling supernatants from individually grown species. (* p<0.05, ** p<0.01 compared to control, One-way ANOVA with Dunnett's multiple comparison test).
**S. salivarius** Modulation of Inflammatory Factors

### 3.1.6 Pro-inflammatory Cytokine Release *in vitro*

The three periodontal pathogens (*P. gingivalis*, *A. actinomycetemcomitans*, and *F. nucleatum*) were tested individually and in combination for their ability to stimulate the production of pro-inflammatory cytokines in primary human gingival fibroblasts *in vitro*. After co-incubation with the fibroblasts for 8 hours, all three significantly increased the release of both IL-6 and IL-8 (Figure 8) compared to the no bacteria control. The combination of three strains stimulated the most IL-6 production, while the triple combination was not significantly different from *P. gingivalis* production in the case of IL-8. In both cases *A. actinomycetemcomitans* was the least effective periodontal pathogen at stimulating the release of these pro-inflammatory cytokines. In contrast, exposing the fibroblasts to *S. salivarius* K12 or M18 did not result in any significant differences in IL-6 or IL-8 (Figure 8) production compared to the control. The concentration of cytokines produced was similar between both *S. salivarius* strains tested.
Figure 8. Stimulation of Cytokine Production From Human Gingival Fibroblasts. Concentration of IL-6 (A) and IL-8 (B) following 8 hour co-incubation of gingival fibroblasts with various oral bacteria (* p<0.05, ** p<0.01 compared to control, One-way ANOVA with Dunnett's multiple comparison test).
3.1.7  *S. salivarius* Reduction of Pathogen Driven Cytokine Release

Using the same *in vitro* model system with primary human gingival fibroblasts, the oral probiotics *S. salivarius* K12 and M18 were tested for their ability to reduce pathogen induced IL-6 and IL-8 production. The *S. salivarius* strains were applied either simultaneously with the pathogens, or pre-incubated with the fibroblasts 30 minutes before the pathogens were added. *S. salivarius* K12 and M18 were able to inhibit pathogen induced IL-6 secretion (Figure 9A). Significant reductions were seen under all conditions, with the exception of K12 pre- or co-incubated with *P. gingivalis*, and K12 or M18 co-incubated with *F. nucleatum*. The *S. salivarius* strains were also effective at reducing IL-8 secretion (Figure 9B), with significant reductions demonstrated for all conditions with *P. gingivalis*, *F. nucleatum*, and the three pathogen combination. Pretreatment with M18 also significantly reduced IL-8 secretion from *A. actinomycetemcomitans* stimulated fibroblasts. Importantly, under no circumstances did the addition of the *S. salivarius* K12 or M18 increase the production of IL-6 or IL-8 from pathogen stimulated fibroblasts.
Figure 9. *S. salivarius* Reduction of Pathogen Stimulated Cytokine Release. Ability of *S. salivarius* K12 and M18 to inhibit the production of IL-6 (A) or IL-8 (B) from human gingival fibroblasts when co-incubated (K12/M18) or administered prior to (K12P/M18P) oral pathogen exposure (* p<0.05, ** p<0.01 compared to control within each pathogen group, One-way ANOVA with Dunnett's multiple comparison test).
3.1.8 Mechanism of IL-8 Reduction

Attempts were made to elucidate the mechanism behind the ability of *S. salivarius* K12 and M18 to reduce the production of pro-inflammatory cytokines from pathogen stimulated human gingival fibroblasts. Based on patterns observed in previous experiments, the choice was made to focus on *S. salivarius* K12 inhibition of *F. nucleatum* induced IL-8 secretion. Once again, co-incubation of fibroblasts with *F. nucleatum* resulted in significantly higher IL-8 production compared to the no bacteria control, while K12 alone resulted in no significant changes (Figure 10). Likewise, adding *S. salivarius* K12 to *F. nucleatum* reduced the IL-8 concentration back to a level not significantly different than the control. Supernatant collected from an overnight *S. salivarius* K12 culture had no effect on *F. nucleatum* induced IL-8 levels, nor did the >10 kDa fraction of this supernatant alone. However, the <10 kDa fraction of the supernatant was effective at significantly reducing the IL-8 concentration. A freeze/thaw extract of *S. salivarius* K12 grown as a lawn overnight on agar plates was also able to accomplish this. While heat treatment of this freeze thaw extract had no effect on its activity, treatment with trypsin abolished its anti-inflammatory properties.
Figure 10. Mechanism of *S. salivarius* K12 Mediated IL-8 Reduction. Concentration of IL-8 produced by human gingival fibroblasts after 8 hours under various conditions. "Sup"= Supernatant, "FT"=Freeze/Thaw, "FN"=Stimulated with *F. nucleatum* (*p*<0.05 compared to control, One-way ANOVA with Dunnett's multiple comparison test).
3.2 Effect of Probiotic Gum Use in the Healthy Oral Cavity

3.2.1 Oral Microbiome Changes in Response to Chewing Gum

After filtering and clustering sequenced reads at 97% identity, there were 476 OTUs > 0.01% total abundance. A total number of 2,774,309 sequenced reads were included for analysis, with a median of 29,779 ± 13,630 reads per sample. These OTUs were further clustered by taxonomic lineage into 38 family groups of at least 0.05% abundance across all samples. The most abundant taxonomic families detected at >10% of total classified reads were: Porphyromonadaceae (17.95%), Pasteurellaceae (15.97%), Prevotellaceae (15.85%), and Veillonellaceae (11.22%), apparent in Figure 11.

The weighted UniFrac distance is one method to determine the relatedness of different microbiota. The distances between samples is relative to how similar the composition of the microbiota are as weighted by the abundance of each organism detected. Weighted uniFrac distances can be interpreted and plotted using principal coordinate analysis (PCoA) as shown in Figure 12. The samples do not segregate by group (probiotic vs control) in examination of the first 3 components, which explain 81.01% of the total variance in the data (top row, Figure 12). However, there is a distinct shift in the first component over time, with many of the 7 day and 14 day samples differentiating from the earlier time points (bottom row, Figure 12).
Figure 11. Oral Microbiota Profiles Over Two Weeks of Gum Chewing. Bacterial composition using V4 16S rRNA gene sequencing of saliva collected at five time points for healthy volunteers chewing either *S. salivarius* K12 probiotic gum (top plot) or non-probiotic (bottom plot) control gum. Each cluster of bars is an individual identified by a subject ID number, and each bar in a cluster is an individual saliva sample ordered as baseline, 4 hours, 24 hours, 7 days, and 14 days. The black bar indicates an uncollected sample. Colours correspond to the proportion of assigned taxonomic family (listed in the legend on the right), ordered according to total abundance from bottom of the plot to top. Groups of sequences that are less than 0.05% abundance across all samples, or less than 1% of sequences in an individual are grouped as “rem”. Unclassified families are labeled by their lowest classified taxonomic rank.
Figure 12. PCoA Plots for All Saliva Samples Based on Weighted UniFrac Distance.

Two dimensional PCoA plots representing the first three components of variation between all saliva samples in the dataset. The first component in this analysis represents the most variation explained in the data (in this case 60.49%), with subsequent components representing the next largest variance in the data. Distances between points on the plot represent how similar samples are in terms of microbiota composition and abundance. Points on the plot that are closer in space are therefore more similar in their taxonomic distribution. The top and bottom row plots are identical, differentially coloured based on variable of interest (top - study group; bottom - sample collection time point).
To further examine the apparent time-dependent change in microbiota, the weighted UniFrac distance of all time points from individuals in both treatment groups compared to their baseline sample before treatment were plotted (Figure 13). The median weighted UniFrac distance increases over treatment time indicating a shift in the microbiota. Notably, a subset of the samples at 7 days and 14 days are very distinct from the others (outlier points at the top of the plot in Figure 13, and to the right of the plot in Figure 12, bottom row).
Figure 13. Change in β-Diversity Measured by Weighted UniFrac Over Time. Weighted UniFrac distance of each saliva microbiota sample compared to that individual's baseline sample at 4 hours, 24 hours, 7 days, and 14 days. A value of 0 would represent identical microbiota composition between samples, with a value of 1 representing maximal microbiota differences. Sample points are coloured by study group (probiotic - red; control - blue). Lines represent the median UniFrac distance of a given time point. Microbiota compositions change over time (regardless of study group), with a subset of individuals changing drastically at 7 and 14 days.
In order to test if there was any differential taxonomic abundances between groups, a compositional data analysis framework was required (75). Therefore the ALDEEx2 toolset was employed to test for significant taxonomic difference between groups at the family level. There were no differences (Benjamini-Hochberg adjusted p-value >0.01) between the probiotic and control groups at baseline, between the probiotic and control groups at study end-point (14 days), nor between these groups at any of the other sample collection time points. Therefore, the treatment groups were pooled to test for differences at end of study (14 days) compared to baseline. Presented in Table 4, there were four family-level taxonomic groups with a relative increase in abundance, and three with a relative decrease in abundance (Benjamini-Hochberg adjusted p-value <0.01 and effect size ≥ 1.5). Examination of the OTUs in the family groups by BLAST to the HOMDB revealed that most of the OTU sequences in Erysipelotrichaceae were similar (>80% sequence identity) to *Erysipelothrix tonsillarum* (HOT_484) or *Solobacterium moorei* (HOT_678).
Table 4. Taxonomic Groups With Significant Changes in Relative Abundance.

<table>
<thead>
<tr>
<th>Relative increase</th>
<th>Family-level taxonomic group</th>
<th>Wt-BH*</th>
<th>Effect Size^</th>
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<tbody>
<tr>
<td>Firmicutes;Erysipelotrichia;Erysipelotrichales;Erysipelotrichaceae</td>
<td>1.85E-08</td>
<td>2.04</td>
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<tr>
<td>Bacteroidetes;Bacteroidia;Bacteroidales;Porphyromonadaceae2</td>
<td>4.48E-08</td>
<td>1.87</td>
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<tr>
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<td>5.25E-06</td>
<td>1.57</td>
<td></td>
</tr>
<tr>
<td>Bacteroidetes;Bacteroidia;Bacteroidales;Porphyromonadaceae</td>
<td>6.86E-07</td>
<td>1.51</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Relative decrease</th>
<th>Family-level taxonomic group</th>
<th>Wt-BH*</th>
<th>Effect Size^</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fusobacteria;Fusobacteria;Fusobacteriales;Leptotrichiaceae</td>
<td>1.39E-05</td>
<td>-1.77</td>
<td></td>
</tr>
<tr>
<td>Actinobacteria;Actinobacteria;Actinomycetales;Actinomycetaceae</td>
<td>1.74E-05</td>
<td>-1.61</td>
<td></td>
</tr>
<tr>
<td>Bacteroidetes;Bacteroidia;Bacteroidales;Prevotellaceae</td>
<td>1.34E-05</td>
<td>-1.51</td>
<td></td>
</tr>
</tbody>
</table>

*Corrected p-value from a paired Welch’s t-test using Benjamini-Hochberg procedure
^The median effect size as estimated by ALDEx2
3.2.2 Changes in Pro-Inflammatory Cytokine Levels

Concentrations of four pro-inflammatory cytokines linked with periodontal disease were measured in the collected saliva of the nine subjects in the probiotic gum group at each time point. These were all healthy individuals with no overt oral diseases, however it has been demonstrated that everyone has some degree of these inflammatory cytokines present in their saliva, as they are important for regular immune regulation. Although cytokine concentrations were only measured for subjects chewing the probiotic gum, the fact that a baseline saliva sample was taken allowed us to determine if exposure to the probiotic would either raise or lower the amount of these cytokines. IL-8 levels were the highest in the saliva samples (mean concentration 426.72 pg/mL for all samples combined), while both IL-6 (8.32 pg/mL) and TNF-α (3.27 pg/mL) were present at much lower concentrations (Figure 14). No significant differences were observed between the concentration of a given cytokine when compared to another time point.

To determine if overall cytokine concentration variation between individuals was obscuring the effects of probiotic gum consumption, fold changes in cytokines within individuals were examined (Figure 15). However, no statistically significant differences were observed when the fold changes for all probiotic gum chewers were compared for any cytokine between any two time points.
Figure 14. Salivary Levels of Pro-Inflammatory Cytokines. Concentration (pg/mL) of IL-1β, IL-6, IL-8 and TNF-α (A-D) in collected saliva samples for the probiotic group at five time points. Cytokine levels for each sample were determined individually, with the mean concentration for all individuals plotted on these graphs. No significant differences were observed for a given cytokine when comparing concentrations at any two time points (One-Way repeated measures ANOVA).
Figure 15. Fold Changes in Cytokine Concentrations. Baseline saliva cytokine levels of IL-1β, IL-6, IL-8 and TNF-α (A-D) for every individual in the probiotic group were set to 1, with fold-changes from baseline at each subsequent time point calculated and plotted. No significant differences were observed for a given cytokine when comparing concentrations at any two time points (One-Way repeated measures ANOVA).
Chapter 4

4 Discussion

This thesis has demonstrated that oral strains of *Streptococcus salivarius* are capable of interacting with other microbes known to cause disease in the oral cavity through a variety of mechanisms, and can also influence pathogen-stimulated production of inflammatory mediators. These are important findings, as in addition to characterizing basic properties of this species central to its ability to colonize and persist in the oral cavity, it included the first test of an *S. salivarius* probiotic strain's ability to modulate salivary markers of inflammation in humans.

Bacteria in the oral cavity are known to adhere to surfaces and to each other in the form of coaggregates and plaque biofilms. Given that no human has a sterile mouth, the question is how do most individuals ensure inflammation and infection is minimal? For many years, the simple presence, or increased abundance of certain bacterial species was believed to be the driving cause behind many oral diseases. For example, *Streptococcus mutans* was long presumed to be the primary etiological agent of caries (76), while *Porphyromonas gingivalis, Tannerella forsythia*, and *Treponema denticola* (categorized together as the "red complex") were closely linked with periodontal disease (77). However, recent studies using high throughput sequencing techniques have shown that these assumptions are over simplistic, with diseases often being polymicrobial in nature (78, 79), and can even vary in microbial compositions between individuals (80). Furthermore, a vast range of species are common constituents of both a healthy and diseased oral cavity, including members of Pasteurellaceae and Prevotellaceae (10, 81).
We confirm the high prevalence of these species in the saliva in our study performed on 19 orally healthy adults. Notably, Streptococcaceae were relatively abundant in these subjects, and it is reasonable to assume that *S. salivarius* strains were among the species detected in this family, as this species is the predominant commensal *Streptococcus* in the oral cavity (82). While not conducted as part of this thesis, it would be possible to detect and quantify *S. salivarius* K12 specifically by qPCR with targeted, validated primers, in a manner similar to Burton *et al.* (83). This presence in healthy adults, as well as its known early colonization of the oral cavity and the existence of various bacteriocins with potent activity against common Gram-positive oral pathogens, has made it a species of potential to recalibrate an aberrant oral microbiota, and thus suggests its use as a probiotic. Indeed, the world's first two *S. salivarius* commercial probiotic strains, K12 and M18, were selected for their ability to adhere, coaggregate and inhibit the growth of various *Streptococcus pyogenes* species known to cause pharyngitis (84, 85).

Malodour, dental caries and gingivitis are symbolic of a dysbiotic oral microbiome whose metabolic activities eventually lead to disease symptoms. Perhaps even more concerning, it is the ability of these colonizing microorganisms to induce high levels of inflammation that leads to pain, discomfort, and severe forms of periodontal disease with subsequent loss of alveolar bone (86). We hypothesized that *S. salivarius* may confer some immuno-modulatory activity in addition to its other beneficial characteristics. To explore this, we used primary human gingival fibroblasts as an *in vitro* model cell line, as they are in close contact with bacteria in subgingival plaque in humans, and have been demonstrated as crucial for sustaining inflammation in periodontal disease (87). We did indeed show that strains K12 and M18 were capable of
reducing oral pathogen-induced IL-6 and IL-8 levels, two of the most notable indicators of inflammation in this disease (25). This adds to a previous finding that showed anti-inflammatory activity of K12, albeit against *Salmonella* and *E. coli* co-cultured with human bronchial epithelial cells (64).

There are several means by which this activity could be beneficial in the oral cavity. Firstly, the direct bacterial interaction with the epithelium could modulate cytokines and reduce inflammation. This has been demonstrated for a *S. salivarius* strain in the context of interaction with intestinal epithelial cells, with administration of live bacteria also managing to alleviate inflammation in a mouse model of induced colitis (62, 63). Such an effect may be mediated by how the organism interacts with receptors such as TLR2, or by interfering with the signal transduction pathways of the host cells (88), either directly or through soluble by-products. We showed that a heat-stable, trypsin-labile component of the supernatant of K12 did indeed down-regulate pathogen-stimulated IL-8 production. More work remains to be done to further isolate and characterize this compound, as well as to determine what other molecules produced by *S. salivarius* K12 may enhance or interfere with this compound's activity. This early work is encouraging, and while this phenomenon has so far not been described elsewhere for *S. salivarius* K12 specifically, it supports a finding by Sliepen *et al.* that supernatants from *S. salivarius*, *Streptococcus mitis*, and *Streptococcus sanguinis* species were capable of a similar effect (65). Importantly, we demonstrated this anti-inflammatory ability of K12 supernatant against *F. nucleatum*, which has traditionally been recognized as a periodontal pathogen, and indeed has many pathogenic characteristics such as invasion of epithelial cells and induction of inflammatory cytokines from host oral tissues (89). It
might be argued that as *S. salivarius* is often located on the dorsal surface of the tongue (58), it would not readily gain access to sub-gingival sites or be in high abundance there, but its secreted by-products could still have an impact. If released into saliva, the proteinaceous compound would be expected to be present in saliva for sufficient time to reach different areas of the mouth. Proteins of similar size have been shown to persist in the saliva and interact with a variety of bacteria and host cells (90). Of course, altering IL-6 and IL-8 is not necessarily the only way that inflammation is driven, and we did not test TNF-α or IL-1β, nor evaluate cellular immunity in this *in vitro* model. In addition, some levels of IL-6 and IL-8 are necessary to maintain an immune system that protects against infection (86), so the intent would not be to completely abolish them.

The ultimate test of these effects can only be achieved by human studies. In that regard, we showed that the net effect on healthy subjects of exposure to K12 was to maintain normal individual levels of IL-6 and IL-8, as these pro-inflammatory cytokines did not increase or decrease in concentration compared to baseline levels when individuals chewed the probiotic gum. This is an indicator of maintaining homeostasis, and also safety of this probiotic. A future study could test this intervention on subjects with known inflammation (for example, patients with moderate gingivitis or fixed orthodontic appliances), and whose condition has not been alleviated by antimicrobial agents.

Another way that these substances produced by K12 could be beneficial, is through the organism's interaction with other bacteria. We showed that K12 and M18 coaggregate with pathogenic strains that are known stimulators of inflammation. It is possible this coaggregation also played a role in reducing IL-6 and IL-8 levels. In other
words, the probiotics either inhibited production of the inflammatory stimulants, or they blocked their ability to trigger the host's reaction to them. It was outside the scope of this thesis to examine these possibilities, but they are both worthy of investigation. K12 or M18 could not inhibit actual production of lipotechoic acid or lipopolysaccharides, but it may interfere with their binding to host cells. One way to test this would be to add LTA or LPS to the streptococcal supernatant to see if there is inhibition.

In the human study, the volunteers brushed their teeth one hour before lozenge use; it would have been interesting to see if there was an effect by not brushing, and therefore allowing the K12 time to coaggregate with the existing biofilms, then see if an anti-inflammatory effect was conveyed. This might have more aptly simulate our in vitro experiments.

Another test of the mechanisms involved would be to allow the subjects to brush their teeth then immediately apply the probiotic. This was done previously with strain M18 in 75 volunteers (57). While this approach displaced the indigenous S. salivarius in some subjects, Illumina sequencing of the V6 region of the 16S rRNA gene showed that the overall composition of the oral microbiome was not modified. This is not unexpected since brushing does not remove all plaque biofilms, but by displacing indigenous strains, it may provide a more anti-inflammatory S. salivarius the opportunity to integrate into the niche and reduce overall inflammation. This remains to be tested. If a more thorough dental cleaning was undertaken (rather than simple brushing), it may be possible to have S. salivarius probiotic strains better colonize, and then deplete re-adhesion of pathogens, thereby also reducing the inflammatory processes. This is more of a competitive
exclusion approach against the oral pathogens, and its potential has been demonstrated for *S. salivarius* by others (61).

The findings with *Candida albicans* were also insightful for several reasons. Up until now, the *S. salivarius* K12 strain had been shown to inhibit adhesion of *C. albicans* to plastic surfaces and lower colonization in the mouth of rats (91). Our studies showed that the interference with the yeast is not due to reducing growth, but rather it is due to inhibiting hyphal formation and adhesion to surfaces. This makes sense ecologically, as yeast are common inhabitants of a healthy mouth (17), but their abundance and pathogenesis are kept in check by the indigenous microbiota and host immune status. Although we did not use primers to detect *C. albicans*, none of the human volunteers had evidence of yeast infection before or after probiotic use, indicating that the treatment did not disrupt the inherent protective nature of the microbiota. However, our *in vitro* studies showed that the K12 and especially M18 supernatant disrupted the yeast biofilms and prevented transition to infectious hyphal form. This aligns with studies in the vagina, where application of probiotic lactobacilli affected *C. albicans* metabolic activity, and increased expression of stress-related genes (92).

The ability of oral probiotics to affect metabolic processes involved in infection remain to be thoroughly studied. It remains unknown which metabolites are released en masse by oral biofilms from healthy and non-healthy individuals, and how they influence health and inflammation. If species such as *S. salivarius* somehow control the amount and types of metabolic products, this would potentially make them a useful intervention in people whose plaque biofilms are recalcitrant. Metabolic by-products have been described in the oral cavity, for example hydrogen sulfide (H$_2$S) gas produced by
degradation of proteins in the subgingival pocket, which is highly toxic and believed to have pro-inflammatory properties (93). It is one of the compounds associated with halitosis. The alleviation of this condition by probiotic *S. salivarius* K12 (48) indicates that the metabolism of pathogens is indeed affected. A range of amino acids, peptides, lipids and carbohydrates can be detected in the mouth and are potentially markers for decay (94). The culprits producing toxic metabolites in periodontitis appear to be *P. gingivalis*, *T. forsythia*, and *T. denticola* (95). Ultimately, the concentrations of bacterial metabolites are what causes symptoms and signs of disease, and stimulation of inflammation. Therefore, a better understanding of which metabolites (microbial and/or host) are influenced by probiotic application, and how these changes affect health of the host, represent a key next series of studies that are warranted. Studies using metatranscriptomics (96) along with metabolomics will be particularly insightful to identify the organisms producing the toxic metabolites, and whether their genes are suppressed by various treatments. This is the approach taken in our lab, where we showed that *Lactobacillus iners*, present in the healthy vagina, adapts to the infectious bacterial vaginosis (BV) emergence and higher pH, by altering its metabolic pathway (97), while *Gardnerella vaginalis* produces compounds specific to the BV condition (McMillan et al. submitted).

The human study provided two other interesting observations. Firstly, it confirmed other recent studies showing a wide range of bacterial types present in the saliva (10, 12, 81). This is transforming our view of the oral cavity and dental practice. It was not long ago that *S. mutans* was taught in university classes to be the exclusive etiological agent of caries, and that only a few pathogens were responsible for the
majority of oral diseases. These had essentially followed the old microbiology concept of Koch's Postulates (98), whereby a single organism caused a disease and this could be reproduced by adding it back into the site. Rather, it is clear now that a wide range of organisms, in most cases acting as a collective, create environmental conditions (low pH, high sugars, toxins, inflammatory mediators) that result in diseases which present in a clinically similar fashion.

Secondly, although the application of probiotics daily for a short seven days did not appear to modulate the microbiota profiles of the volunteers, as has also been shown with probiotic yogurt and the gut microbiota (99), there was some signs of shifts at day 14 regardless of whether the probiotic or control gum was being chewed. This was surprising because it suggests that regular gum use can impact the oral microbiota, above and beyond whether it contains sugar or not. It also demonstrated that the chewing action per se over- rode the probiotic effect. We postulate that the xylitol present in both gums, was responsible, as it has been shown to modulate the populations of cariogenic streptococci (100, 101). Xylitol is a sugar alcohol which does not kill the probiotic strain directly, but it may have caused a change in the coaggregated biofilms, such that it affected adherence of the probiotic strain, and dislodged the clumps containing the probiotic. Thus, it may be better to chew a xylitol gum, dislodge the biofilms, then chew a probiotic gum (without xylitol) to re-calibrate the microbiota. Such a study is worthy of investigation, although its practical implementation may be difficult since consumers are not likely to do such a double-use of a gum product.

Alternatively, the human study simply showed that the probiotic application was not effective, and the xylitol effects were artifacts. The latter is supported by a study of
children in which xylitol did not disrupt the oral microbiota when chewed twice daily (102). Clearly, other human studies are warranted, particularly in patients with chronic diseases. If K12 or M18 were found not to be effective, it would question the importance of bacteriocin production in inhibiting growth of pathogens. If studies confirmed that K12 or M18 were effective, it need not be because they produce the bacteriocins. As these bacteriocins are on mega-plasmids, removal of the plasmids would allow such testing to occur. This has actually been achieved, and both K12 and M18 have been produced without their plasmids (53). It would now be useful to assess the supernatants of these strains to see if they affect pathogenic biofilms and inflammation. In that case, it would be best to mimic plaque, caries or periodontal conditions, using a grouping of say the ten most abundance pathogens found in these conditions. These are being identified now through sequencing methodologies.

4.1 Overall Conclusions

This thesis has utilized a range of in vitro, high throughput sequencing and human approaches to understanding how probiotic streptococci can be considered to improve and maintain oral health. Both S. salivarius K12 and M18 appear to have characteristics capable of interfering with bacterial and yeast pathogens in the mouth, and in addition to modulating host inflammation responses. Both these strains are now sold in Canada and other countries without any adverse effects. Given this, opportunities exist for dental practitioners to monitor use of these products in a randomized, placebo-controlled manner, to determine if clinical benefits are indeed accrued. From a mechanistic perspective, more studies, as suggested herein, are warranted.
References


74. **R Core Team.** 2014. R: A Language and Environment for Statistical Computing. Vienna, Austria.

75. **Fernandes AD, Reid JN, Macklaim JM, McMurrough TA, Edgell DR, Gloor GB.** 2014. Unifying the analysis of high-throughput sequencing datasets: characterizing RNA-seq, 16S rRNA gene sequencing and selective growth experiments by compositional data analysis. Microbiome **2:**15.


Appendix 1. Western University Ethics Approval Notice

Principal Investigator: Dr. Jeremy Burton
File Number: 104641
Review Level: Full Board
Protocol Title: Investigating the ability of a probiotic gum to induce a protective anti-inflammatory effect in saliva
Department & Institution: Schulich School of Medicine and Dentistry/Microbiology & Immunology, Lawson Health Research Institute
Sponsor:
Ethics Approval Date: January 03, 2014
Ethics Expiry Date: January 31, 2014

Documents Reviewed & Approved & Documents Received for Information:

<table>
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<th>Document Name</th>
<th>Comments</th>
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<tr>
<td>Advertisement</td>
<td>Recruitment Poster (Received Dec 18, 2013)</td>
<td></td>
</tr>
<tr>
<td>Western University Protocol</td>
<td>including Study Design Flowchart</td>
<td>2013/12/18</td>
</tr>
<tr>
<td>Letter of Information &amp; Consent</td>
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This is to notify you that the University of Western Ontario Health Sciences Research Ethics Board (HSREB) which is organized and operates according to the Tri-Council Policy Statement: Ethical Conduct of Research Involving Humans and the Health Canada/CIH Good Clinical Practice Practices: Consolidated Guidelines; and the applicable laws and regulations of Ontario has reviewed and granted approval to the above referenced study on the approval date noted above. The membership of this HSREB also complies with the membership requirements for REB’s as defined in Division 5 of the Food and Drug Regulations.

The ethics approval for this study shall remain valid until the expiry date noted above assuming timely and acceptable responses to the HSREB’s periodic requests for surveillance and monitoring information. If you require an updated approval notice prior to that time you must request it using the University of Western Ontario Updated Approval Request form.

Member of the HSREB that are named as investigators in research studies, or declare a conflict of interest, do not participate in discussions related to, nor vote on, such studies when they are presented to the HSREB.

The Chair of the HSREB is Dr. Joseph Gilbert. The HSREB is registered with the U.O. Department of Health & Human Services under the IRB registration number IRB 00000540.

Signature

Ethics Officer to Contact for Further Information

This is an official document. Please retain the original in your files.

Western University Research Support Services Bldg. Room 5350
www.uwo.ca/research/services/ethics
Appendix 2. Lawson Health Research Institute Ethics Approval Notice

LAWSON FINAL APPROVAL NOTICE

LAWSON APPROVAL NUMBER:  R-13-523

PROJECT TITLE:  Investigating the ability of a probiotic gum to induce a protective anti-inflammatory effect in saliva

PRINCIPAL INVESTIGATOR:  Dr. Jeremy Burton

LAWSON APPROVAL DATE:  January 9, 2014

Health Sciences REB#:  104641

Please be advised that the above project was reviewed by the Clinical Research Impact Committee and Lawson Administration and the project:

Was Approved

Please inform the appropriate nursing units, laboratories, etc. before starting this protocol. The Lawson Approval Number must be used when communicating with these areas.

Dr. David Hill
V.P. Research
Lawson Health Research Institute

All future correspondence concerning this study should include the Lawson Approval Number and should be directed to Sherry Paiva, Research Administration Officer, Lawson Approval, Lawson Health Research Institute.

cc: Administration
Appendix 3. Letter of Information and Consent

Investigating the ability of a probiotic gum to induce a protective anti-inflammatory effect in saliva

I. .................................................. have read the Letter of Information, have had the nature of the study explained to me and I agree to participate. All questions have been answered to my satisfaction.

Date ____________________________ Signature of Participant ____________________________

Name of Participant (Please Print) ____________________________

Date ____________________________ Signature of Person Obtaining Informed Consent ____________________________

Name (Please Print) ____________________________

Note: the participant must initial each page of this letter and agreement and must be provided a photocopy of all six pages.

12/18/2013
Initials _______ 6 of 6

The research institute of London Health Sciences Centre and St. Joseph’s Health Care, London.
Appendix 4. Study Recruitment Poster

VOLUNTEERS NEEDED FOR RESEARCH STUDY
Looking for healthy volunteers to take part in a two-week trial aimed at determining the effects of chewing a probiotic gum

Researchers at the Lawson Health Research Institute and The University of Western Ontario are looking for healthy volunteers to take part in a two-week long study to examine the benefits of chewing a probiotic gum. We are interested in determining if consuming a specific probiotic gum will result in the generation of a protective anti-inflammatory effect in saliva. Participants will be given a supply of either regular gum or probiotic gum (gum provided free of charge by the researchers) to chew twice daily over the course of two weeks. Participants will supply saliva samples at four points during the study, which will be tested to determine if an anti-inflammatory effect has been generated.

If you are a healthy individual 20 to 60 years and have no oral health problems, you might be interested in participating in this study.

If interested, please contact:

Dr. Jeremy Burton
Lawson Health Research Institute,
St. Joseph’s Health Centre,
Tel:

Dr. Jeremy Burton
Dr. Jeremy Burton
Dr. Jeremy Burton
Dr. Jeremy Burton
Dr. Jeremy Burton
Dr. Jeremy Burton
Dr. Jeremy Burton
Dr. Jeremy Burton
Appendix 5. Study Design Flowchart

Day 0
- Participants randomized into two study groups
- Participants receive supply of gum tablets

Day 1
- 8 AM: Brush teeth
- 9 AM: Collect saliva (baseline)
- Chew tablet 10 minutes
- 1 PM: Collect saliva (4 Hours)
- 8 PM: Brush teeth
- Chew tablet 10 minutes

Day 2
- 8 AM: Brush teeth
- 9 AM: Collect saliva (24 Hours)
- Chew tablet 10 minutes
- 8 PM: Brush teeth
- Chew tablet 10 minutes

Days 3-7
- 8 AM: Brush teeth
- 9 AM: Chew tablet 10 minutes
- 8 PM: Brush teeth
- Chew tablet 10 minutes

Day 8
- 8 AM: Brush teeth
- 9 AM: Collect saliva (7 days)

Days 9-14
- Wash-out period
- Normal oral hygiene routine with no tablets chewed

Day 15
- 8 AM: Brush teeth
- 9 AM: Collect saliva (7 day wash-out period)
Curriculum Vitae

EDUCATION:  09/2011 – Present
Graduate Studies - MSc
Department of Microbiology and Immunology, University of Western Ontario

09/2007 – 04/2011
Undergraduate - BMSc
Honors Specialization in Microbiology & Immunology,
University of Western Ontario

Ontario Secondary School Diploma
Kincardine District Secondary School

RESEARCH EXPERIENCE:  05/2011 – 09/2011
Research Assistant
LHRI
Supervisor: Dr. Peter Cadieux

09/2010 – 04/2011
Microbiology & Immunology Honors Research Project
LHRI
Supervisor: Dr. Peter Cadieux

TEACHING/SUPERVISORY EXPERIENCE:

09/2012-12/2012
Teaching Assistant
Biology of Prokaryotes - Lab Course
Department of Microbiology and Immunology – UWO

09/2010-Present
Laboratory Co-Supervisor
Cadieux/Burton Lab – LHRI

Fourth Year Thesis Students
Department of Microbiology and Immunology – UWO
09/2011-04/2012 – Amanda Ruprecht

Partners in Experiential Learning Program

Schulich Summer Dental Awards Program
Department of Dentistry - UWO
06/2012-09/2012 – Ryan Lum-Tai
06/2011-09/2011 – Michael Lung

Schulich Research Opportunities Program
Department of Medicine – UWO
06/2011-09/2011 – Melissa Huynh

Endourology Fellow and Residency Laboratory Training Program
10/2010-06/2011 – Dr. Petar Erdeljan
10/2010-06/2011 – Dr. Alfonso Carreno
04/2011-06/2012 – Dr. Andrew Fuller
06/2012-07/2013 – Dr. Nader Fahmy

VOLUNTEER EXPERIENCE AND MENTORSHIP:

Lawson Association of Fellows and Students
Co-President
09/2012-09/2013

Laboratory Workshop Supervisor - "Antibiotics: Susceptibility Versus Resistance"
Discovery Days - Canadian Medical Hall of Fame
St. Joseph's Foundation/LHRI
05/2012 - Morning and Afternoon Sessions

Laboratory Workshop Supervisor - Byron Southwood Public School
LHRI
05/2012

Poster Judge – Junior Sciences Division
London District Science and Technology Fair
03/2012
Poster Judge – Junior Sciences Division
Orchard Park Elementary School Science Fair
03/2012
Poster Judge – Senior Sciences Division
Canada Wide Science Fair – Seneca College, Toronto Ontario
05/2011

Laboratory Workshop Supervisor – “Studying Antibiotic Resistance”
Discovery Days - Canadian Medical Hall of Fame
Conducted at LHRI
05/2011

Laboratory Demonstrator – Annual Cornerstone Event
St. Joseph’s Foundation/LHRI
02/2011

Microbiology and Immunology Graduate Social Committee
2011-2013

Laboratory Demonstrator – “Probiotics and Antibiotics”
Jack Chambers Elementary School – Grade 6 Classes
10/2010

SCHOLARSHIPS AND AWARDS:

- Canadian Institutes of Health Research: 2013
  - Master's Award: Frederick Banting and Charles Best Canada Graduate Scholarships ($17500)

- Ontario Graduate Scholarship – 2012-2013
  - Ontario Government Award ($15000)

- London Health Research Day: First Prize Poster Presentation (Infection and Immunity Section) – 03/2012
  - Travel award ($500)
• The University of Western Ontario: Microbiology and Immunology Graduate Entrance Scholarship – 2011
  • Awarded for academic excellence ($2000)

• The University of Western Ontario Gold Medal: Honors Specialization in Microbiology and Immunology - 2011
  • Highest overall 4-year average in the Microbiology and Immunology module.

• Endourology Fellowship Manuscript Award: (Basic Science Division) – 2011
  • Erdeljan P, MacDonald KW, Goneau LW, Bevan T, CARRIERE R, Razvi H, Denstedt JD, Cadieux PA. Effects of Subinhibitory Concentrations of Ciprofloxacin on *Staphylococcus saprophyticus* Adherence and Virulence in Urinary Tract Infections.
  • Presented by the International Endourological Society
  • Third Prize ($750)

• The University of Western Ontario: In-Course Scholarship Year IV (2010-2011)
  • Awarded for high academic achievement in the previous year of study ($700)

• The University of Western Ontario: Dean’s Honor List – 2008, 2009, 2010, and 2011

• Bruce Power 10th Anniversary Scholarship (2011)
  • Awarded to 200 students from Bruce, Grey, or Huron Counties attending post-secondary school ($250)

• Bruce County In-Course Scholarship (2009-2010)
  • Awarded to the man and woman from Bruce County who stand highest in any year of any regular program at the University of Western Ontario ($200)

• The University of Western Ontario: In-Course Scholarship Year III (2009-2010)
  • Awarded for high academic achievement in the previous year of study ($700)

• Dr. G. E. Hall Scholarship - 2010
• Awarded to a student with the highest average in third year, who is entering fourth year of an Honors Specialization offered by the Departments of Biochemistry, Medical Biophysics, Microbiology and Immunology, Pharmacology and Toxicology and Physiology. ($450)

• Bruce County In-Course Scholarship (2007-2008)
  • Awarded to the man and woman from Bruce County who stand highest in any year of any regular program at the University of Western Ontario ($200)

• The University of Western Ontario Scholarship of Excellence (2007-2008)
  • Awarded to students with an admission average between 90-94.9% ($600)

PEER REVIEWED PUBLICATIONS:


**PUBLISHED COLLECTIVE WORKS**


**ACCEPTED PEER REVIEWED ABSTRACTS:**


**POSTER PRESENTATIONS:**


4. Goneau LW, MacDonald KW, Cadieux PA. 2011. Sub-Inhibitory Antibiotic Treatment Induces High Density Growth and Improves *Staphylococcus saprophyticus* Survival Against Bactericidal Concentrations. The Annual Infection and Immunity Research Forum, Department of Microbiology and Immunology, Schulich School of Medicine and Dentistry, University of Western Ontario. (Co-author).


**PODIUM PRESENTATIONS**