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Characterization Of Oral Microbiota In Xerostomic Versus Non-Xerostomic Volunteers And In Daily Samples Following Standard Oral Hygiene Practices

Chelsea Myers
Wayne State University,

CHARACTERIZATION OF ORAL MICROBIOTA IN XEROSTOMIC VERSUS NON-XEROSTOMIC VOLUNTEERS AND IN DAILY SAMPLES FOLLOWING STANDARD ORAL HYGIENE PRACTICES

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CHELSEA ANN MYERS

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DEDICATION

I dedicate my thesis to my parents, Melissa and Harry Myers, and my grandparents, Marie and John Beverly. They have been my number one fans since I was born. They have done nothing but love, support and push me to be the very best I can be. There are not enough words or thanks that I can write to explain how glad I am to have you as my role models and family members. Thank you for never losing faith in me.

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CHAPTER 1: INTRODUCTION

1.1 Oral Microbiology Background

The oral microbiome is quite complex; from the extreme numbers of oral microbiota that colonize the oral cavity, to the different combinations of groups of oral microbiota that colonize specific areas and surfaces of the oral cavity. The NIH Human Microbiome Project (HMP) has recently revealed that the oral microbiome, as compared to the gut or skin, has the largest core of commonly shared microbes among unrelated individuals [1, 2]. More specific to the oral microbiome than the HMP, the Human Oral Microbiome Database (HOMD) (www.homd.org) was developed using a provisional taxonomic scheme for unmatched oral bacterial isolates and phylotypes. A total of 36,043 16s rRNA gene oral clone sequences were analyzed to identify additional taxa not included in the initial set up of the HOMD. The HOMD consists of 619 Oral taxa with 6 major phyla *(Firmicultes, Bacteroidetes, Proteobacteria, Actinobacteria, Spirochaetes, and Fusobacteria*) that make up 96% of the taxa among all oral bacteria [3].

There are many different groups of microbiota that dominate depending on their location in the oral cavity, such as the hard palate, saliva, subgingival plaque or throat. These groups can be found in Table 1 below. For instance, in the healthy oral cavity, saliva contained the major core genera of *Prevotella, Streptococcus, Veillonella, Pasteurellaceae, Fusobacterium, Porphyromonas, Neisseria,* and unclassified bacteria (*Uncl)* [2]. The most abundant that dominated nearly all oral mucosal sites was *Streptococcus: Streptococcus oralis, Streptococcus mitis,* and *Streptococcus peroris* [4].

Table 1. The core bacterial taxa in the oral cavity from over 200 healthy individuals participating in HMP

Sample type	High abundance core genera in >75% samples at >10% abundance	Other major core genera in >80% samples at >1% abundance	Minor core genera in >50% samples
Buccal mucosa	Streptococcus (2)	Uncl. Pasteurellaceae (16, 19), Gemella (11)	Atopobium, Uncl. Prevotellaceae, Uncl. Bacilli, Catonella
Hard palate	Streptococcus (2, 6)	Uncl. Pasteurellaceae (16), Veillonella (4), Prevotella (10), Uncl. Lactobacillales (13), Gemella (11)	Mogibacterium Catonella
Keratinized gingiva	Streptococcus (2), Uncl. Pasteurellaceae (19)		Uncl. Bacilli
Palatine tonsils		Streptococcus (2, 6), Veillonella (4), Prevotella (10), Fusobacterium (9), Uncl. Pasteurellaceae (16)	Mogibacterium, Uncl. Firmicutes
Saliva		Prevotella (10), Streptococcus (2, 6), Veillonella (4), Uncl. Pasteurellaceae (16), Fusobacterium (9), Porphyromonas (7), Neisseria (−)	Uncl. Actinomycetales, Tannerella, Kingella
Subgingival plaque		Streptococcus (2), Fusobacterium (9), Capnocytophaga (−), Prevotella (−), Corynebacterium (−), Uncl. Pasteurellaceae (−)	Uncl. Firmicutes
Supragingival plaque		Streptococcus (2), Capnocytophaga (−), Corynebacterium (15), Uncl. Pasteurellaceae (−), Uncl. Neisseriaceae (21), Fusobacterium (9)	Uncl. Betaproteobacteria
Throat	Streptococcus (2, 6)	Veillonella (4), Prevotella (10), Uncl. Pasteurellaceae (16), Actinomyces (−), Fusobacterium (9), Uncl. Lachnospiraceae (−)	Mogibacterium, Uncl. Firmicutes
Tongue dorsum	Streptococcus (2, 6)	Veillonella (4), Prevotella (10), Uncl. Pasteurellaceae (16), Actinomyces (14), Fusobacterium (9), Uncl. Lactobacillales (13), Neisseria (8)	Uncl. Actinomycetales, Uncl. Bacilli, Peptostreptococcus

HMP [2, 4]. In the parentheses is the corresponding operational taxonomic unites in the genus or family. Uncl is the abbreviation used for unclassified.

There are a large number of different species, both bacterial and fungal, found in the healthy. A study done by Kumar et al. in 2005 found that phylotypes significantly associated with healthy patients were: V*eilonella, Campylobacter gracilis, Abiotrophia adiacens, Eubacterium saburreum, Capylobacter showae, Gemella, Streptococus sanguis, Capnocytophaga gingivalis, Streptococcus mutans, Abiotrophia, Rothia dentocariosa, Eubacterium,* and *Selenomonas* [5]. In healthy individuals, 5 genera of fungi have been found. They are consisted of *Candida species, Cladosporium, Aureobasidium, Saccharomycetales,* and *Aspergillus* [6].

Many studies have shown that specific oral bacteria are associated with disease. The most prevalent worldwide chronic infectious disease, dental caries, has been found to have changes in the abundance of genera depending on the stage of the caries [7]. Dental caries was previously thought to be associated with *Streptococcus mutans*, however, molecular analysis has shown that there is a predominance of *Atopobium, Propionibacterium* or *Prevotella*, with *Streptococcus* or *Actinomyces* in carious dental lesions [8]. Other genera found to be associated with dental caries are *Lactobacillus, Atopobium, Olsenella, Propionibacterium, Bifidobacterium, Dialister, Sphingomonas and Parascardovia* [9]. The "Red complex", consisting of *Porphyromonas gingivalis, Tannerella forsythensis* (formerly *Bacteroides forsythus*), and *Treponema denticola,* have been found to be elevated in patients with chronic periodontitis [10] [5]. *Aggregatibacter actinomycetemcomitans* has also been found to be a periodontal pathogen [11]. Whereas, *Tannerella forsythia, Peptostreptococcus micros (Parvimonas micra), Fusobacterium nucleatum subsp., Haemophilus paraphrophilus* and *Capnocytophaga sp*. have been found to be associated

with gingivitis [12]. Taxa also found to be associated with gingivitis included *Fusobacterium nucleatum subsp. polymorphum, Lachnospiraceae [G-2] sp. HOT100, Lautropia sp. HOTA94,* and *Prevotella oulorum,* whilst *Rothia dentocariosa* was associated with periodontal health [13]. Halitosis bacteria are *Porphyromonas gingivalis, Fusobacterium nucleatum, Prevotella intermedia* and *Solobacterium moorei* [14]. Particular *Candida species* have been found to be involved in oral mucosal disorders in patients with xerostomia, such as *Candida albicans, Candida glabrata, Canadida tropicalis,* and *Candida krusei* [15].

The analysis of the association of specific or groups of microbes with oral disease and health have been a great insight to causes and treatment. The methods of determining the microbiota include culture, PCR or qPCR after microbial DNA extraction, DGGE and Next Generation Sequencing [5, 16, 17]. The continuation of further microbial associations with oral diseases and building of the HOMD through microbial DNA extraction, directly from saliva, and sequencing needs to be done, especially those that have not been done already, such as xerostomia.

1.2 Xerostomia

Approximately 5.5% to 46% of the population suffers from the burden of xerostomia [18]. The prevalence and diagnosis of xerostomia and salivary gland hypofunction is very difficult to determine with certainty owing to the limited number of epidemiological studies and differences in how the two conditions have been defined. Xerostomia is the subjective feeling of dry mouth, a symptom that may or may not be accompanied by hyposalivation, an objective decrease in salivary flow. The stimulated salivary flow rate for healthy, non-hyposalivation averages 1.5–2.0

mL/min and the unstimulated salivary flow rate is approximately 0.3–0.4 mL/min [18, 19]. For xerostomia, however, the individual has a stimulated flow rate below 0.5 mL/min, and an unstimulated flow rate below 0.12-0.16 mL/min [20]. Chronic xerostomia has significant negative implications that may affect the comfort of the oral cavity, bad oral hygiene and general well-being; salvia is very important for lubrication and oral health; negative oral health such as dental caries, oral fungal infections, halitosis, or burning mouth [19-21]. The cause of xerostomia can be induced from both salivary and non-salivary reasons (such as mouth breathing, psychological disorders, and dehydration) [20]. There are several types of treatments and ways to manage the negative side effects of the disease, however, further understanding at the microbial level may be helpful in understanding the disease as a whole and may be helpful in developing better treatments.

1.2.1 Causes

There are several possible causes for the development of xerostomia. The most frequent cause of hyposalivation is the use of certain medications, radiotherapy to the head and neck, and certain diseases. Other factors include salivary gland trauma or tumors, depression, anxiety and stress, mouth breathing, psychological disorders, or malnutrition [18, 20]. Medications that have been associated with dry mouth are anticoagulants, antidepressants, anti-hypertensives, anti-retrovirals, hypoglycemics, levothyroxine, non-steroidal anti-inflammatory drugs, and steroid inhalers [18]. Diseases that have been thought to cause xerostomia are Sjögren's syndrome, autoimmune disorders, diabetes mellitus, HIV, sarcoidosis, herpes virus, hepatitis C and end-stage renal disease. Sjögren's syndrome (SS), a chronic, autoimmune, inflammatory disorder

characterized by lymphocytic infiltration of the exocrine glands in multiple sites, most commonly the lacrimal and salivary glands. It can occur alone (primary SS), or in conjunction with another autoimmune rheumatic disease (secondary SS). Clinically, patients with SS most often present with a complaint of dry eyes (keratoconjunctivitis sicca) and dry mouth [20].

1.2.2 Current Diagnosis Methods

The diagnosis of xerostomia is done through by assaying the individuals reported symptoms, medication use, and past medical history. To help identify whether patients are experiencing xerostomia or hyposalivation, several dental health questionnaires have been proposed and used. These questionnaires ask questions about comfort of mouth, dryness of entire internal and external oral cavity, if there is any difficulty talking, swallowing or eating dry foods, and similar questions [18]. Medications that may reduce saliva flow should be noted when identifying whether it could be a cause of the chronic xerostomia or hyposalivation. Finally, the medical history should be examined for any radiation treatment of the head and neck region, and other systemic diseases that have be found to induce xerostomia. In addition, an oral examination can be used to identify clinical signs pathognomonic for hyposalivation. These signs include: sticking of an intraoral mirror to the buccal mucosa or tongue, frothy saliva, no saliva pooling in floor of mouth, loss of papillae of the tongue dorsum, altered/smooth gingival architecture, glassy appearance to the oral mucosa (especially the palate), lobulated/deeply fissured tongue, cervical caries (more than two teeth); and/or mucosal debris on palate (except under dentures) [22]. Another indication of xerostomia is found through a stimulated and unstimulated salivary flow tests. Most of the tests are easy to perform and require little time. As previously mentioned,

xerostomia patients tend to have a stimulated flow rate below 0.5 mL/min, and an unstimulated flow rate below 0.12-0.16 mL/min [20]. More extreme xerostomia diagnosis measures include salivary imaging by ultrasonography or magnetic resonance imaging or salivary biopsy of the major or minor salivary glands [20].

1.2.3 Current Treatment Methods

There are several treatment strategies that can be used for the management of xerostomia. These strategies aim to reduce patients' symptoms and/or increase salivary flow. Xerostomia symptoms can be managed by diet modifications, proper hydration, increase in humidity at night-time, avoidance of crunchy/hard foods and use of salivary substitutes and lubricants (such as rinses, gels, sprays, toothpastes, and artificial saliva) [20]. Patients can increase salivary flow by using sugar-free, xylitol-containing mints, candies and gum, sialagogues (drugs that increase saliva flow) and acupuncture. Two systemic US Food and Drug Administration-approved sialagogues are pilocarpine and cevimeline [18]. Following the bad oral hygiene that come from xerostomia, individuals may have to treat these oral conditions by the restoration of dental caries, antifungal medications to treat oral candidiasis, antibiotics for bacterial infections, and denture adjustments and or denture adhesives [20]. However, there are some preventative measures that can be used to avoid some of the bad oral hygiene effects from xerostomia. By increasing the frequency of oral/dental evaluation by a dentist and use of topical fluoride applications the status of oral hygiene can be closely monitored and maintained [20].

1.2.4 Characterization of Xerostomia Microbiota

There is a great understanding of the implications of xerostomia, but there are few studies that analyze the microbial environment by qPCR (quantitative polymerase chain reaction) or NGS (next-generation sequencing), while others were done by culturing followed with qPCR for identification. A recent culture study on xerostomia found total numbers of *Candida albicans* were significantly higher in patients with xerostomia (67%) than in the controls (13%) [23]. Irradiated, dentate, xerostomia individuals' oral rinses were cultured and *Acinetobacter*, *Neisseria*, *Chryseomonas*, *Flavimonas*, *Pseudomonas*, *Citrobacter*, *Enterobacter*, *Escherichia*, *Klebsiella*, *Flavobacterium* and *Weeksella* species were prevelent [24]. *Klebsiella pneumoniae* subsp. *pneumoniae* was found to be significantly more prevalent in the irradiated subjects, and *Enterobacteriaceae* were found more frequently in aged irradiated subjects, where *Citrobacter freundii* was also significantly elevated in the culture based study [24]. Another culture study with the addition of PCR identification showed mean levels of *Streptococcus mutans, Lactobacilli spp*. and *Candida spp*. to be significantly higher in medicated hypertensive xerostomia patients and primary Sjögren's syndrome [17, 25].

Several studies that assay the microbial composition of dry mouth/hyposalivation. For instance, a culture study done on complete denture wearers, found that as the salivary flow rate (mL/5 min) decreased, it was found that the number of anaerobic bacteria and *Candida species* increased 1.4 x 10^9 CFU/mL [26]. Patients with Sjögren's syndrome contained significantly higher proportions of cultures of *Streptococcus mutans, Candida albicans, Actinomyces naeslundii* and *Lactobacillus spp.* (specifically L. *acidophilus*) [27]. Head and neck radiotherapy patients' saliva

was assayed by high-throughput sequencing, and 11 genera were found in all subjects: *Streptococcus, Actinomyces, Veillonella, Capnocytophaga, Derxia, Neisseria, Rothia, Prevotella, Granulicatella, Luteococcus* and *Gemella* [28].

Further research needs to be done with PCR and Next Generation Sequencing on DNA of oral microbiota in xerostomia patients. Culture methods can be limiting in types of microbiota growth and methods of non-specific oral microbial DNA extraction combined with PCR and Next Generation Sequencing could be a better method for analysis. By getting a better, and full understanding of xerostomia at the microbial level could help with better diagnosis and treatment methods.

1.3 Standard Oral Hygiene Practices

Recent findings have found that douching is counterproductive in maintaining a healthy collection of vaginal microbes [29]. With this idea in mind, are certain mouthwashes counterproductive in keeping the healthy groups of oral microbes? The primary use for antimicrobial mouthwashes is to reduce plaque and gingivitis. The American Dental Association (ADA) puts an ADA-Accepted seal on antimicrobial mouthwashes that reduce plaque and gingivitis better than brushing and flossing alone [30]. Mouthwashes can be composed of various active ingredients that may or may not have antimicrobial agents. Available mouthwashes may include the following active ingredients: antibiotics, bisbiguanide, chlorine derivatives, essential oils, fluorides, oxygenating compounds, phenols, plant extracts, or quaternary ammonium compounds [31]. Products that have earned the ADA Seal are those that contain 0.12% chlorhexidine gluconate (CHG) or a fixed combination of essential oils (EO) such as Listerine®

[30]. Several studies have been conducted on the effectiveness of different active ingredients on oral health; however, there are few studies that actually characterize the changes of the oral microbiota due to the main active ingredients in these products.

1.3.1 Effectiveness of Active Ingredients

When choosing a mouthwash or toothpaste to buy, the effect of it on its oral hygiene can be a main deciding factor. Depending on its main active ingredients, it can act as an antiplaque, antigingivitis, anticaries, desensitizing, or whitening agent. In order to know which mouthwashes or toothpastes are any of these agents they must be tested. Mouthwashes including essential oils have strong clinical evidence for efficacy against different oral biofilms bacteria [31, 32]. They also have been shown to effectively act as both an antiplaque and anti-gingivitis agent [33]. Studies testing various chlorhexidine varnishes as prevention for caries/biofilms were inconclusive; they were effective against different oral biofilms, but, present a number of unwanted side effects and should be prescribed with caution. It was recommended that further well-conducted randomized trails be completed before being recommended for caries/biofilm prevention [31, 34]. Mouthwashes with 0.12% chlorhexidine or essential oils, and dentifrices containing triclosan with 2% Gantrez copolymer or stannous fluoride reduce gingivitis [33]. Stannous fluoride/sodium hexametaphosphate provides antiplaque, antigingivitis, anticaries, and antisensitivity benefits [35].

The composition of oral microbiota can change based on the main active ingredients of the mouthwash or toothpaste. These changes can possibly alter the composition of oral microbiota, possibly eliminating both bad and good bacteria. What researchers need to determine is what populations of oral microbiota flourish after use of these chemically different hygiene

applications. Several recent studies, mostly using culture techniques, have found reduction in various select oral microbiota. For example, chitosan mouthwash interferes with the adherence of all microorganisms *in vitro* [36]. Xylitol in mouthwashes was found to reduce the number of *Streptococci mutans* [37]. The Green Tea Extract in mouthwashes was also found to decrease the colony counts of *Streptococci mutans* and *Lactobacilli* [38]. Cetylpyridinium chloride mouthwashes reduce plaque accumulation, gingival inflammation and also reduce *Porphyromonas gingivalis* and *Fusobacterium nucleatum* associated with halitosis [14, 39]. Chlorhexidine (0.12%) has been found to reduce *Aggregatibacter actinomycetemcomitans*. *Escherichia coli* and *Streptococcus mutans* counts were also reduced when a combination of chlorhexidine gluconate and sodium fluoride were the main active ingredients in a mouthwash [11, 40]. Triclosan in toothpaste was found to significantly reduce the number of *Candida albicans* and *Streptococci mutans*, however its efficacy as a mouthwash had limited data [40]. Antibacterial toothpastes containing stannous fluoride were found to significantly reduce bacterial viability in plaque left behind after brushing for up to 12 hours [41]. Biotene, a dry mouth wetting agent containing lysozyme, lactoferrin, glucose oxidase, and lactoperoxidase, exhibited antimicrobial activity against *Streptococcus mutans* and *Lactobacillus acidophilus*, but was not effective on *Candida albicans* [42].

Analysis of previous studies provides some insight into which ingredients act as preventative and whitening agents, and which species are decreased in numbers and counts. Most of these studies only assayed specific species, so that alterations in the vast repertoire of other species went unmeasured. Further research should examine the effects of the entire normal

microbiota and determine which species colonize and flourish after a specific treatment. My thesis research addresses this gap.

1.4 Specific Aims

The first specific aim was to design a human subject's research proposal and questionnaire that met Wayne State University (WSU) requirements. This research thesis requires the participation of the WSU Institutional Review Board (IRB) committee, and therefore, protocols, consent, and case report forms were developed for expedited approval. A Dental Health Questionnaire (Appendix A) form was also approved and obtained for statistical analysis. This specific aim was completed and accepted by the IRB committee (IRB protocol number: 075914). The second specific aim was to optimize sample storage and DNA extraction. Proper storage and DNA extraction of spit samples required the testing of several storage buffers and lysis combinations. This aim was completed and a Saliva Storage Buffer (SSB) solution combined with Fungal Lysis Buffer (FLB) confirmed no cell growth or DNA degradation. The third and fourth specific aims include bacterial and fungal microbiome characterizations. Saliva DNA samples from the xerostomia versus healthy study arm were completely characterized by phylogenetic branch specific qPCR [24]. DNA from samples were stored for analysis by next-generation sequencing. Saliva DNA sample from the nightly oral hygiene practice study arm were partially characterized. Fungal species in the xerostomia and healthy samples were identified by qPCR and melt curve analysis, coupled with selective sequencing, as described [25]. Statistical analysis to correlate compositions with patient data was performed with GraphPad Prism 6 and other software, and with Microsoft Excel add-on tools.

1.5 Significance

The primary purpose of this investigation is to define differences in oral microbiota between healthy and xerostomia patients. We hypothesize that there will be differences between the groups; if so, these differences will provide a basis for dealing with the problems associated with xerostomia. A better understanding of the altered microbiota of xerostomia patients should provide insights into more rational therapies. Also, characterization of combined bacterial and fungal biomes is novel and may provide insight into oral candidiasis in these and in other groups of patients. This will be done by microbial DNA extraction directly from saliva, using the mouth as an incubator for normal microbial compositions per individual.

Secondary goals are to define day-to-day changes in oral microbiota in healthy individuals, and to measure the impact of nightly oral hygiene practices on oral microbiota the following day. We hypothesize that commercial mouthwashes/toothpastes alter the oral microbiome in ways that may not foster the outgrowth of potentially beneficial species, based on counter-productive use of douches in vaginal studies. A better understanding of the composition of the oral microbiota as a whole, based on nightly oral hygiene practices, should provide insights to which practice is most beneficial for fostering growth of beneficial oral microbiota. These oral hygiene practices are done at night, before bed and without any food or water between the nightly routine and the morning sample, to reduce the variables that change the oral microbiota throughout the day. The mouth is then used as an over-night incubator for primary colonies to become established based on their ability to flourish after the specific nightly oral hygiene practice.

CHAPTER 2: MATERIALS AND METHODS

2.1 Preliminary Trials & Saliva Storage/Lysis Buffers Methodology

2.1.1 Sample Collection Strategy

The xerostomia versus control study arm and the nightly oral hygiene study arm's DNA were collected the same way. To achieve extraction of DNA directly from saliva from all the oral microbiota, a stable storage buffer combined with a productive DNA extraction method must be carefully chosen. For the convenience of the volunteers and the number of daily samples needed in the nightly oral hygiene study, saliva should be collected and stored until all samples are completed and returned to the lab for DNA extraction. The saliva sample could possibly sit in the buffer for at least 30 days. This buffer must not degrade cells and/or DNA from the saliva but also should not promote new cell growth either. The buffer chosen must be stable across several individuals, not inhibit or interfere with DNA extraction, and combined with the DNA extraction method of choice, they must allow maximum DNA extraction across several oral species. The storage buffer must also be non-toxic and chemically safe, in case small traces were to get on lips during collection. However, volunteers will be strongly advised to not pour the buffer in their mouths or consume it, no matter what buffer is chosen for the study.

To find this ideal storage buffer and lysis combination, several storage buffers/lysis methods were tested from an aliquot of saliva from a single volunteer. Following the results of this comparison, the 2 best storage buffers were tested with lysis across 4 different individuals. Finally the best storage buffer was further tested on the prevention of cell growth and extraction across several species.

2.1.2 Initial Saliva Storage/DNA Extraction Testing

A single volunteer's aliquot of saliva was tested across 7 storage buffers: Qiagen RNAlater (Qiagen RNAlater RNA stabilization reagent, Qiagen Sciences, Maryland, USA), 91% isopropyl alcohol (IPOH), 95% ethanol (EtOH), 1 x Fungal Lysis Buffer A (FLBA) (contents listed in Appendix B) without potassium hydroxide (KOH), 1 x FLBA without KOH plus ethylenediaminetetraacetic acid (EDTA), saliva with no buffer, and regular 1 x FLBA with KOH, or a high SDS/alkaline lysis-phenol extraction [43].

To compare the longevity of bacterial DNA in saliva in the storage buffers, each was tested at three time points: day 0 or same day processing, day 7, and day 30. Approximately 1.5 mL of each of the storage buffers was added to 500 µL aliquots of saliva. On the day of processing, these were centrifuged at 16,060 x g, supernatants were discarded, and pellets were suspended in 500 µL FLBA with 5 µL KOH (100:1). All of the storage buffers were then incubated at 65°C for 2 hours and followed with centrifugation at 16,060 x g and collection of the supernatant. Approximately 250 µL of Fungal Lysis Buffer B (FLBB) (contents listed in Appendix B) was then added to the collected supernatant for neutralization (pH of approximately 7.8). Half of the FLB supernatant collected was placed in a new sterile 2 mL tube and purified with buffer QG (Qiagen buffer QG buffer from Qiagen's QIAquick Gel Extraction Kit, Qiagen Sciences, Maryland, USA). Buffer QG was added to fill the 2 mL tube, then centrifuged 10 min at 16,060 x g; the supernatant was bound to and washed with a Qiagen QIAquick spin column, according to the manufacturer's protocol. The column was then left to air dry for 10 minutes followed by the addition of 200 µL of 1 x Tris-EDTA buffer (10 mM Tris pH 8.3, 1 mM EDTA;

TE). After the TE was in the column for 5 minutes, the eluate was collected through centrifugation at 16,060 x g and stored at -20°C until analysis. The remaining half of the neutralized FLB supernatant was stored at -20°C until analysis.

An aliquot of purified DNA from each of the storage buffer variations with time periods (total of 42 variations) was assayed by qPCR with a few primers: a 16s bacterial universal primer, a primer to test for inhibition, and a phylogenetic branch-inclusive (PB) 16s *Lactobacillus* primer. *Lactobacillus* was chosen since it is a gram positive bacterium, more difficult to lyse and therefore a more stringent test of the buffers. Molecules per reaction or Cq values were then compared for the top 2 storage buffers, those with best overall and best *Lactobacillus* titers and with least inhibition; these were then tested on across a few different individuals.

2.1.3 Secondary Saliva Storage/DNA Extraction Testing

After eliminating unacceptable saliva storage/DNA extraction methods from the initial experiment, one buffer from the initial testing and two additional new buffers were tested on their stability across multiple individuals. Four volunteers each provided an aliquot of saliva for testing stability across 3 storage buffers: 91% IPOH, Saliva Storage Buffer (SSB; Appendix B) and Qiagen's buffer QG. Stability of DNA and extraction efficiencies were compared as described above.

An aliquot of purified DNA from each of the storage buffer variations and time periods from each individual (total of 20 variations) were assayed by qPCR with several primers: a 16s bacterial universal primer, a primer to test for inhibition of the purified DNA in its buffer, and

phylogenetic branch-inclusive (PB) 16s *Lachnospiraceae* primers [43]. Molecules per reaction (calculated by CFX Manager™ Software v3.1) or Cq values were compared for the type of storage buffer/processing method to use in both arms of the project.

2.1.4 Cell Viability Testing in SSB & IPOH

The viability of the saliva cells in the select buffers was tested to determine whether any bacterial growth was occurring what might alter the initial populations, over a 28 day time period. The buffers of choice, determined from the previous experiments, were IPOH and SSB and the control buffer was 1x PBS (Appendix B). These three buffers were tested on two different individuals' saliva. For direct comparison of the three buffers across the 28 days a single 36 ml aliquot of each buffer was compared for each subject. An aliquot of 4.5 ml of each subject's saliva was added to each of the buffer tubes. These tubes were then tested by CFU on Trypticase Soy Agar (TSA; Appendix B). and aliquots were lysed with FLB for 8 different time points (t = 0 [initial], 1 hour, 1 day, 5 days, 9 days, 13 days, 21 days, and 28 days) during the 28-day testing. The initial time period (t = 0) is samples taken immediately after the saliva sample is added to the buffer. These tubes were tested using two separate analyses. The first was cell counting by streaking on select media. This experiment will show if there is any growth or death of the saliva cells. The DNA was extracted (FLB method) from cells still alive in either the IPOH or SSB buffer, amplified and sent out for sequencing. The second experiment requires extraction of the saliva's microbial DNA and qPCR to test for any changes in the species of the salvia sample.

When counting cells or colony-forming units (CFU), samples were serially diluted in PBS (-1, -2, and -3) and 10 µL of each were spread uniformly with a sterile glass rod in a quadrant on TSA. The plates were then incubated anaerobically in a candle jar at 37°C overnight and colonies were counted in the quadrant that had the closest to 100 colonies, and calculations were done to get the CFU/mL in each buffer at each time point. Pictures of each of the plates were also taken. Cells still alive in either buffer were lysed using the FLB method described previously in Section 2.1.1, except cells were scraped from agar using a sterile pipet tip and mixed in the FLB solution. Then the DNA of these cells were amplified using qPCR with the bacterial universal primer (16s) and prepped to be sent out for sequencing (method described in Section 2.3.5).

During the DNA extraction portion, an aliquot of 4 mL was removed from the original aliquot and centrifuged for 30 minutes at 16,060 x g. The buffer was then discarded and 500 µL of FLB-A + KOH was added to the pellet, and processed using the FLB method (mentioned above in section 2.1.2). Samples that originally contained SSB, however, did not need any FLB-B because they were already neutral. To directly compare the three samples, each sample was adjusted to a final volume of 850 µL with appropriate amounts of 1 x TE. Each of the samples was then assayed by qPCR with two broad-spectrum bacterial primers: one spanning variable domains 3-5 of the 16S ribosomal RNA gene, (Bu10) and the other spanning the internal transcribed spacer between the 16S and 23S ribosomal RNA genes (BuITS). Melt peak results were compared to monitor for overt changes in bacterial compositions.

2.1.5 Species DNA Extraction with SSB/FLB Test

To determine if our extraction protocol and buffers compared well with commercial kits, the chosen storage/lysis buffer (SSB/FLB) was tested against FastDNA™ KIT (MP Biomedicals). The FastDNA™ Kit quickly and efficiently isolates high quality genomic DNA from plants, animals, bacteria, yeast, algae, and fungi using Lysing Matrix A (garnet and one 1/4 inch ceramic bead) for cell lysis and a silica-based method for the purification process. To compare these two extraction methods against each other, equal volumes of 10 different species' DNA were extracted using both methods. The 10 species and collection methods are listed in Table 5 in Section 3.1.4. Notably, we included several species of *Streptococcus*, which has a reputation for poor extraction efficiency using commercial kits. The extracted DNA was compared using the 16s bacterial universal primer and an inhibitory primer (DR3) in qPCR.

The 10 species were collected and grown by incubation for 48 hours in 37°C in a 2 mL tube with 500 µL of LIB + supplements broth (Appendix B). A control tube was also made with the same 500 µL aliquot that the other tubes received and also processed with both methods. After incubation for 48 hours, an aliquot from each species tube adjusted to an optical density (A600) and then at similar cellular densities species' aliquots were individually prepped for extraction. To do this, the cells were diluted 50-fold in new LIB media broth (196 µL of LIB media and 4 µL of cells grown in LIB) and read in a Model 25 spectrophotometer (Beckman, CA), blanking against LIB media only. Volumes were then adjusted, by adding small amounts of LIB to get an A600 reading of 0.079 ± 0.0043.

Samples were processed with the FastDNA™ Kit as outlined in its manual with the modifications below. Aliquots of 100 µL of cells suspended in LIB (Appendix B) were transferred into a new tube and centrifuged. The supernatants were discarded and 100 µL of deionized water was added and the cells were re-suspended. This combination of cells and deionized water were then moved into Lysing Matrix A tube. Then 1.0 mL of CLS-TC Cell Lysis Solution was added to the Lysing Matrix A tube and was homogenized on a Krafttech™ (1/4 Sheet Palm Sander, PS160CA), because lack of FastPrep Instrument, for 40 seconds at 200 oscillations/second. Each tube was then centrifuged at 16,060 x g for 8 minutes to pellet debris and the supernatant was transferred to a 2.0 mL micro-centrifuge tube. Equal volume of Binding Matrix (1.0 mL) was then added to the tube and it was then incubated with gentle agitation for 5 minutes at room temperature on a rotator. After agitation the tube was centrifuged at 16,060 x g for 10s to pellet the Binding Matrix and then the supernatant was discarded. In the next step, 500 µL of prepared SEWS-M was added and the pellet was re-suspended gently using the force of the liquid from the pipet tip. This followed with centrifuging at 16,060 x g for 1 minute and discarding supernatant, and then centrifuging at 16,060 x g for 10s and removing residual liquid with a small pipet tip. The DNA was eluted by gently re-suspending Binding Matrix in 100 µl of DES and incubating for 5 minutes at 55°C in water bath. The tube was then centrifuged at 16,060 x g for 1 minute. Finally, eluted DNA was transferred to a clean tube and appropriate amounts of 1 x TE was added to give a final volume of 800 µL and then stored at -70°C. The addition of TE to get a final volume of 800 µL is done so that all samples have the same final volume.

DNA extraction protocol via SSB/FLB: an aliquot of 100 µL of each of the species' cells grown in LIB were added to a 15 mL tube containing 2 mL of SSB and incubated at room temperature for 1 week. After the cells sat in SSB for 1 week, the tube was then centrifuged at 16,060 x g for 15 minutes and the supernatant was discarded. The pellet was then left to air dry in the tube for 10 minutes, and then processed with FLB (method described in section 2.1.2). After being processed, TE was added to give a final volume of 800 µL and the sample was then stored at -70°C.

2.2 Quality Control Methods

2.2.1 Saliva Storage Buffer Contaminants Methodology

The long-term storage of saliva in 4 mL of SSB buffer in the 15 mL tubes could introduce contamination. To test whether this is an issue or not, 500 µL of SSB was evenly distributed across the plate via a sterile glass rod, on two types of Agar: YPD+AMP and MLT Max (both contents can be found in Appendix B). The aliquot of SSB came from the same lot used for patient samples and was incubated for more than 30 days. A total of 6 plates were tested, 3 of each type using 3 different tubes of SSB. The YPD + AMP plates were incubated at room temperature and the MLT Max plates were incubated in candle jars at 37ºC for 5 days. There was no growth on any of the plates.

2.2.2 DNA Extraction Contaminants Methodology

The process of extracting DNA from saliva that was stored in the SSB buffer for 30 days could contaminate the samples. To demonstrate that contamination was unlikely, 12 mock samples, leftover tubes of 4 mL of SSB that never received a saliva inoculum and held SSB from

more than 30 days, were processed with FLB using the same process mentioned in section 2.1.2. FLB samples were then tested at several dilutions in qPCR using the 3 universal primers and an inhibitor testing primer.

2.2.3 Air Contaminants Methodology

The universal primers are very sensitive and could potentially detect air contaminants introduced during the qPCR setup. Working in a lab where bacterial and fungal species are constantly being streaked for growth, there can be a lot of airborne species, especially fungal and mold. To test the possible air contaminants at my bench, 3 YPD +AMP Agar plates sat on with lids off on my lab bench for several days. Only two colonies grew; DNA amplified from these with FungalITS primers were sequenced to compare with targets derived from patient samples.

2.2.4 DNA Degradation Test

Each plate of samples endured multiple freeze-thaw cycles in order to complete different primer qPCRs. This cycling could degrade the DNA templates. To test whether the DNA was degraded, one of the master plates, XC1, was assayed with the same Bu10 primer after all of the other primers were completed. Therefore, the initial Bu10 run molecules/reaction is directly compared to the values obtained from a run at the end of the study.

2.3 Molecular Characterization Methods

2.3.1 Phylogenetic branch-inclusive qPCR Primers Construction

Bacterial and fungal compositions were initially analyzed by qPCR [43, 44 45], using, methods similar to those described by Lambert et al. for Phylogenetic branch-inclusive (PB) qPCR [44]. Phylogenetic branch-inclusive (PB) qPCR uses PB primers that target a specific

phylum or family or genus, making them far more inclusive than species-specific primers [44]. The collection was initially validated with target and non-target single species [43]. An aliquot of purified DNA was assayed by qPCR with 3 universal (2 bacterial and 1 fungal) and phylogenetic branch-inclusive (PB) primers and PCR conditions as described previously. Additional primers used were *Betaproteobacteria* ITS in anticipation of oral *Neisseria* and *Derxia*, *Streptococcus* ITS in anticipation of oral *Streptococcus*, *Bacteroidetes* ITS phylum primer in anticipation of *oral Capnocytophaga, Bacteroides, Flavobacteria* and *Prevotella, Actinobacteridae* 16s in anticipation of oral *Actinomyces* and *Luteococcus, Megashaera* 16s in anticipation of oral *Veillonella, Lactobacillaceae* 16s and ITS in anticipation of oral *Granulicatella,* and *Enterococcaceae* 16s in anticipation of oral *Gemella*. All primers with additional information about each primer can be found in Appendix B.

Whether contaminants in a given sample inhibited or reduced the efficiency of qPCR was determined by testing each with exogenous template and primers. We used an amplicon derived from *Deinococcus radiodurans*, chosen because it amplifies with broad spectrum primers but not with any PB primers, since it is a member of a distinct phyla (Deinococcus-Thermus). The species are not normally found in the human body or our saliva samples [46]. Inhibition of a DNA sample in qPCR was determined using primer DR3 (Appendix D), specific for the species *Deinococcus radiodurans*. In this inhibition testing qPCR, a fixed amount of amplicon (7.5 ng/ 1 µL), made by ampifying *D. radiodurans* genomic DNA with DR3, was added to the mastermix. This was distributed to all wells, except negative control wells. Standard wells received no sample, only the amplicon mastermix. Samples were then added to all experimental

wells. qPCR was performed with DR3. The distribution of Cq values of wells receiving both sample and amplicon were compared to those with amplicon only. Wells whose Cq values were more than 2 standard deviations from the average amplicon-only wells were considered to have some level of inhibition, and would require retesting after further purification or after diluting out contaminants causing the inhibition.

2.3.2 Biorad's CFX Program-Cq Call Methods

The Biorad's qPCR CFX program has three different methods for calling Cq values for each qPCR run: Regression, Single Threshold-Auto, and Single Threshold-Custom. To determine which method was the best for calling Cq values across multiple experiments, I calculated the average and standard deviation of the Cq values from each dilution of the spike amplicon, from 21 separate qPCR runs with Bu10, for each of the three methods. Then these dilution averages were plotted against the log of its molecules/µL, giving the new averaged standard slope per Cq call. The slope was then used to calculate the percent of efficiency for the qPCR, %E = (10^ (-1 / slope) -1) * 100.

2.3.3 Determining Molecules/µL of DNA Methods

The Biorad's CFX program allows the input of serially diluted standards (spike, usually dilutions 10^{-4} to 10^{-9} containing 7.10 x 10^4 to 2.10 x 10^6 molecules/µL for each qPCR run. The program then generates titers (molecules/reaction) for each sample individually derived from Cq values. The percent compositions of target microbial groups for each sample can then be calculated and compared between xerostomia and non-xerostomia groups using GraphPad

Prism® 6 statistical software. I did not convert molecules per sample to cells per sample, because the copy number of ribosomal RNA genes per genome varies from 1-15 depending on species [47].

The number of molecules/µL of the undiluted spike was calculated using by determining the values of the mass of the spike in ng/µL and the length of the spike in bp, and converting those values using the New England Biolabs Inc.'s dsDNA: Mass to Moles Convertor Calculator (http://nebiocalculator.neb.com/#!/dsdnaamt). Once the molecules/µL of the spike was found, it was easily converted into the correct dilutions used in qPCR (10^{-4}). The mass of the spike (ng/µL) was determined using gel electrophoresis and a 100 bp ladder (GoldBIO.COM 100 bp Plus TM DNA Ladder, CAT#D003-500). 10 µL of two-fold dilutions of amplicon was loaded into 2 % Agarose LE Gel (contents can be found in Appendix B) and electrophoresed in 1 x SB Buffer (contents in Appendix B) at 100 Volts for 45 minutes. The dilution of spike the intensity of a similar size band of the ladder was used to approximate its mass in ng/5µL of spike.

2.3.4 Oral Bacterial and Fungal Library Construction

I assembled a repository of 364 live colony purified cultures from saliva of both healthy and xerostomia volunteers, with matching DNA freezer stocks, listed in Appendix C. To start this process, several 10 µL aliquots from both healthy and xerostomia saliva flow test tubes less than 24 hours old, were streaked using the quadrant streaking technique on three types of Agar: Human Blood Trypticase Soy Agar (TSA), Rogosa Agar, and MesLib (MLT) Agar (all agar contents can be found in Appendix B). Plates were incubated anaerobically in 37°C for 48 hours. In the next step referred to as the "pie plates", single isolated colonies were picked from the first streaked plate onto a fresh agar plate of the same media, which is divided into several "pie

slices", one for each isolated colony, and picked colony is then re-streaked using the quadrant form and incubated anaerobically in 37°C for 48 hours. After cell growth, individual isolated cell colonies were picked for making "nickels"; nickel-sized circles were drawn on the bottom of the agar plates and each nickel is a different isolated colony. Rubbing the single isolated colony all over the circle, overlapping parts of the circle multiple times, results are in confluent growth. Plates were then incubated anaerobically in 37°C for 48 hours. Once confluent nickels were grown, FLB and Milk/Yeast Extract/Glycerol's (MYEG) (Appendix B) were made. First using a p10 pipette tip, a barely visible amount of the nickel is picked and put into 100 µL of FLBA and KOH (100:1) in a 96 well plate, to make the FLB sample for qPCR. Then the rest of the nickel was harvested and suspended in a 1.5 mL tube with 1 mL of MYEG mixture (Appendix B). The nickel in the MYEG mixture is then mixed using the pipette and 100 µL aliquot is taken out and put into the MYEG 96 well plate that matches the FLB plate. All nickels were photographed and colony morphologies were recorded. Once all the nickels were made into FLB and MYEG stocks, the MYEG plates and tubes were stored in a -70°C freezer. The FLB is processed as described in Section 2.1.2. The finished FLB product is then stored in the freezer until qPCR analysis. FLB's of saliva cell colonies were diluted into 50 µL of 1 x TE using a pin replicator (Scinomix, MO), which transfers ~0.5 to 1 µL, sterilized with a 5 min exposure to germicidal ultraviolet light at a distance of 21 cm. These dilution master plates were then tested using 3 broad-spectrum primers (both bacterial universal primers and the fungal universal primer). Samples from qPCR that had unique melting temperatures for combined bacterial universal

primers were prepped and sent out for sequencing (method described in next section, Section 2.3.5).

2.3.5 Sequencing Methods

Two to three samples representing unique melt profiles across several experiments for each primer were selected for sequencing. Samples were tested to see if they were positive and composed of single discrete bands by gel electrophoresis: 10 µL of each post qPCR sample with dye was loaded into individual wells of 2% Agarose LE Gel and ran in 1 x SB buffer at 100 Volts for 30 minutes. If samples showed no bands or multiple bands, they were not sent out for sequencing. Samples with single discrete bands were enzymatically "cleaned" by the Exo-Sap method [48]. Depending on band intensity, 3-5 µL of sample was adjusted to 5 µL with sterile deionized water and added to 5 µL of the Exo-Sap. This was made for an entire 96 well plate as follows: 2.4 µL of Shrimp Alkaline Phosphatase (SAP) (Tested User Friendly™, 1UN/µl, Lot: 114511), 24 µL Exonuclease 1 (Thermo Scientific, 20,000 U, Lot. 00132863), 48 µL of 10 x PCR Buffer minus Mg (GIBCO-BRL, Lot No. 1090571), 192 µL of 25 mM $MgCl_2$ (Promega, Madison, WI, USA, A351B), 216 µL of Reverse Osmosis Water (ROW). Samples were incubated at 37ºC for 15 minutes, and then heat-inactivated at 65ºC for 30 minutes. Primers were then added (25 pmoles in 5 µL TE, adjusted with 25 mM MgCl2 to counter the EDTA in the TE buffer. Samples were stored in a -70ºC freezer until they were shipped to GenScript USA Inc. for Sanger DNA sequencing [49] using "Big dye" chemistry [50, 51].

Sequences that were returned from GenScript were then matched using two online microbial sequence databases: RDP Seqmatch (https://rdp.cme.msu.edu/seqmatch) and the NCBI

BLAST® (http://blast.ncbi.nlm.nih.gov/Blast.cgi). For analysis of the 16S ribosomal RNA gene amplicons, zipped folders containing the fas and ab1 files were uploaded into the RDP pipeline for processing. Aligned sequences were seqmatched to the top 3 database entries (>1200, Quality: Good). For the NCBI BLAST® sequence analysis, the "Nucleotide Blast" program was used to find the species matches, testing several databases (nr), Whole-genome shotgun contigs (WGS). For primers Bu10, EntC, Lachno2, LbITS, StaphITSO, and StrepITSO, the WGS database was limited to *Firmicutes*. For primers Actino, BProITS, and OidesITS, the target was limited to *Actinobacteria*, *Proteobacteria*, and *Bacteroidetes*, respectively. Once results were given, samples matches with species that had a greater than or equal to 97% identity and query coverage were accounted for within each primer set. For the remaining primer, Mega, amplicons that were sent out for sequencing, the NCBI BLAST® was used for analysis of the sequenced species. The "Nucleotide Blast" program was used to find the species matches. Once directed to "Nucleotide Blast", the database was "nr", limited to Bacteria. Once results were given, samples matches with species that had a greater than or equal to 97% identity and query coverage were accounted for within each primer set.

The fungal ITS sequences were analyzed by aligning the reads using Mega 6 Muscle Alignment. This identified poor base calls particularly at the ends. Ends were trimmed to conservative shared calls; poorly aligning or short reads were discarded. Remaining sequences were Megablasted at the NCBI website against both nr and WGS databases to identify closest matches.

2.4 Study Setups & Experimental Plans

2.4.1 Volunteer Enrollment

Xerostomia and healthy volunteers will be identified and enrolled with the help of a participating dental office, and additional healthy volunteers will be self-enrolled by paper and Internet postings on Wayne State University Pipeline. Nightly oral hygiene practice volunteers were enrolled by paper and Internet postings on Wayne State University Pipeline. The enrollment lasted approximately 35-45 minutes. During enrollment, volunteers were asked to sign a consent form, fill out a dental health questionnaire (Appendix A) and perform a saliva pH test and two five-minute Saliva flow tests (unstimulated and stimulated). Because a focus of this project is xerostomia, we used stimulated and whole saliva (Saliva) as samples as our diagnostic of xerostomia. In the pH Saliva test, volunteers stuck an end of short-range pH paper (Hydrion Papers 4.5 to 7.5, Micro Essential Laboratory, N.Y.) in their mouth to get it moist and then compare the color to the pH color standards. To perform the unstimulated saliva flow test, volunteers were asked to try to not create any saliva flow and to drool into a 2.0 mL tube instead of swallowing for duration of 5 minutes. To perform the stimulated saliva flow test, volunteers were asked to chew on a 2” by 2” piece of sterile Parafilm® (Menasha, WI) and to saliva in a 15 mL tube instead of swallowing for a duration of 5 minutes. The unstimulated and stimulated saliva flow tests were both recorded in mL/5 minutes.

2.4.2 Xerostomia vs. Control Study Arm Sampling Method

Volunteers were given instructions and materials to collect three Saliva samples on three consecutive days at their homes, upon awakening and before eating, drinking, or brushing, to

minimize those variables. Saliva is collected in a 15 mL sterile tube with Saliva Storage Buffer (SSB), safe to participants. Saliva is stable to sit in buffer at room temperature for up to 30 days. The inclusion criteria for xerostomia participants were as follows: 18 years or older, participants must self-report subjective "dry-mouth" feeling over 3 months, and salivary flow test indicative of hyposalivation: stimulated whole saliva flow rate below 0.5 mL/min [20]. The inclusion criteria for healthy control volunteers were: participants must not have self-reported subjective "dry-mouth" feeling in the previous 3 months or any of the following diseases: Patients with the following diseases are excluded: Sjögren's Syndrome, or enrollment visit symptoms of xerostomia, head-and-neck radiation therapy, or trauma to salivary glands, and saliva flow tests must report values denoting no xerostomia: Salivary flow test indicative of non-hyposalivation (measurement of unstimulated whole saliva (UWS) above 0.12-0.16 mL/min and stimulated whole saliva flow rate above 0.5 mL/min[20]. The exclusion criteria for both groups of the study included the use of antibiotics 3 months prior to enrollment in the study.

2.4.3 Nightly Oral Hygiene Practices Arm Sampling Method

The exclusion criteria for volunteers of the study included the use of antibiotics 3 months prior to enrollment in the study. Volunteers were given instructions (listed below) and materials to collect 30 Saliva samples on at their homes. It is stressed that the participants do not eat, drink, or brush after the specific nightly oral hygiene procedure is performed until the morning sample is taken, upon awakening and before eating, drinking, or brushing, to minimize those variables. The nightly variations in oral hygiene practices instructions:

a. Days 1-5: no nightly mouthwash or brush, then morning 2 min brush with provided Crest Pro-Health toothpaste and brush after sample collection.

b. Days 6-10: nightly mouthwash with 15- 20 mL Listerine Total Care Mouthwash without brushing (for 60 seconds of vigorous swishing), then morning 2 min brush with provided Crest Pro-Health toothpaste and brush after sample collection.

c. Days 11-15: nightly mouthwash with 15- 20 mL Crest 3D White Luxe Mouthwash without brushing (for 60 seconds of vigorous swishing), then morning 2 min brush with provided Crest Pro-Health toothpaste and brush after sample collection.

d. Days 16-20: nightly toothpaste with a 1-inch strip of Crest 3D White Toothpaste and provided brush (brushing for 2 minutes), then morning 2 min brush with provided Crest Pro-Health toothpaste and brush after sample collection.

e. Days 21-25: nightly toothpaste with a 1-inch strip of Colgate Optic White Toothpaste and provided brush (brushing for 2 minutes), then morning 2 min brush with provided Crest Pro-Health toothpaste and brush after sample collection.

f. Days 26-30: no nightly mouthwash or brush, then morning 2 min brush with provided Crest Pro-Health toothpaste and brush after sample collection.

After the sample is taken, volunteers are asked to brush their teeth with the provided toothpaste and toothbrush. Saliva is collected in a 15 mL sterile tube with Saliva Storage Buffer (SSB), safe to participants. Saliva is stable to sit in buffer at room temperature for up to 30 days. If participants forget on any given night to perform the indicated rinse, non-rinse, or brush, they will simply skip sampling the next morning and resume the schedule the following night. They

will perform the full 5 days of sampling, even if that requires 6 or more days because of skipped days. If any product causes irritation, or any signs of oral infection or pain, participants will be instructed to stop using it, and to see their dentists.

2.4.4 Sample DNA Extraction Method

After the Saliva samples sit in the SSB for at least 30 days the DNA is extracted. To process the saliva samples and extract their bacterial and fungal DNA a Fungal Lysis Buffer (FLB) was used. The samples are centrifuged and supernatant discarded. Pellet is suspended in fungal lysis buffer A (FLBA) mixture with KOH, a high SDS/alkaline lysis-phenol extraction, and then incubated at 65°C for 2 hours and followed with centrifugation at 16,060 x g and collection of the supernatant. Addition of fungal lysis buffer B (FLBB) was not needed for neutralization (pH of approximately 7.8), because it was already neutral. The samples were stored at -20°C until assayed with qPCR.

CHAPTER 3: RESULTS

3.1 SSB/FLB for Saliva Storage/Lysis Buffer

3.1.1 91% IPOH/FQ Prevailed in Initial Storage/Lysis Combinations

The 14 different saliva storage buffers/DNA extraction combinations (listed in Table 2) were tested from a single volunteer's aliquot of saliva, to compare the stability and longevity of the saliva microbial DNA in each buffer by running a qPCR using a bacterial universal primer (Bu10), a primer to test for inhibition (DR3), and a branch-inclusive 16s primer specific for *Lactobacillus species* (LbITS). The goals for this experiment is to pick a combination or two that show high titers (amount of DNA) for this individual tested for multiple time periods and the sample must not be inhibited. Any samples that may show inhibition to samples or may be negative at day 30 should not be considered for the study.

The total bacterial titers for each of the combinations, using a 16s bacterial universal primer (Bu10), combined with the inhibitory primer data will help eliminate undesirable storage/DNA lysis buffers. Table 2 presents the total bacterial titer, inhibitory values, and *Lactobacillus* Cq values for the 14 combinations tested in the primary salivary storage/DNA extraction buffer test on a single aliquot of saliva from one individual. The best dilution of the combinations with the highest number of molecules/reaction was used compared for analysis. There were 5 storage/extraction combinations that did amplify day 30 samples with the universal primer: 91% IPOH/FLB, 95%EtOH/FLB, FLBA-KOH/FLB, FLBA+KOH/FLB, and RNAl/FQ. From these, only FLBA+KOH/FLB showed large inhibition with a dCq value of 8.83, this inhibition could have given a false negative. The combinations that had high bacterial titers and

low inhibition (<2.00) at day 30 are: 91%IPOH/FQ (7.49 and 0.25, respectively), 95%EtOH/FQ (7.05 and 1.23, respectively), RNAl/FLB (5.37 and 0.30, respectively), and Spit/FLB (7.92 and 1.97, respectively). Those combinations also gave desirable Cq values (<24) for *Lactobacillus*. Overall from the 14 saliva storage/DNA extraction buffers tested, 91%IPOH/FQ showed the lowest amount of inhibition, had a high return on bacterial molecules per reaction, and had a Cq value <24 for *Lactobacillus.*

Table 2. Total bacterial titers and inhibition values for day 30 of 14 storage/DNA lysis combinations tested on a single individual's saliva aliquot.

Storage/Lysis	Dilution of Sample	Bacterial 16s Universal Primer	Bacterial 16s Universal Primer	Lactobacillus 16s Primer	Inhibition Primer
		Molecules/ Reaction	Molecules/ mL Saliva	Cq Value	dCq Value
91%IPOH/FLB	10^{-1}	1.31E+01	3.9E+05	27.49	0.44
91%IPOH/FQ	undiluted	2.88E+07	4.6E+10	23.08	0.25
95%EtOH/FLB	10^{-1}	1.31E+01	3.9E+05	26.60	2.22
95%EtOH/FQ	undiluted	1.12E+07	1.8E+10	21.68	1.23
FLBA-KOH/FLB	10^{-1}	1.31E+01	3.9E+05	26.10	2.51
FLBA-KOH/FQ	undiluted	6.31E+05	1.0E+09	21.64	2.05
FLBA+EDTA/FLB	10^{-1}	7.19E+06	2.2E+11	19.86	2.25
FLBA+EDTA/FQ	undiluted	1.12E+06	1.8E+09	19.39	2.31
FLBA+KOH/FLB	10^{-1}	1.31E+01	3.9E+05	24.47	8.83
FLBA+KOH/FQ	undiluted	2.56E+06	4.1E+09	30.47	2.55
RNAl/FLB	10^{-1}	2.36E+05	7.1E+09	22.62	0.30
RNAl/FQ	undiluted	1.31E+01	2.1E+04	18.90	0.10
Spit/FLB	10^{-1}	8.34E+07	2.5E+12	22.81	1.97
Spit/FQ	undiluted	9.08E+06	1.5E+10	20.06	2.43

Values 1.31E+01 (molecules/reaction) and 3.9E+05 (molecules/mL saliva) were used for samples that were below detection level in qPCR. The full names of the storage/lysis combinations are as follows: 91% isopropyl alcohol with FLB lysis (91% IPOH/FLB), 91% isopropyl alcohol with FLB lysis followed by QG prep (91% IPOH/FQ), 95% ethanol with FLB lysis (95% EtOH/FLB), 95% ethanol with FLB lysis followed by QG prep (95% EtOH/FQ), 1 x Fungal Lysis Buffer A without potassium hydroxide with FLB lysis (FLBA-KOH/FLB), 1 x Fungal Lysis Buffer A without potassium hydroxide with FLB lysis followed by QG prep (FLBA-KOH/FQ), 1 x FLBA without KOH plus ethylenediaminetetraacetic acid with FLB lysis (FLBA+EDTA/FLB), 1 x FLBA without KOH plus ethylenediaminetetraacetic acid with FLB lysis followed by QG prep (FLBA+EDTA/FQ), 1 x Fungal Lysis Buffer A with potassium hydroxide with FLB lysis (FLBA+KOH/FLB), 1 x Fungal Lysis Buffer A with potassium hydroxide with FLB lysis followed by QG prep (FLBA+KOH/FQ)Qiagen RNAlater RNA stabilization reagent with FLB lysis (RNAl/FLB), Qiagen RNAlater RNA stabilization reagent with FLB lysis followed by QG prep(RNAl/FQ), Saliva with no buffer with FLB lysis (saliva/FLB), and Saliva with no buffer with FLB lysis followed by QG prep (saliva/FQ).

3.1.2 Saliva Storage Buffer is Comparable to IPOH

The buffers 91%IPOH, Saliva Storage Buffer (SSB), and Qiagen's buffer QG (QG), combined with DNA extraction methods, were assayed amongst 4 individuals' saliva samples by using qPCR with a universal primer, inhibition testing primer, and a *Lachnospiraceae* species primer. The goals for this experiment is to pick a combination or two that show high titers (amount of DNA) for this individual tested for multiple time periods and the sample must not be inhibited. The universal bacterial primer, Bu10, QPCR results from day 30 samples (Figure 1) suggests that SSB combined with FLB is the best method, giving high average log titer values and the smallest standard deviation from the 4 individuals 9.60 ± 0.59, respectively. The next best buffer/lysis combination is 91% IPOH/FLB with average log titer values of 9.70 ± 0.73. SSB/FLB has a lower standard deviation than the 91% IPOH/FLB combination.

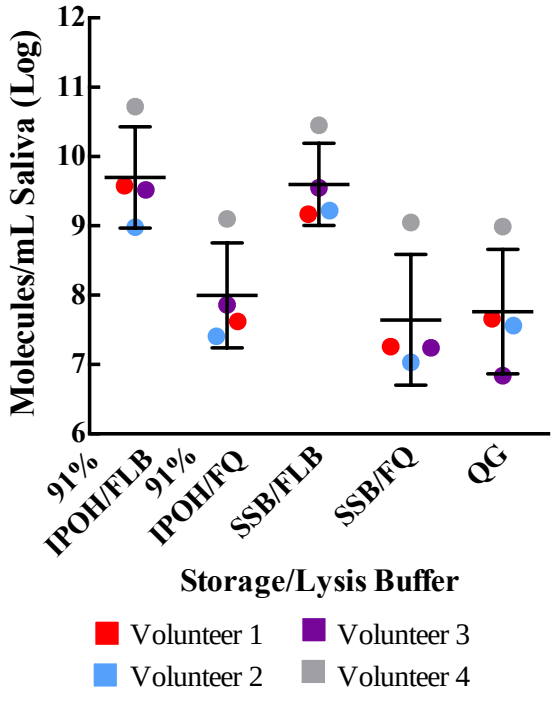

Figure 1. Bacterial titers of possible storage/lysis buffers from day 30 samples. qPCR was ran using a 16s universal bacterial primer, Bu10. An aliquot of Saliva from 4 different subjects sat in buffer for 30 days before prepped for lysis.

The day 30 buffer/lysis saliva samples were checked for inhibition by QPCR with primer DR3 (Section 2.3.1; Appendix D). A table with the dCq values and average dCq values of each buffer across individuals is located in Table 4. The total average dCq across all day 30 samples is 0.82 ± 0.52. All of the buffer/lysis preps, except QG, have average dCq values <1.0, indicating that they were free of PCR inhibitors. A 1-way ANOVA Friedman test gave a P value of 0.0124 and the Dunn's multiple comparisons test showed that QG was significantly different than the standard.

Table 3. Levels of inhibition of qPCR of saliva DNA among five types of storage and extraction buffers.

	IPOH/FLB	IPOH/FQ	SSB/FLB	SSB/FQ	QG
Volunteer 1	0.6	0.3	0.7	0.9	1.2
Volunteer 2	0.6	0.3	0.4	0.7	2.3
Volunteer 3	0.8	0	0.4	0.7	0.7
Volunteer 4	0	0.3	1.1	0.8	1.1
Average	0.50 ± 0.35	0.23 ± 0.15	0.65 ± 0.33	0.78 ± 0.10	1.33 ± 0.68

An aliquot of Saliva from 4 different subjects sat in buffer for 30 days before prepped for lysis. All samples are a 10^{-1} dilution.

3.1.3 Both SSB & IPOH Prevent New Cell Growth

The two best saliva storage buffers (SSB and IPOH) and a control buffer, sterile saline buffer (PBS), were tested for new cell growth (via colony forming-unit [CFU]) and by qPCR analysis with bacterial universal primers for 8 time periods across 28 days. The percentage of cells alive, Table 4, was calculated by counting the number of colonies in the quadrant that had approximately 100 cells, times the dilution factor, times 100 to bring the 10 by amount of µL plated to get the cells per mL still alive. Then the number of cells per mL still alive was divided by the initial value of cells per mL alive and then multiplied by 100 to bring the 10 µL plated to

Table 4. The percentage of cells alive in the potential saliva storage buffers across 28 days.

	Subject #1			Subject #2		
Time in buffer (days)	SSB	IPOH	PBS	SSB	IPOH	PBS
0	100	0	100	100	0	100
0.04	8	0	104	54	0	111
1	79	0	642	30	0	147
5	2	0	123	7	0	350
9	2	0	172	3	0	361
13	0.2	0	97	1	0	TNTC
21	0	0	5218	0	0	TNTC
28	0	0	TNTC	0	0	TNTC

Percentages were based off of the initial time (0) and were calculated using the colony forming-units.

1 mL. IPOH immediately kills all cells and prevents growth from happening. In SSB cells did not die immediately, but in fact slowly die off. The cells that remained alive in SSB after several days were processed, amplified with qPCR using the bacterial universal primer (16s), sequenced, revealing that they were *Streptococcus salivarius* and *Staphylococcus aureus subsp. aureus.*

Comparing the melt peaks from qPCR for each of the time points, listed previously, is a good indication on whether the dominant species are changing over time. Both of the bacterial universal primers were used. The 16s bacterial universal primer showed promising results for both buffers tested. SSB, Figure 2 (A & B), had little to no variation in melt temperatures across the days for both subjects. IPOH, Figure 2 (C & D), also had no variation between melt peaks for both subjects tested.

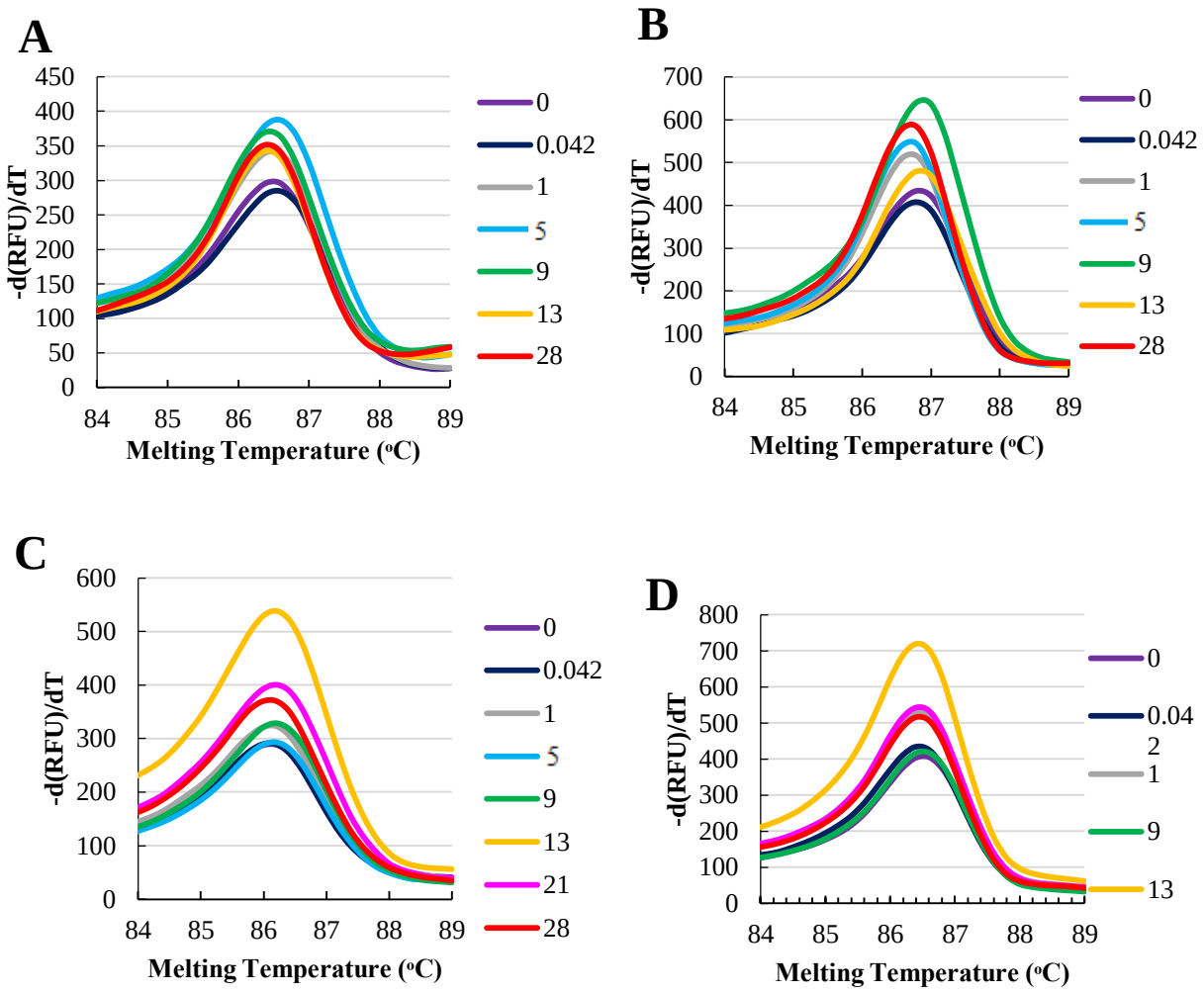

Figure 2. Bu10 melt curves for cells lysed from SSB and IPOH across 28 days. SSB curves for Subject 1 and Subject 2 are A and B, respectively. IPOH curves for Subject 1 and Subject 2 are C and D, respectively. Bacterial universal primer Bu10 was used in this qPCR. Each melt curve on the graph represents the aliquot taken and lysed at the specific time period, the number corresponds to the time in days. The sample dilution used in this qPCR reaction for SSB was 10^{-1} dilution and pin replication dilution for IPOH. Day 21 samples for both subjects from SSB and day 5 samples from Subject 2 from IPOH were excluded from analysis due to contamination in its original sample.

The melt curve results from the BuITS bacterial universal primer run determined which storage buffer was going to be used in the study. Trial storage buffer SSB, shown in Figure 3, gave very consistent melt peaks with Subject 1 (Figure 3A), providing evidence that there were no changes in the dominant species across this samples time in the buffer. Subject 2, Figure 3B, may look deceiving, however, all of the samples have two distinct matching melt peaks, showing relative consistency. The average Cq values for each of the subjects are 21.7 ± 1.18 and 19.9 ± 1.00, respectively. These values are below 24, indicating that there were high titers of bacteria in these samples. Unfortunately, IPOH melt peaks data was rejected because they failed for an unknown reason; the DNA from the preps were previously shown to be good with the Bu10 primer.

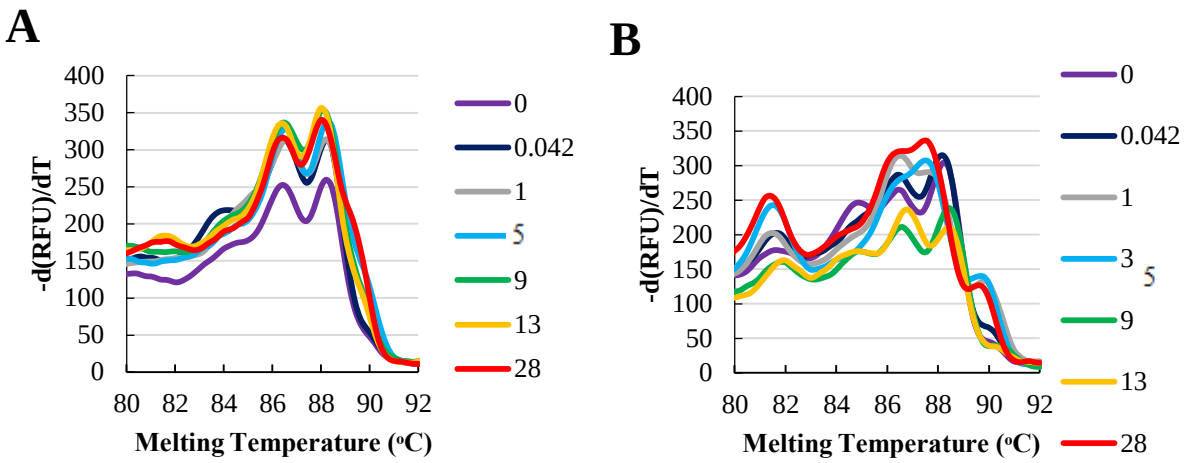

Figure 3. BuITS melt curves for cells lysed from SSB across 28 days for Subject 1 (A) and Subject 2 (B). Bacterial universal primer BuITS was used in this qPCR. Each melt curve on the graph represents the aliquot taken and lysed at the specific time period, the number corresponds to the time in days. The sample dilution used in this qPCR reaction for SSB was 10^{-1} dilution and pin replication dilution for IPOH. Day 21 samples for both subjects from SSB were excluded from analysis due to contamination in its original sample. Curves from cells stored in IPOH were rejected on the basis of qPCR quality, not a reflection of the storage.

3.1.4 Species DNA Extraction more efficient with SSB/FLB than commercial FastDNA™ Kit

SSB/FLB and FastDNA™ KIT extraction method extraction efficiency's across 10 different species were compared using a spectrophotometer, and universal primers (Bu10 and BuITS) and an inhibitory primer (DR3) in QPCR. The OD readings of the samples in the spectrophotometer, shown in Table 6, were used to verify that the samples had approximately the same number of cells. The OD readings combined had an average of 0.079 ± 0.0043, and only 3 samples (*E.coli*, *L. crispatus*, and *L. gasseri*) were outside the standard deviation (0.075 - 0.083).

The samples were also tested for inhibition to make sure that this was not a problem in the analysis. There was little to no inhibition in the species samples what were stored and extracted with SSB/FLB. All were well below 0.15 except *Lactobacillus jensenii*, which is outside two standard deviations (slightly inhibited). *Lactobacillus jensenii* is also outside two standard deviation for FastDNA™ samples (slightly inhibited). The bacterial universal primer also confirms that the *Lactobacillus jensenii* did show this slight inhibition of molecules.

Table 5. Information about the 10 species compared in the extraction efficiency using SSB/FLB and FastDNA™ Kit.

Species	Strain	Stock Origin	OD Read	FastDNA Molec/ mL	SSB/FLB Molec/ mL	SSB/FLB: FastDNA
Escherichia coli	XL1-Blue*	frozen cells	0.087	6.0E+08	6.8E+09	11
Enterococcus faecalis	RK22***	freshly grown agar cells	0.079	3.4E+07	1.1E+09	32
Lactobacillus crispatus	HM-370**	freshly grown broth cells	0.074	1.8E+08	1.9E+09	11
Lactobacillus gasseri	HM-642**	freshly grown broth cells	0.084	1.8E+07	4.9E+09	272
Lactobacillus jensenii	HM-646**	freshly grown broth cells	0.078	5.6E+08	2.0E+09	4
Streptococcus agalactiae	31***	frozen cells	0.075	6.0E+06	1.9E+08	32
Streptococcus cristatus	HM-163**	frozen cells	0.075	1.3E+06	2.2E+08	169
Streptococcus downei	HM-475**	frozen cells	0.079	1.3E+06	1.3E+07	10
Streptococcus mitis	HM-262**	frozen cells	0.076	6.0E+06	1.6E+09	267
Streptococcus vestibularis	HM-561**	frozen cells	0.082	1.7E+04	1.7E+08	10000

*Agilent Technologies, Inc (CA)
**BEI Resources, NIAID, NIH as part of the Human Microbiome Project (VA)
***Lab strain, identified by 16s Sequencing
Molecules/mL were calculated from the qPCR with the 16s bacterial universal primer Bu10 and dCq values were from qPCR with the inhibitor primer. 0.5 µL of undiluted FastDNA and 10^{-1} diluted SSB/FLB samples were used for both primers.

The qPCR using the universal primer Bu10 comparing the two methods, shown in Table 6 and presented in Figure 4, gave some interesting results. The SSB/FLB method was more efficient at extracting amplifiable DNA than the FastDNA™ Kit. On average the SSB/FLB method extracted 8.8 ± 0.8 molecules/mL sample (log) for the 10 species test, whereas FastDNA™ only extracted 7.1 ± 1.4 molecules/mL sample (log). The FastDNA™ Kit results support the theory that some commercial DNA extraction methods do not extract *Streptococcus* species well, and in this case the small titer of *S. vestibularis* extracted was not recognizable by qPCR. FastDNA™ is appropriate for extracting species other than *Streptococcus*, but SSB/FLB is a better solution across all species tested and has 10-100 times more molecules of DNA from most of the species tested.

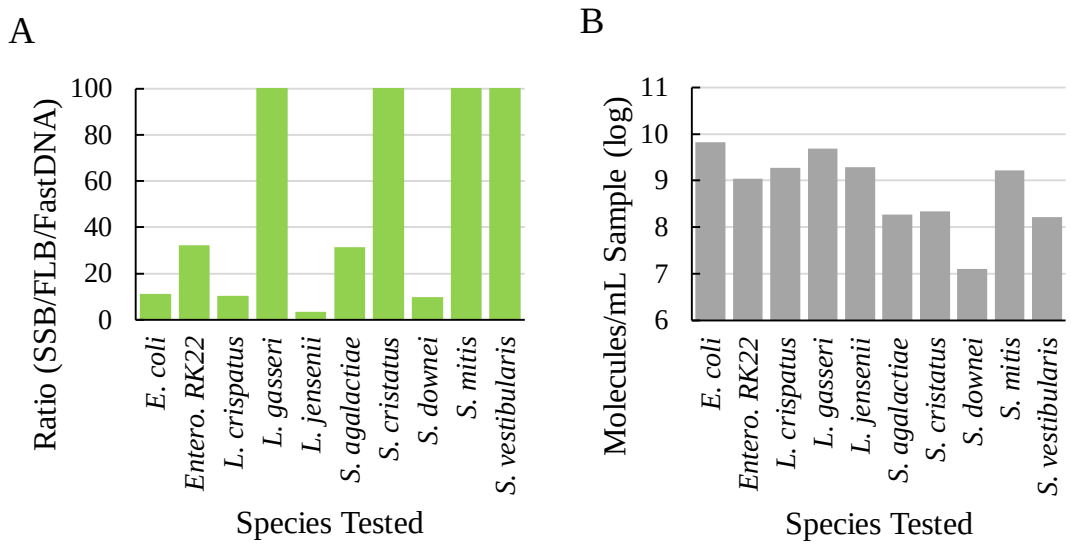

Figure 4. Comparison of FastDNA™ Kit to SSB/FLB across 10 species. (A) Ratio of SSB/FLB to Fast DNA extraction methods. Data presented is from 16s bacterial universal primer Bu10 qPCR using undiluted FastDNA™ and 10-1 diluted SSB/FLB samples. The SSB/FLB samples molecules/reaction was converted to undiluted samples for comparison against FastDNA™. (B) Log of molecules/reaction for species extracted using SSB/FLB. The values used in this figure are the converted undiluted SSB/FLB Bu10 data.

3.1.5 Bu10 and BuITS Combined Give Unique Species Melt Temperatures

The bacterial universal primers melting temperatures can be used to decipher between various bacterial species. Figure 5 is a digital representation of the melting temperatures of both universal bacterial primers. Together they can help us distinguish between known species. There is not enough variation between the Bu10 single melt temperatures. However, combined with the multiple temperatures given from the BuITS primer it gives a unique fingerprint that reflects dominant species in the sample.

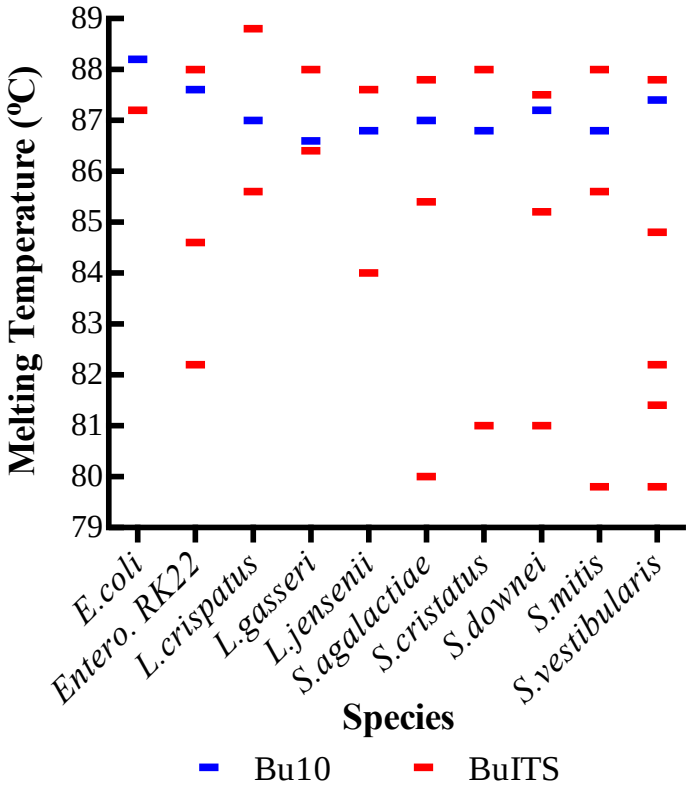

Figure 5. Composite melting temperatures using Bu10 and BuITS bacterial universal primers. Species were processed by the SSB/FLB method. Bu10 is blue, and BuITS is red.

Using the known sequenced or BEI species' melting temperatures, whether it is universal or one of the branch inclusive primers, to help ID prevalent species in the saliva samples is one of the key analysis methods. However, only the SSB/FLB identified species melting temperatures can be used when identifying SSB/FLB saliva samples. This is because the melting temperature is different between SSB/FLB and FLB only by an average standard deviation of ± 0.72 °C, seen Figure 6 (below). The FLB only cells were processed directly from frozen BEI (BEI Resources, NIAID, NIH as part of the Human Microbiome Project [VA]) stocks into FLB. The SSB/FLB samples were stored in SSB from frozen BEI stocks then processed with FLB (detailed process was already mentioned). This large shift causes concern that species will not be identified correctly, if any FLB only samples were used for standards. Therefore, only sequenced or known samples that were processed with SSB/FLB will be used to identify the unknown melt temperatures.

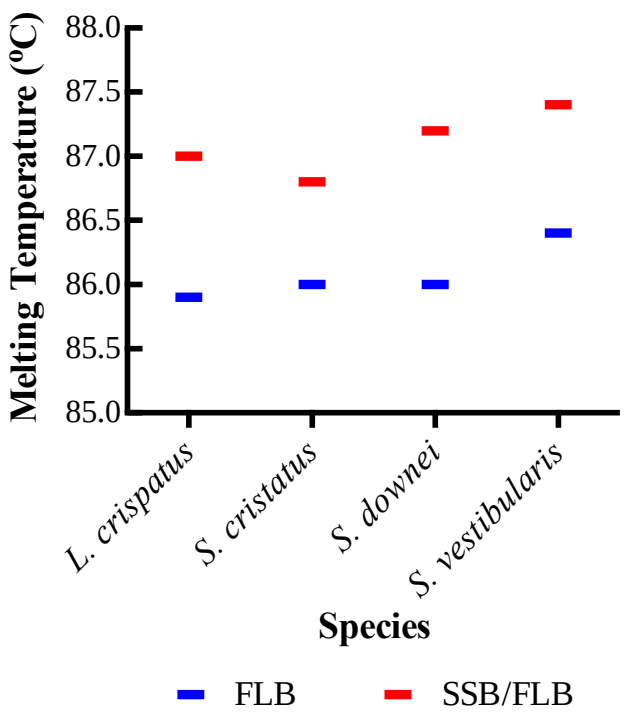

Figure 6. Changes in melting temperatures of species that were processed with FLB versus SSB/FLB. Melting temperatures are from a bacterial universal Bu10 primer.

3.2 Contamination control results

3.2.1 No Saliva Storage Buffer Contaminants

The distribution of 4 mL of SSB buffer into the 15 mL tubes for morning saliva samples could induce contamination to the samples if not done properly. A total of 6 plates were tested, 3 of each type using 3 different tubes of SSB incubated for more than 30 days, from the same lot as used for study patients. There was no growth on any of the plates.

3.2.2 No DNA Extraction Contaminants

The process of extracting DNA from saliva that was stored in the SSB buffer for 30 days could have induced contamination to the samples. All samples showed no inhibition of a spiked template, with an average of 0.09 ± 0.07 dCq relative to template alone. Two samples had

positive melt peaks at 87°C with the bacterial 16s universal primer, however these samples had very low titers of 19.3 and 14.7 molecules/reaction similar to those of the negative wells in the qPCR. Also the duplicates of these samples did not show melt peaks and were also very low in titer (1.50 and 4.37 molecules per reaction). Therefore, no high titer contaminants were present to alter the titers of saliva samples.

3.2.3 Puccinia striiformis f. sp. tritici is an Air Contaminant

The universal primers are very sensitive and can amplify possible air contaminants. Working in a lab where bacterial and fungal species are constantly being streaked for growth, there can be a lot of airborne species, especially fungal and mold. Two colonies grew. FLB's of the colonies were made and tested with the fungal universal primer and were prepped to be sent out for sequencing. The FLB's of the two colonies had two identical melt peaks with averages and standard deviations of 89.09 °C ± 0.07 °C and 85.36 °C ± 0.07 °C. Sequences came back as *Puccinia striiformis f. sp. tritici* and none of the saliva samples tested have these melt temperatures. Therefore it is unlikely that species detected in samples derive from the environment in which the samples were processed.

3.2.4 DNA prepared by SSB/FLB DNA was not degraded during the study interval

Each plate of samples did endure multiple freeze thaws in order to complete different primer qPCR's. The concern is that these multiple freeze thaws would cause DNA template degradation. I compared results from 108 samples assayed at the beginning of the study with the same samples re-assayed 84 days later at the end of the study, after approximately 13 freeze-thaws (Figure 7). A Wilcoxon matched-pairs signed rank test had a P Value of 0.4298 and is

considered not significantly different. Therefore, there was no significant DNA template/sample degradation.

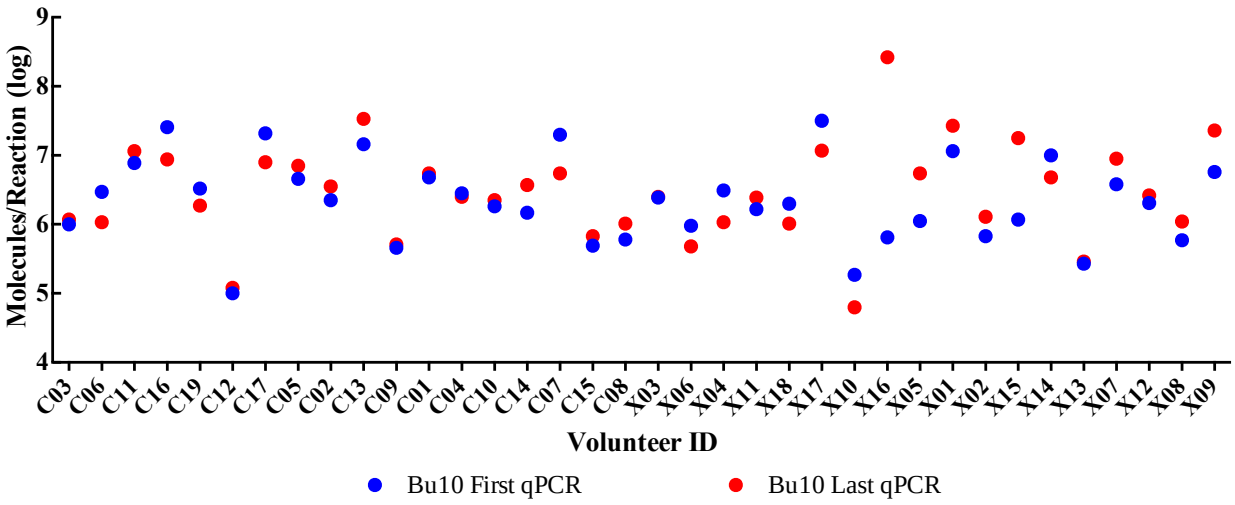

Figure 7. Variation in molecules/reaction of first Bu10 qPCR run vs. last Bu10 qPCR run for XC Study Arm. Molecules/reaction are the 16s bacterial universal Bu10 primer.

3.3 Molecular Characterization

3.3.1 qPCR Primers Biorad Cq Call Based on Highest %E

All three methods of calculating Cq values showed impressively low average standard deviations among all serial dilutions (Figure 8). Therefore it is important to compare the percent efficiencies to choose the best call. In this case with the Bu10 primer the Regression Cq call is shown to be the highest, with an efficiency of 94.1%. Single Threshold-Custom and Single Threshold-Auto follow with 82.8% and 82.7% respectively. For all other primers, the percent efficiencies given by the CFX will be compared for each Cq call type for several runs and the

highest % efficiencies will be used. Again, all of the primers used in this study can be found in Appendix D, along with additional information regarding primer make-up and qPCR. The qPCR run-to-run variation for each primer can be seen in the average and standard deviations of the percent effiencies, Appendix D.

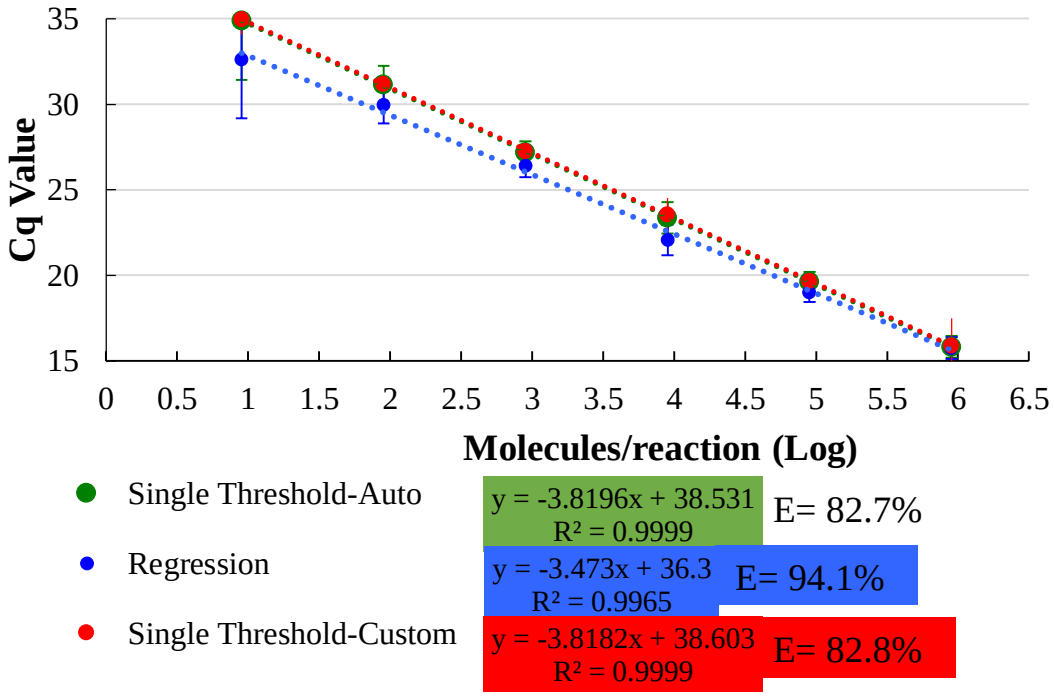

Figure 8. The average standard slopes for each of the three methods of determining Cq values in the CFX Biorad program. The Bu10 primer standard was compiled from 34 runs and graph equations and percent efficiencies were calculated using excel; no outliers were excluded.

3.3.2 Oral Bactria and Fungal Library Composition

All of the information on the 364 saliva cells species that were grown for the oral lab cell library can be found in Appendix C. A total of 11 volunteers' saliva was used to create this library and 30.6% (110/364) of the oral cell library has been identified through sequencing. The

library, chosen from different combined Bu10 and BuITS representative melting temperatures, consists of 3 major phyla: *Firmicute, Actinobacteria,* and *Proteobacteria*. Figure 9 contains the break-down of the contents of the oral library based on the sequencing results. 70% of the species come from the families: *Lactobacillaeae, Actinobacteridae,* and *Streptococcus*. The oral cell library consists of 230, or 63.2%, xerostomia saliva cells and 134, or 36.8%, healthy saliva cells. From the identified saliva cultures, 51.8% were from xerostomia patients.

Species identified only found in these volunteers were (prevalence in parenthesis): *Lactobacillus fermentum* (3), *Rothia mucilaginosa* (4), *Staphylococcus epidermidis* (3), and *Streptococcus salivarius* (5). This data will be combined with all sequencing data and further analyzed for differences among the groups in the next section, Section 3.3.3.

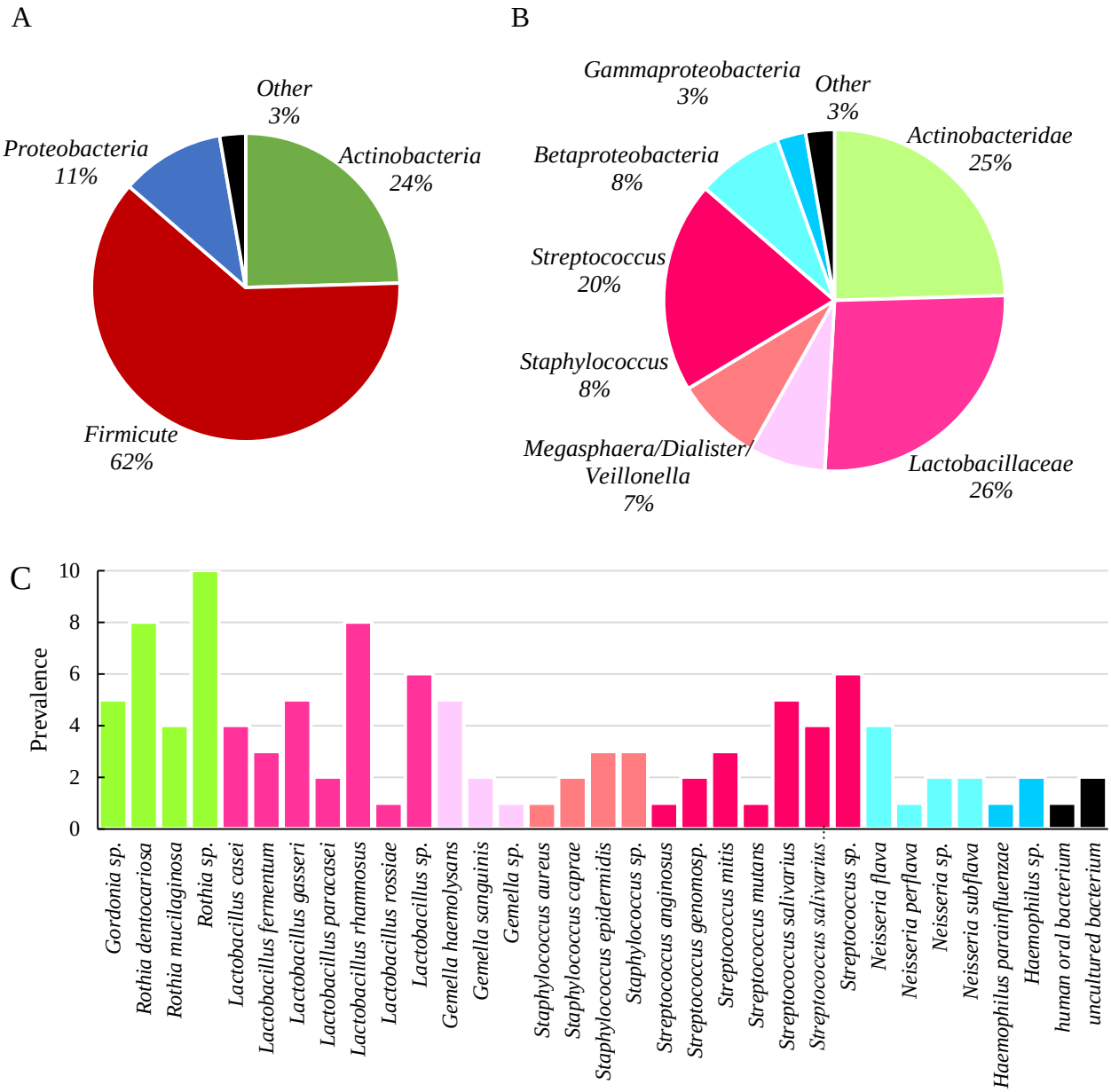

Figure 9. Oral cultured library composition based on sequencing. (A) Phyla distribution of oral cell library. (B) Family distribution of oral cell library. (C) Species of oral cell library with prevalence, within 97 % identity over 97% read length. The different shades of colors in the figures represent the phyla of microbes: green (*Actinobacteria*), red (*Firmicutes*), blue (*Proteobacteria*), and black (other). All cells' DNA sequenced were from 16s bacterial universal primer qPCR.

3.3.3 Composition of Sequenced qPCR Samples

A total of 401 DNAs amplified with a variety of primers (Table 6) were sequenced. Only 244 (60.8%) of the amplicons (from 12 primers) that returned readable sequences had species matches with 95% coverage and identity. Any primers that did not return with sequences matched with at least 95% coverage and identity and/or a majority of the amplified samples matched species outside its target were rejected. Therefore, two primers were rejected, ChlamITS (*Chlamydia*) and MycoUreaITS (*Mycoplasmatales*). All information on the amplicons sequenced, including DNA sequence and melting temperatures, can be found in Appendix E. 157 (39.2%) were mixed template reads, which could be a result from codominant species. Overall, 49.2% of the sequenced amplicons were from the study salvia samples, 46.6% were from the Oral Cell Library (Section 3.3.2), and the remaining 4.2% of the matched sequences came from other previous experiments from Chapter 2, such as the air contamination experiment. The saliva samples that matched species sequences came from 14 xerostomia volunteers, 15 control volunteers, and 8 nightly routine volunteers.

A total of 68 species were identified across all the matched sequences. The distribution of these species from each target primer is presented in Table 7 A & B. Only the *Staphylococcus* primer amplified a sequence outside of its target branch; it had two reads with 99% coverage and 97-99% identities for *Streptococcus pneumonia*, likely resulting from the close relationship of the two targets and the dominant titers of *Streptococcus*. The melting temperatures for each species matched, listed in Appendix E, suggests what the dominant species of each sample is in the study. However, it is important to note that the melting temperature for each species is primer

specific. Further analysis on which species are more dominant in each saliva sample will be done in Sections 3.4 and 3.5.

Table 6. Breakdown of sequenced matches

Primer Target	Primer	Primer Name	Total Samples	Total Target	Total Non-target	Mixed template reads*	Percent Target
Actinobacteridae	ActinoR3	Actin-R	16	11	0	5	100
Betaproteobacteria	BProt16SITS	BPro-F	3	0	0	3	100
Bacterial Broad-spectrum	Bu4L Bu4LC Bu4L2 Bu4L3	Bact-F	157	129	0	28	100
Enterococcaceae	EntcR	Ent-R	2	1	0	1	100
Fungal Broad-spectrum	RT2	Fung-R	74	49	0	25	100
Fusobacterium	Fuso-R6	Fuso-R	8	7	0	1	100
Lachnospiraceae uc & incertae sedis, Clostridium IVa, Roseburia	Lachno-R3	Lach-R	19	6	0	13	100
Lactobacillaceae	LB16SITSfvr LB16SITSvsp LB16SITSj LB16SITSsc	Lacto-F	44	13	0	31	100
Megasphaera/Dialister/ Veillonella	MegaR869	Mega-R	4	4	0	0	100
Bacteroidaceae/ uc Prevotellaceae	OidiesITSFa OidiesITSFb OidiesITSFc	Oides-F	16	9	0	7	100
Staphylococcus	Staph16sITS	Staph-R	44	14	2	30	88
Streptococcus	Strep16SITSL	Strep-R	12	1	0	11	100

Table 7 A. Sequence Read Summary												
A	Target Primer Name											
Bacterial species detected	Bact-F	Actin-R	Ent-R	Fuso-R	Lach-R	Lacto-F	Mega-R	Oides-F	Staph-R	Strep-R	Fung-R	Total
Actinomyces graevenitzii		2										2
Actinomyces odontolyticus		1										1
Actinomyces viscosus		3										3
Atopobium sp.		2										2
Rothia dentocariosa	8	2										10
Rothia mucilaginosa	4	1										5
Rothia sp.	10											10
Gordonia sp.	5											5
Gemella haemolysans	5		1									6
Gemella sanguinis	3											3
Gemella sp.	1											1
Fusobacterium nucleatum subsp.				1								1
Fusobacterium periodonticum				6								6
Oribacterium sinus					3							3
Stomatobaculum longum					3							3
Abiotrophia defectiva						10						10
Lactobacillus casei	4											4
Lactobacillus fermentum	3					2						5
Lactobacillus gasseri	5											5
Lactobacillus paracasei	2											2
Lactobacillus rhamnosus	8											8
Lactobacillus rossiae	1											1
Lactobacillus sp.	6											6
Veillonella dispar							4					4
Prevotella melaninogenica								7				7
Prevotella veroralis								2				2
Staphylococcus aureus	1								3			4
Staphylococcus epidermidis	3								9			12
Staphylococcus aureus subsp. Aureus	5											5
Staphylococcus caprae	2											2
Staphylococcus sp.	3											3
Neisseria flava	4											4
Neisseria perflava	1											1
Neisseria sp.	2											2
Neisseria subflava	2											2

Table 7 B. Sequence Read Summary												
B	Target											
Bacterial species detected	Bact-F	Actin-R	Ent-R	Fuso-R	Lach-R	Lacto-F	Mega-R	Oides-F	Staph-R	Strep-R	Fung-R	Total
Streptococcus pneumoniae									2			2
Streptococcus anginosus	1											1
Streptococcus australis	2											2
Streptococcus genomosp.	2											2
Streptococcus mitis	5											5
Streptococcus mutans	1											1
Streptococcus salivarius	13									1		14
Streptococcus salivarius subsp. Null	4											4
Streptococcus sp.	6											6
Streptococcus thermophilus	1											1
Haemophilus parainfluenzae	1											1
Haemophilus sp.	2											2
human oral bacterium	1											1
uncultured bacterium	2											2
Fungal species detected												
Aureobasidium proteae											1	1
Aureobasidium pullulans											1	1
Candida albicans											17	17
Candida dubliniensis											2	2
Candida glabrata											1	1
Candida parapsilosis											2	2
Candida tropicalis											1	1
Cladosporium perangustum											1	1
Cryptococcus sp.											1	1
Malassezia restricta											4	4
Metarhizium brunneum											1	1
Puccinia striiformis f. sp. Tritici											3	3
Rhodotorula mucilaginosa											1	1
Saccharomyces cerevisiae											1	1
Uncultured basidiomycete ITS region											4	4
Uncultured compost fungus 18S rRNA gene											1	1
Uncultured eukaryote clone											1	1
Uncultured fungus clone											6	6
Uncultured Glomus clone											1	1
Total	129	11	1	7	6	12	4	9	14	1	50	244

3.4 Xerostomia vs. Control Study Arm

3.4.1 Volunteer Enrollment

Each volunteer enrolled filled out a dental health questionnaire, the blank questionnaire can be found in Appendix A, each enrolled volunteers answers are presented in Appendix F. A total of 36 volunteers were enrolled in this study, 18 xerostomia and 18 healthy controls. A detailed characterization of the xerostomia and control volunteers is found in Table 8.

Table 8. Characterization of xerostomia and control volunteers

		Xerostomia	Control
Total	Enrolled	18	18
	Male	4	10
	Female	14	8
	White	16	16
	Black	1	1
	Asian	1	1
	Diagnosed xerostomia	4	0
	Symptomatic xerostomia	14	0
	Periodontal or Gingivitis	3	2
	Wear Dentures	2	3
	Cosmetic Dentistry	4	1
Average	Age (years)	54.3 ± 22.9	55.7 ± 23.8
	Cavities (#)	10.3 ± 8.0	5.8 ± 5.5
	Root canals (#)	0.9 ± 1.3	0.8 ± 0.9
	Crowns (#)	2.7 ± 2.8	1.4 ± 2.4
	pH	5.6 ± 0.5	5.9 ± 0.4
	unstimulated (mL)	0.6 ± 0.4	1.4 ± 0.4
	stimulated (mL)	3.4 ± 1.7	6.4 ± 2.5
	flow sum (mL)	4.0 ± 1.9	7.9 ± 2.5

Comparing the volunteer's saliva tests, in the groups that they enrolled as (control or xerostomia), can be seen in Figure 10. There was no significant difference between controls or xerostomias' saliva pH, due to definite overlaps in these groups. However, even though there are several overlaps also seen between the groups in their unstimulated and stimulated saliva flow tests, both of their group means were significantly different. Mann Whitney t-

tests gave a P value of <0.0001 for unstimulated and a P value 0.0002 for stimulated flow rates. By combining the unstimulated value with the stimulated value the overlap between the two groups was minimized but not eliminated, and their new means were significantly different with a P value <0.0001 from a Mann Whitney t-test. However, because of the overlap between these groups, one cannot use this test to solely determine whether they are xerostomic or not.

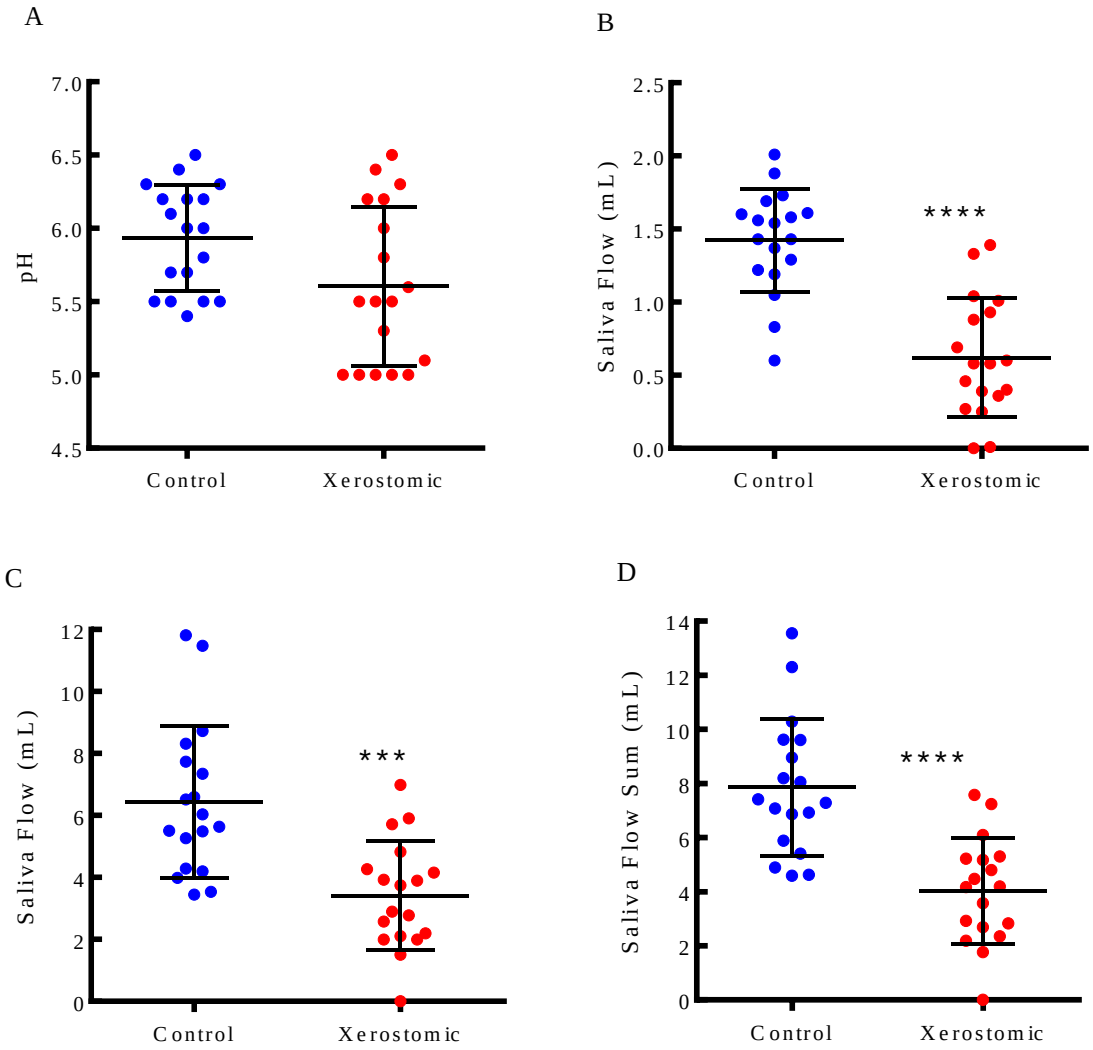

Figure 10. Saliva tests' results. (A) pH of enrolled control vs. xerostomia volunteers. (B) Unstimulated saliva flow (mL) of enrolled control vs. xerostomia volunteers. (C) Stimulated saliva flow (mL) of enrolled control vs. xerostomia volunteers. (D) Saliva flow sum of enrolled control vs. xerostomia volunteers.

3.4.2 Xerostomia patients' saliva samples do not have higher bacterial loads than controls

The total bacterial titers (16s) for each volunteer varied up to 100 fold from one day to the next, although the 3 sample limit did not allow a determination of whether these differences were significant (1-way ANOVA Dunn's multiple comparisons test, P value >0.9). The variations in the total fungi titers also failed the ANOVA test for significance. However, a 1-way ANOVA test on the volunteer's enrolled in the xerostomia vs. control study gave a P value of <0.0001, indicating that the means vary significantly between individuals. Figure 11 A. presents the total bacterial titers (16s) in log form for each individual's three samples given. These titers are at least partially confounded by variations in spit volumes, which ranged from 0.25 mL to 3 mL.

Individual differences in melting temperatures (dominant species) were found when comparing samples from each individual sample, (Figure 11 B). This reflects daily changes in which species are dominant. There is less deviation from day to day within individuals (average standard deviation = 0.10 °C) than the deviation among all samples (standard deviation = 0.23 °C). These data also indicate that healthy oral samples almost always have several co-dominant species, each contributing to the Tm values. Diagrams for each primer are found in Appendix F.

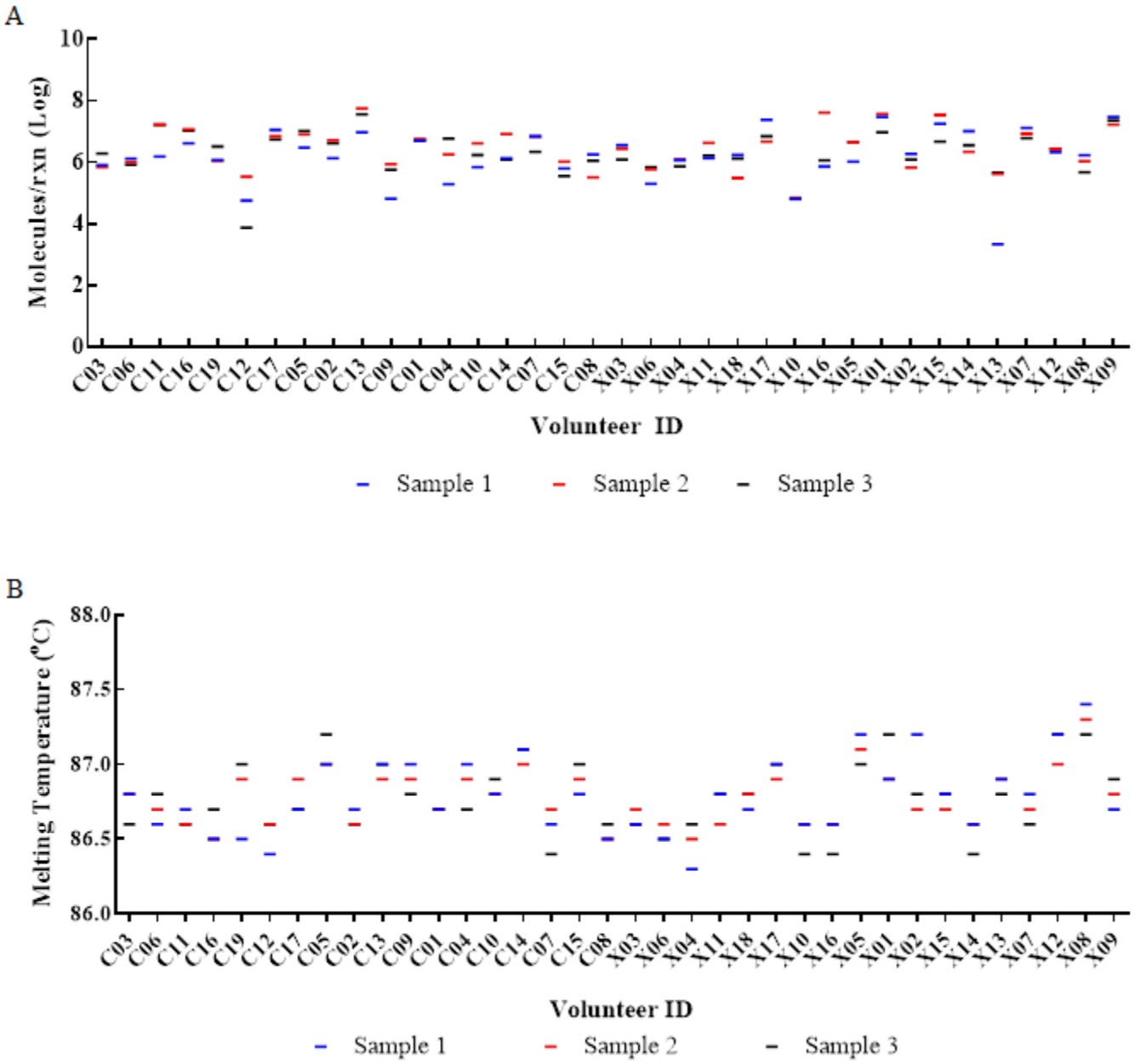

Figure 11. Bacterial titers from xerostomia and control three sequential samples. Total titers are found in (A) and melting temperatures can be found in (B). Data was generated from the qPCR run using the bacterial universal primer (16s).

3.4.3 Oral microbial composition for each volunteer

The composition of dominant species for each volunteer varies, but there are two phyla that outnumber the others and compete for dominance: *Streptococcus* and *Megasphera/ Dialister/ Veillonella* (Fig. 12; Appendix L). The 2-way ANOVA test on the volunteer's average titers for each primer showed no significant difference of any individual compared to an overall average titer per target, nor between average titers per target between control and xerostomic groups. At the level of resolution depicted in Figure 12, there is not a dramatic difference in magnitudes of titer changes between controls versus xerostomic groups.

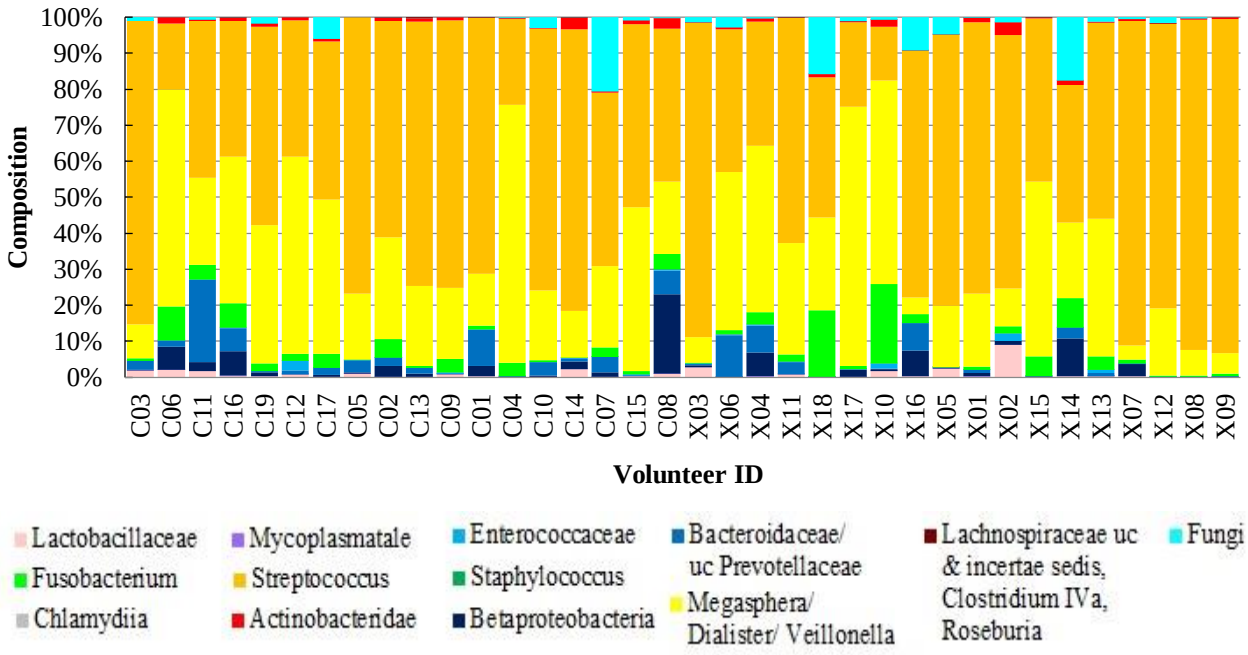

Figure 12. Percent composition of oral microbes for xerostomia and control volunteers. The percentages are based on titers from each primer per individual. Each individuals' three samples were averaged.

3.4.4 Dominant populations of xerostomia and controls

Titer data was analyzed by using a Mann Whitney t-test on log molecules per reaction values for each volunteer's sample. Because there was no tight control of saliva volumes, I compared titers of subgroups as percentages of total titers of the sample; the latter was determined both by Bu10 titers and by summing titers from all PB qPCR reactions. Even with overlap between the groups, there were significant decreases in a subset of bacterial branches' average titers in xerostomia as compared to controls, most dramatically 5 and 18 fold reductions in *Lachnospiracea* and in *Bacteroidaceae*, respectively (Fig. 13). Xerostomia samples were significantly lower in microbial populations of *Lactobacillaceae* (1.3×10^4 in controls, 6.67×10^3 in xerostomia, $P = 0.027$), *Betaproteobacteria* (2.26×10^4 in controls, 8.18×10^3 in xerostomia, $P = 0.014$), *Megasphaera/Dialister/Veillonella* (8.5×10^5 in controls, 4.82×10^5 in xerostomia, $P = 0.0068$), *Lachnospiraceae/ incertae sedis/ clostridum IVa/ Roseburia* (1.75×10^3 in controls, 3.76×10^2 in xerostomia, $P = 0.0003$), and *Bacteroidaceae/ uc Prevotellaceae* (4.05×10^4 in controls, 2.31×10^3 in xerostomia, $P = <0.0001$). All of the figures for every primer of control versus xerostomia can be found in figures in Appendix G.

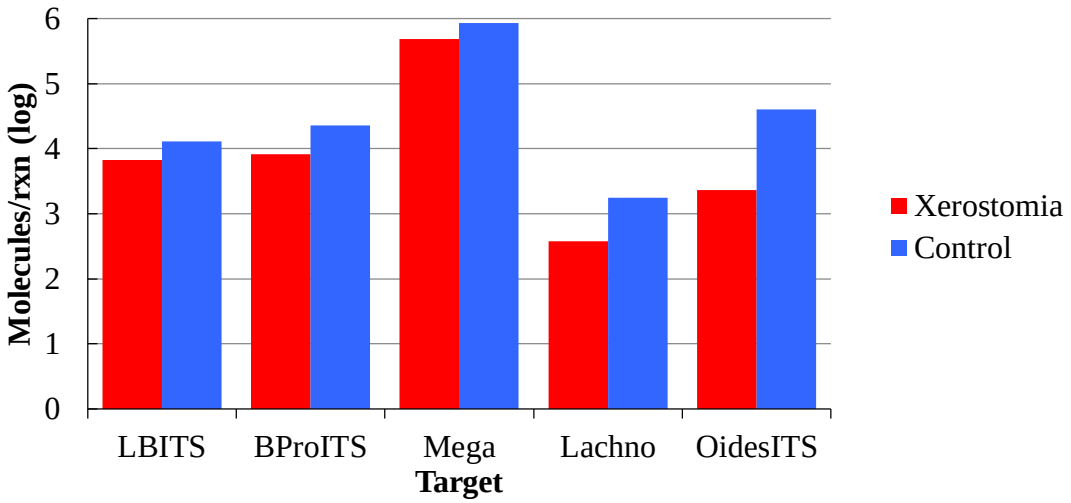

Figure 13. Average titers (log) significantly different for xerostomia and control volunteers. The averages are based on titers from each primer per individual in each group. LBITS= *Lactobacillaceae,* BProITS= *Betaproteobacteria*, Mega= *Megasphaera/Dialister/Veillonella*, Lachno= *Lachnospiraceae/incertae sedis/clostridum Iva/Roseburia,* and OidesITS= *Bacteroidaceae/un Prevotellaceae*

Xerostomia patient samples were depleted of several species relative to controls, as indicated by missing or less prevalent species with BuITS or more specific primers (Figure #...). Samples species were analyzed by uncorrected multiple comparisons Fisher's LSD ordinary two-way ANOVA and significant species differences were seen in the several primers (Appendix H). BuITS detected three species that were significantly lower in prevalence in xerostomia versus control samples. They were BuITS-81.2 (10 in controls, 3 in xerostomia, P = 0.031), BuITS-85.6 (9 in controls, 2 in xerostomia, P = 0.031), and BuITS-88 (15 in controls, 5 in xerostomia, P = 0.0029). These species would reflect dominant bacterial species, either *Streptococcus* or *Megasphaera/Dialister/ Veillonella,* therefore these could be: *Streptococcus sp., Streptococcus*

mitis, Streptococcus salivarius subsp. Null, and *Streptococcus mutans.* Xerostomia also had a significantly lower amount (17 in controls, 8 in xerostomia, P = 0.047) of *Lachnospiraceae/ incertae sedis/ clostridum IVa/ Roseburia* with the melt temperature of Lachno-86.4, based on sequencing results this may be *Oribacterium sinus*.

Other branches that had a significantly lower prevalence in xerostomia were *Staphylococcus* and *Bacteroidaceae/ uc Prevotellaceae*. *Staphylococcus* was also found to be significantly lower (22 in controls, 14 in xerostomia, and 21 in controls, 11 in xerostomia, P = 0.020 and 0.0048) in species StaphITS-80.4 and StaphITS-80.6. Certain species of *Bacteroidaceae/ uc Prevotellaceae* were found to be less prevalent in xerostomia. Xerostomia had a significantly lower amount (15 in controls, 9 in xerostomia, P = 0.032) of OidesITS-81.6. *Bacteroidaceae/ uc Prevotellaceae* also found to be significantly lower (8 in controls, 1 in xerostomia, P = 0.013) in OidesITS-90, and sequencing results suggests this may be *Prevotella melaninogenica*.

Some branches had a significant increase in prevalence for one species but a significant decrease in another for xerostomia, based off of the control. For example, *Lactobacillaceae* primers detected lower prevalence (16 in controls, 8 in xerostomia, P = 0.0043) of LBITS-87.6, likely *Lactobacillus fermentum*, possibly *Abiotrophia defectiva.* In contrast there was higher prevalence (2 in controls, 8 in xerostomia, P = 0.027) of LBITS-90.2. There was lower prevalence of *Betaproteobacteria* among xerostomia patients (10 in controls, 1 in xerostomia, P = 0.0038) of BProITS-86, but higher prevalence (3 in controls, 12 in xerostomia, P = 0.0038) of

BProITS-84. These data indicate compositional changes in the two patient groups that warrant further study by NGS.

Certain species of fungi were found to be more prevalent among xerostomia patients. More prevalent species among xerostomia included Fungi-84.4 (6 in controls, 15 in xerostomia, P = 0.029) likely to be *Candida albicans*, Fungi-84.8 (9 in controls, 23 in xerostomia, P = 0.0011) and Fungi-86.6 (18 in controls, 32 in xerostomia, P = 0.0011), likely *Saccharomyces cerevisiae* and *Candida albicans*. In contrast, no melt/species of fungi was significantly more prevalent in controls than in xerostomia. All of the significant differences in the species noted in the paragraphs above are depicted Figure 14.

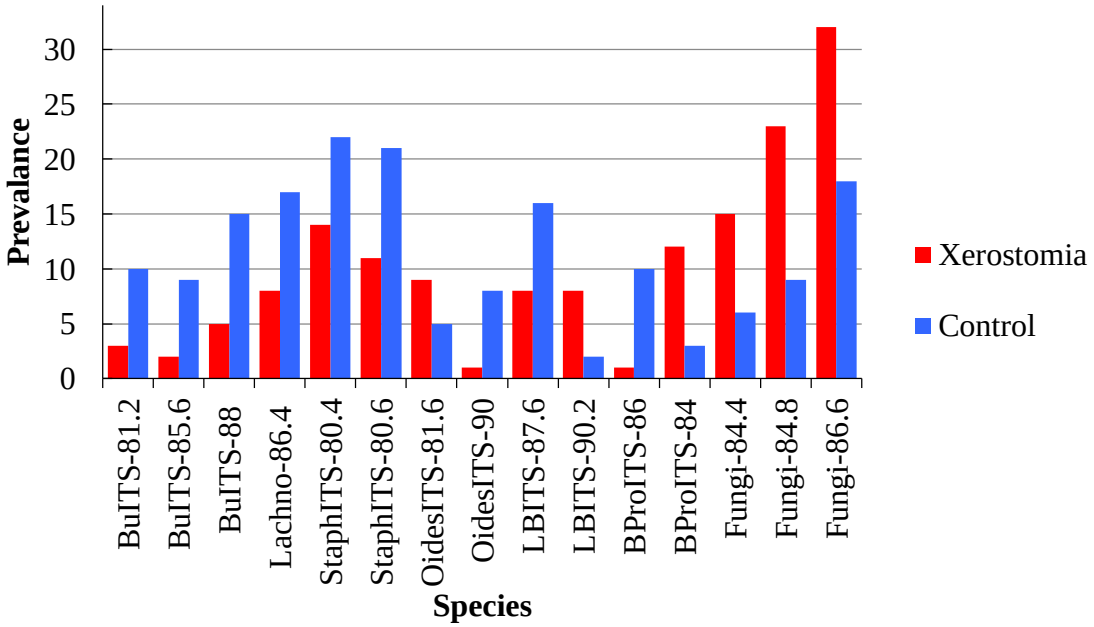

Figure 14. Prevalence of species significantly different for xerostomia and control volunteers. The averages are based on titers from each primer per individual in each group.

3.4.5 Dominant populations of high flow versus low flow

Table 9. Characterization of low flow and high flow sum volunteers

		Low Flow	High Flow
Total	Enrolled	20	16
	Male	5	9
	Female	15	7
	White	18	14
	Black	1	1
	Asian	1	1
	Diagnosed xerostomia	3	1
	Symptomatic xerostomia	12	2
	Periodontal or Gingivitis	4	1
	Wear Dentures	3	2
	Cosmetic Dentistry	5	0
Average	Age (years)	51.1 ± 24.1	59.8 ± 21.4
	Cavities (#)	9.3 ± 7.5	6.8 ± 6.6
	Root canals (#)	0.9 ± 1.3	0.8 ± 0.9
	Crowns (#)	2.3 ± 2.7	1.8 ± 2.6
	pH	5.7 ± 0.5	5.8 ± 0.5
	unstimulated (mL)	0.7 ± 0.5	1.4 ± 0.4
	stimulated (mL)	3.1 ± 1.2	7.2 ± 2.0
	flow sum (mL)	3.9 ± 1.5	8.6 ± 2.1

Although significant differences between xerostomia and controls were seen for some bacterial groups, others may have been overlooked due to the imperfect grouping of the groups by self-reported symptoms. Therefore all volunteers were separated into two groups based on their saliva flow sum values to see if there were any significant differences between microbial populations. Volunteers that had a saliva flow sum <6.0 were put in "low flow" (20 volunteers) and volunteers with a saliva flow sum of 6.0 or greater were grouped into "high flow" (16 volunteers), based on observed gaps in the distribution. This criteria moved three xerostomia individuals into the high flow group and five controls into the low flow group. Table 9 gives a characterization of the two groups.

Titer data was analyzed by using a Mann Whitney t-test. Low flow volunteers were significantly lower in titer averages, as compared to high flow (control), most dramatically a 20 fold lower average titer for *Bacteroidaceae/ uc Prevotellaceae* (Figure 15). The following target groups were lower: *Betaproteobacteria* (low flow 9.58 x 10^3, high flow 2.10 x 10^4, P = 0.0433), *Megasphaera/Dialister/Veillonella* (low flow 4.80 x 10^5, high flow 9.20 x 10^5, P = 0.012), *Lachnospiraceae/ incertae sedis/ clostridum IVa/ Roseburia* (low flow 4.36 x 10^2, high flow 1.76 x 10^3, P = 0.0053), *Fusobacterium* (low flow 2.32 x 10^4, high flow 6.85 x 10^4, P = 0.0047) and *Bacteroidaceae/ uc Prevotellaceae* (low flow 2.30 x 10^3, high flow 5.83 x 10^4, P = 0.0003). All of these differences in average titers per target can be found in figures in Appendix G.

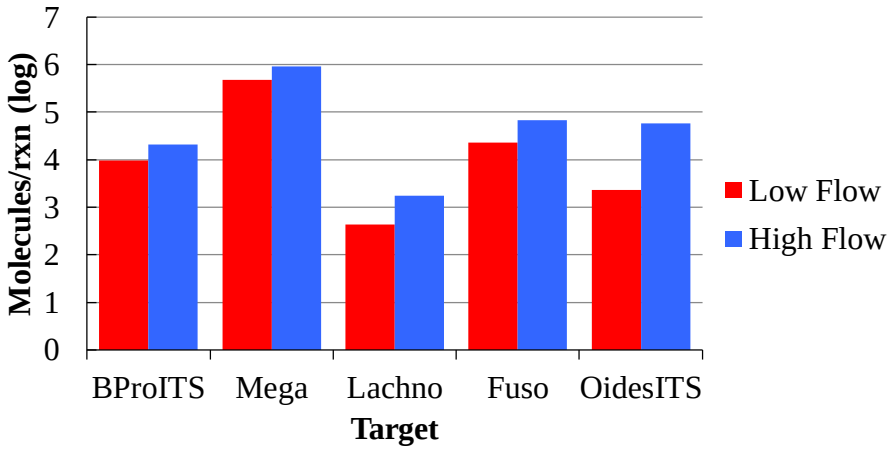

Figure 15. Average titers (log) significantly different for low flow and high flow volunteers. The averages are based on titers from each primer per individual in each group. BProITS= *Betaproteobacteria*, Mega= *Megasphaera/Dialister/Veillonella*, Lachno= *Lachnospiraceae/incertae sedis/clostridum Iva/Roseburia,* Fuso= *Fusobacterium* and OidesITS= *Bacteroidaceae/un Prevotellaceae*

BuITS melt analysis of low versus high flow groups showed two species that differed in prevalence. Melting temperature prevalence data was analyzed by multiple comparisons uncorrected Fisher's LSD ordinary two-way ANOVA and the significant species differences were seen in the several primers (Appendix H). The first species BuITS-88 was found to be significantly lower (low flow 2, high flow 18, P = 0.0022) in low saliva flow, but the second species BuITS-88.4, was found to be significantly higher (low flow 23, high flow 9, P = 0.0066) in low saliva flow. These temperatures would reflect dominant bacterial species, either *Streptococcus* or *Megasphaera/Dialister/ Veillonella*, therefore these temperatures could be: *Streptococcus sp., Streptococcus salivarius subsp. Null,* and *Streptococcus mutans.*

However, in the fungal universal primer ITS, all species were found to be significantly higher in low flow. These are Fungi-84.4 (low flow 17, high flow 4, P = 0.0014; *Candida albicans, Candida tropicalis*) Fungi-84.8 (low flow 22, high flow 10, P = 0.0029; *Candida albicans, Saccharomyces cerevisiae, Aureobasidium proteae*), Fungi-86.4 (low flow 13, high flow 5, P = 0.040; *Candida albicans,* or Uncultured *basidiomycete*), and Fungi-86.6 (low flow 29, high flow 21, P = 0.040; *Candida albicans, Malassezia restricta*). Another branch primers that had species prevalence significantly higher in low flow was *Lactobacillaceae. Lactobacillaceae* were found to be more prevalent at LBITS-87.8 (low flow 12, high flow 6, P = 0.035; *Abiotrophia defectiva*) and LBITS-90.2 (low flow 9, high flow 1, P = 0.0063).

Betaproteobacteria and *Staphylococcus* groups showed a significant increase in prevalence for one species but a significant decrease in another for low flow compared to high flow. Low saliva flow was found to have significantly higher in prevalence (low flow 16, high

flow 5, P = 0.0024) of BProITS-83.6 for *Betaproteobacteria*, but significantly lower prevalence (low flow 1, high flow 10, P = 0.011) in BProITS-86.0. Among *Staphylococcus spp.*, low flow was significantly lower (low flow 2, high flow 6, P = 0.039) in StaphITS-86.6 (*Staphylococcus epidermidis*), but significantly higher (low flow 16, high flow 12, P = 0.039) in StaphITS-87.0 (*Staphylococcus aureus* or *Streptococcus pneumonia*). Among *Bacteroidaceae/ uc Prevotellaceae*, species OidesITS-88.6 (*Prevotella melaninogenica*) was less prevalent in low flow as compared to high flow (low flow 0, high flow 11, P = 0.0008). Figure 16 shows these significant differences between the species in these groups.

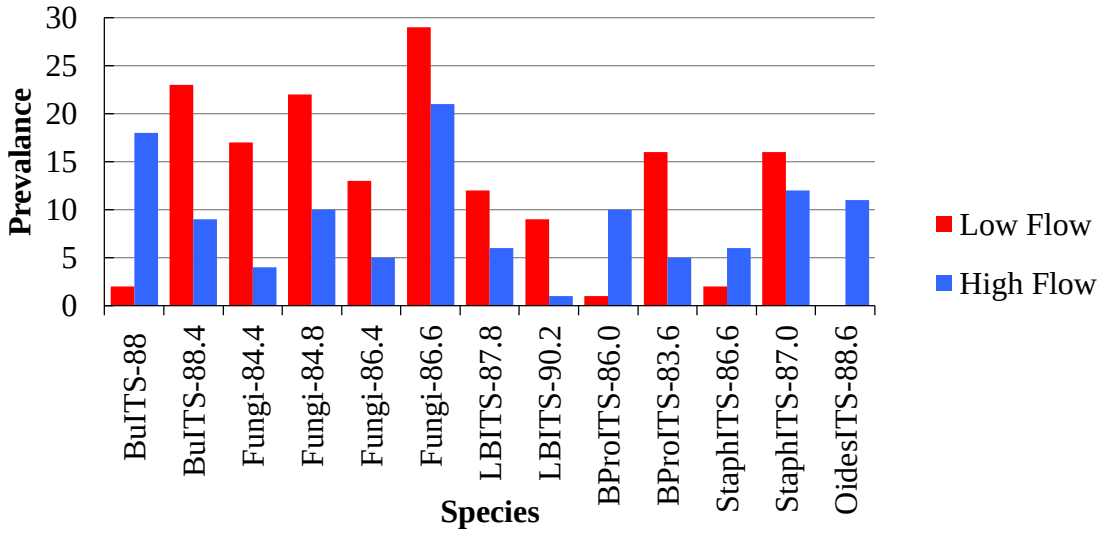

Figure 16. Prevalence of species significantly different for low flow and high flow volunteers. The averages are based on titers from each primer per individual in each group.

The analysis of low flow versus high flow groups was more sensitive for detected differences in prevalence than was the xerostomic versus healthy grouping. Both showed compositional differences that warrant further analysis by NGS.

3.4.6 Dominant populations of control versus medication

The volunteers of the xerostomia and control study were separated into two groups based on their questionnaire answers for medication to distinguish if there were any significant differences between microbial populations. The first group consisted of volunteers that currently took medications that cause dry mouth (xerostomia) and were named the "medication-induced xerostomia" or MIX group. The second group consisted of age-matched volunteers that did not take medications that cause dry mouth and were named "controls".

Table 10. Characterization of MIX vs. control volunteers

		MIX	Control
Total	Enrolled	11	11
	Male	7	5
	Female	4	6
	White	10	10
	Black	1	0
	Asian	0	1
	Diagnosed xerostomia	2	0
	Symptomatic xerostomia	6	0
	Periodontal or Givingivitis	0	1
	Wear Dentures	1	2
	Cosmetic Dentistry	2	1
Average	Age (years)	57.1 ± 23.8	57.5 ± 24.7
	Cavities (#)	9.2 ± 7.1	6.4 ± 6.7
	Root canals (#)	0.9 ± 1.2	0.7 ± 0.8
	Crowns (#)	1.9 ± 2.0	1.8 ± 2.9
	pH	5.5 ± 0.5	6.0 ± 0.4
	unstimulated (mL)	0.8 ± 0.5	1.6 ± 0.3
	stimulated (mL)	4.2 ± 1.5	6.5 ± 2.5
	flow sum (mL)	5.1 ± 1.8	8.0 ± 2.6

Characterization of the volunteers in each of these groups can be found in Table 10.

Titer data was analyzed by using a Mann Whitney t-test. Significant decreases in titer averages in MIX volunteers were seen in these three primers: *Bacteroidaceae/ uc Prevotellaceae* (MIX 5.43 x 10^3, control 3.90 x 10^4, P = 0.0368), *Betaproteobacteria* (MIX 6.77 x 10^3, control 4.59 x 10^4, P = 0.0279), and *Lachnospiraceae/ incertae sedis/ clostridum IVa/ Roseburia* (MIX 5.68 x 10^2, control 2.21 x 10^3, P = 0.0159). These significant differences can be seen in Figure 17. All of the differences from each of the primers tested can be found in figures in Appendix G.

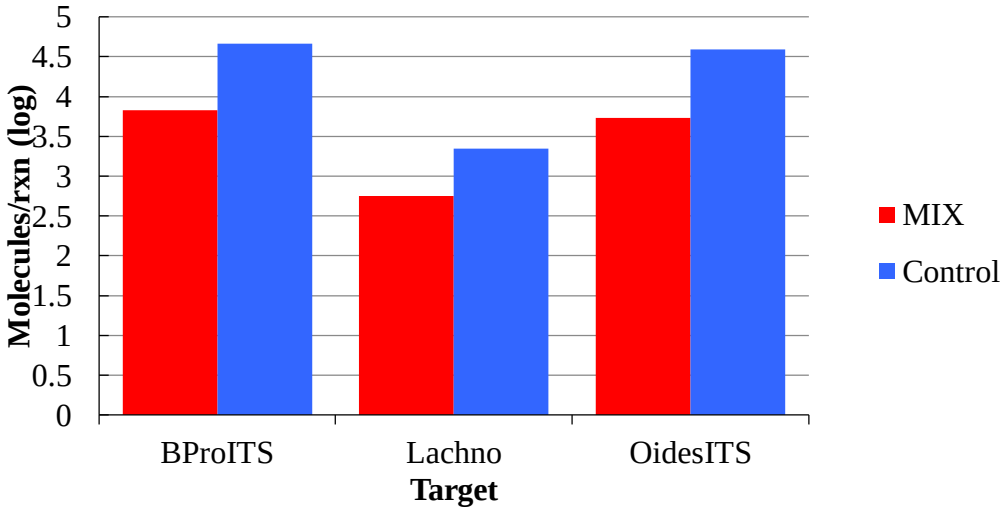

Figure 17. Average titers (log) significantly different for xerostomia and control volunteers. The averages are based on titers from each primer per individual in each group. BProITS= *Betaproteobacteria*, Lachno= *Lachnospiraceae/incertae sedis/clostridum Iva/Roseburia,* and OidesITS= *Bacteroidaceae/un Prevotellaceae*

There were several similarities and few significant differences between the prevalence of the two groups' species. Species prevalence data was analyzed by multiple comparisons uncorrected Fisher's LSD ordinary two-way ANOVA and the significant species differences were seen in the several primers (Appendix H). The bacterial universal primer ITS, was shown to

have three species that were significantly different between MIX and controls. The first two species, BuITS-81.2 and BuITS-84.6, were found to be significantly higher (MIX 7, control 3, and MIX 4, control 0, P = 0.042) in MIX, but the species BuITS-88, was found to be significantly lower (medicated 4, control 9, P= 0.012). These temperatures reflect dominant bacterial species in the *Streptococcus* or *Megasphaera/Dialister/ Veillonella* target group, likely *Streptococcus sp., Streptococcus salivarius subsp. Null,* or *Streptococcus mutans.*

Branches that had a significant increase in prevalence for one temperature but a significant decrease in another temperature for MIX, compared to controls, were found in the fungal universal primer and *Betaproteobacteria. Fungi* species Fungi-84.6 (likely *Cryptococcus sp*., possibly *Candida albicans*) were found to be significantly reduced in MIX (MIX 5, control 11, P= 0.048), but Fungi-86.4 (likely *Candida albicans,* possibly an uncultured *basidiomycete)* were significantly increased in MIX (MIX 11, control 1, P= 0.0018). *Betaproteobacteria* species BProTIS-84.0 were found to be significantly more prevalent (MIX 7, control 2, P= 0.013) in MIX and species BProITS-85.6 and BProITS-86.0 were found to be significantly less prevalent (MIX 0, control 4, and MIX 2, control 6, P= 0.043). Unfortunately, possible species for these temperatures were not matched with DNA sequences from RDP for this primer.

Lactobacillaceae species were found to be more significantly prevalent in MIX volunteers. LBITS-84.6 and LBITS-87.6 (*Lactobacillus fermentum* or *Abiotrophia defectiva*) were found only in MIX (MIX 5, control 0, and MIX 11, control 6, P= 0.043). Species for these temperatures did not closely match with DNA sequences from the RDP database for these primers.

Lastly, MIX volunteers were found to be significantly less prevalent in species from the branches of *Staphylococcus* and *Bacteroidaceae/ uc Prevotellaceae*. *Staphylococcus* species of StaphITS-80.8 (*Staphylococcus epidermidis*) were 10-fold more prevalent in controls (MIX 1, control 9, P= 0.018). *Bacteroidaceae/ uc Prevotellaceae* species OidesITS-81.6 were 5-fold more prevalent in controls (MIX 2, control 10, P= 0.0027) and OidesITS-90.0 was seen only in controls (MIX 0, control 6, P= 0.021, respectively) in these volunteers.

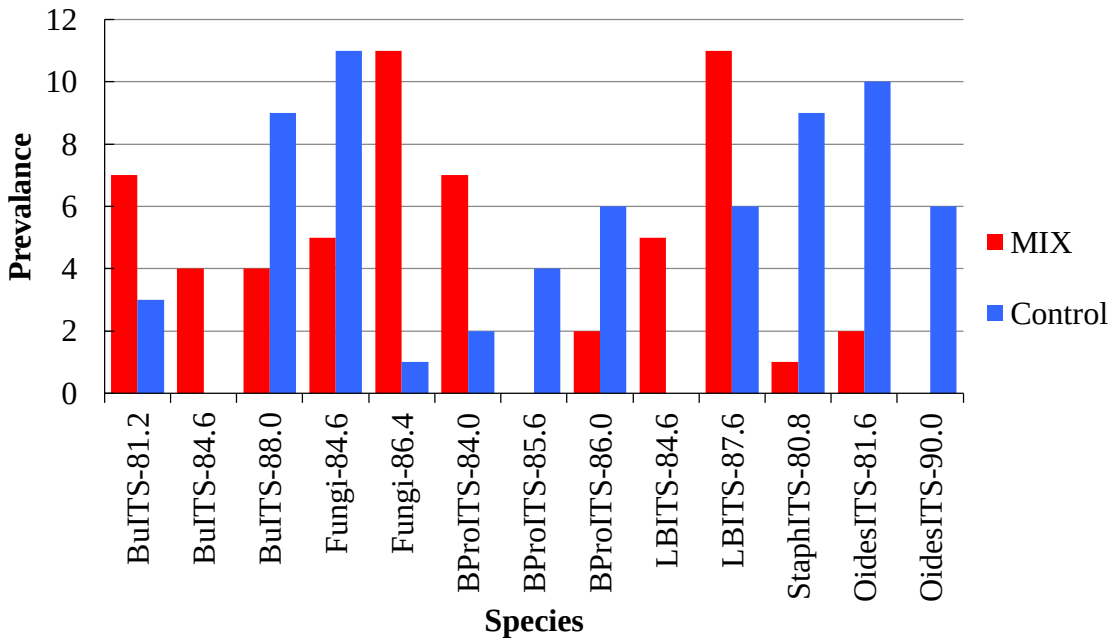

Figure 18. Prevalence of species significantly different for MIX and controls. The averages are based on titers from each primer per individual in each group.

3.5 Nightly Oral Hygiene Practices Arm

3.5.1 Volunteer Enrollment

Table 11. Characterization of nightly routine volunteers

Total	Enrolled	17
	Male	8
	Female	9
	White	14
	Black	0
	Asian	3
	Periodontal or Gingivitis	1
	Wear Dentures	0
	Cosmetic Dentistry	1
Average	Age (years)	27.1 ± 10.5
	Cavities (#)	4.5 ± 3.5
	Root canals (#)	0.4 ± 1.0
	Crowns (#)	0.2 ± 0.7
	pH	6.0 ± 0.5
	unstimulated (mL)	1.0 ± 0.5
	stimulated (mL)	6.3 ± 3.0
	flow sum (mL)	7.3 ± 3.2

A total of 17 volunteers, 8 males and 9 females, were enrolled in this study with an age range of 18-60 years, and an average age of 27.1 ± 10.5 years. A detailed characterization of the xerostomia and control volunteers is found in Table 11.

Titer data was analyzed by using a 1way ANOVA Dunn's multiple comparisons test on log molecules per reaction values for each volunteer's sample per nightly routine (Appendix J). Because there was no tight control of saliva volumes, I compared titers of subgroups as percentages of total titers of the sample. Even with overlap between the groups, there were significant decreases in many bacterial branches' average titers in different nightly routines as compared to no nightly oral hygiene routine (none). Nightly oral hygiene routine samples were depleted of several species relative to controls (none or no nightly routine), as indicated by missing or less prevalent melt temperatures with BuITS or more specific primers (Appendix K). Samples melting temperatures/species were analyzed by uncorrected multiple comparisons Fisher's LSD ordinary

two-way ANOVA and the significant species differences were seen in the several primers (Appendix K).

Analysis in Section 3.5.2- 3.5.5 refer to titers averaged among the 17 patients.

3.5.2 Dominant species after Listerine mouthwash

After use of Listerine mouthwash (Fig. 19), *Lactobacillaceae* (1.03 x 10^4 in none, 4.17 x 10^3 in Listerine mouthwash, P = < 0.0001) and *Fusobacterium* (2.99 x 10^4 in none, 1.79 x 10^4 in Listerine mouthwash, P = 0.01) titers were reduced about two-fold. Other average titers did not show significant reduction.

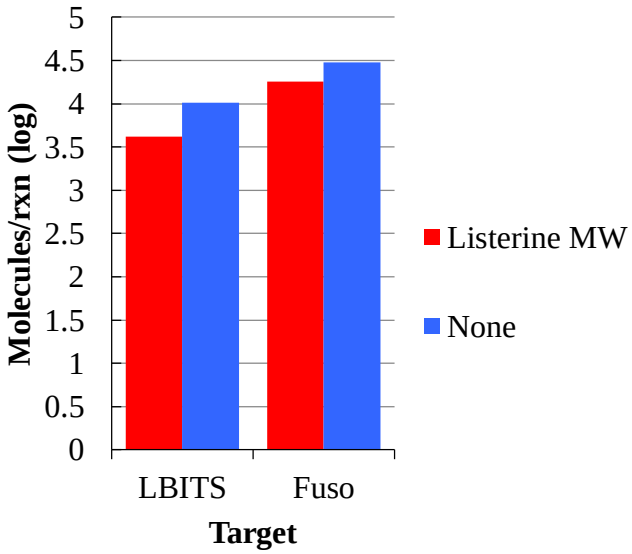

Figure 19. Average titers (log) significantly different for Listerine mouthwash and none across all volunteers. The averages are based on titers from each primer per individual in each group. LBITS= *Lactobacillaceae*, and Fuso= *Fusobacterium*

A number of specific species, as provisionally defined by their melt temperatures, were reduced in prevalence after Listerine (Fig. 20). I use the nomenclature for these unnamed species as follows: the primer being used – melt temperature. BuITS detected seven melt temperatures that were significantly lower in Listerine mouthwash versus control samples: BuITS-80.4 (16 in controls, 2 in Listerine mouthwash, P = 0.0168), BuITS-81 (15 in controls, 2 in Listerine

mouthwash, P = 0.0299), BuITS-84.4 (20 in controls, 7 in Listerine mouthwash, P = 0.0299), BuITS-86.4 (24 in controls, 11 in Listerine mouthwash, P = 0.0299), BuITS-88 (44 in controls, 29 in Listerine mouthwash, P = 0.0091), BuITS-88.2 (41 in controls, 17 in Listerine mouthwash, P = < 0.0001), and BuITS-88.4 (37 in controls, 15 in Listerine mouthwash, P = < 0.0001). Species Bu10-87.0 was more than two-fold less prevalent in Listerine mouthwash (58 in control, 25 in Listerine mouthwash, P = 0.059). *Fusobacterium* (Fuso- 85.0, possibly *Fusobacterium periodonticum*) was also reduced in prevalence by at least two-fold (59 in controls, 25 after Listerine mouthwash, P = 0.001). Fuso-85.2 (possibly *Fusobacterium periodonticum*) was reduced by at least 1.6 fold with 59 in controls, 35 in Listerine mouthwash (P = 0.0229). *Lachnospiraceae/ incertae sedis/ clostridum IVa/ Roseburia* detected species/melts that were significantly lower than the control. The first, Lachno-86.4 (possibly *Oribacterium sinus*), was two- to three-fold lower, 45 in none and 23 in Listerine mouthwash (P = 0.0152). *Lactobacillaceae* detected three species/melts with significantly lower titers: LbITS-87.4, possibly *Abiotrophia defectiva* (39 in controls, 21 after Listerine mouthwash, P = 0.0009), LbITS-87.6 possibly *Abiotrophia defectiva*, 33 in controls, 21 in Listerine mouthwash (P = 0.0429), and LbITS-87.8 possibly *Abiotrophia defectiva*, with 41 in controls, 16 in Listerine mouthwash (P = < 0.0001). One *Lactobacillus* species, LbITS-87.2, was 13-fold lower, but only after Listerine mouthwash (13 in control, 1 in Listerine mouthwash, P = 0.0429). Strep-88 was reduced more than two-fold (51 in controls, 18 after Listerine mouthwash, P = 0.0231).

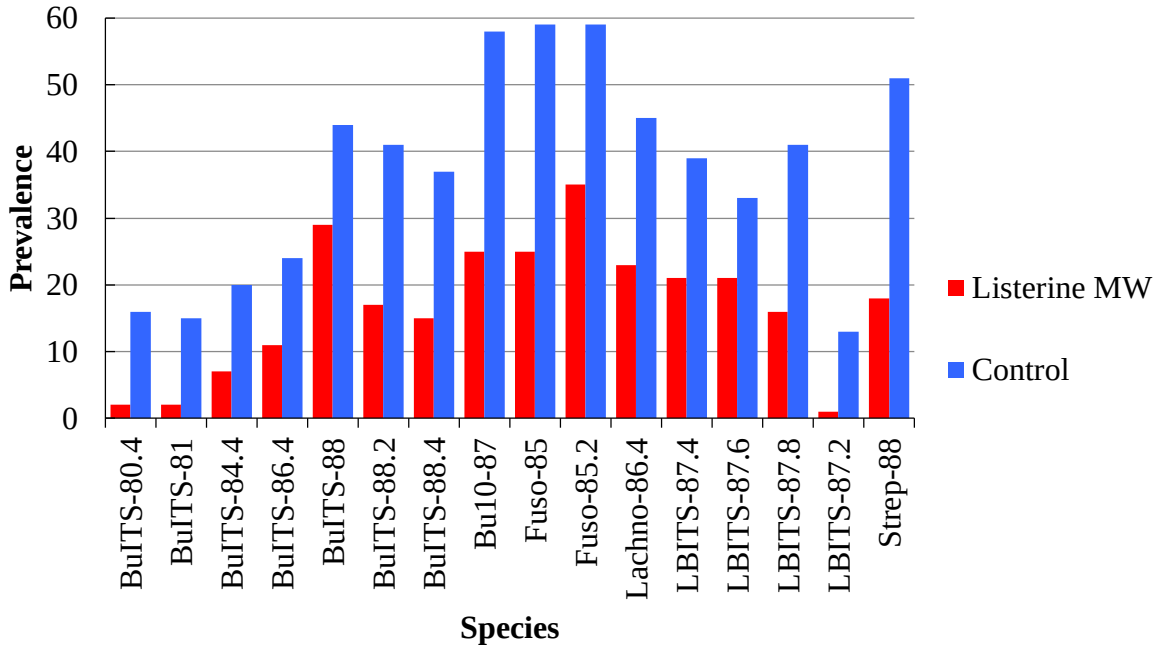

Figure 20. Prevalence of species significantly different for Listerine mouthwash and control across volunteers.

3.5.3 Dominant species of Crest mouthwash

Bacterial populations were significantly decreased after nightly use of Crest mouthwash in all six of the branches tested, by about 2 to 7 fold (Figure 21). This included: bacterial 16s (1.30×10^6 in none, 4.47×10^5 in Crest mouthwash, P = 0.0006), bacterial ITS (6.35×10^5 in none, 2.64×10^5 in Crest mouthwash, P = 0.0077), *Lactobacillaceae* (1.03×10^4 in none, 6.34×10^3 in Crest mouthwash, P = 0.023), *Streptococcus* (4.37×10^5 in none, 1.84×10^5 in Crest mouthwash, P = 0.0445), *Fusobacterium* (2.99×10^4 in none, 1.07×10^4 in Crest mouthwash, P = < 0.0001), and *Lachnospiraceae/ incertae sedis/ clostridum IVa/ Roseburia* (7.69×10^2 in none, 1.15×10^2 in Crest mouthwash, P = < 0.0001).

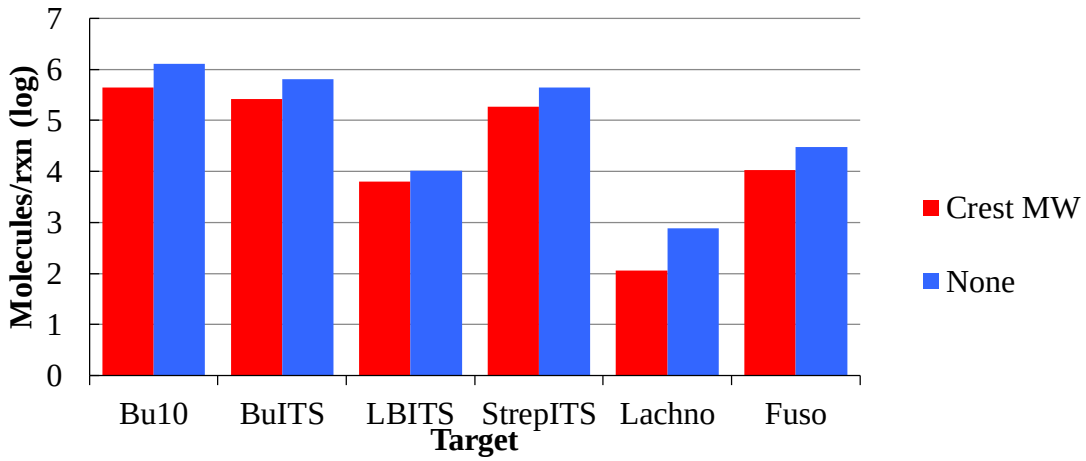

Figure 21. Average titers (log) significantly different for Crest mouthwash and none across all volunteers. The averages are based on titers from each primer per individual in each group. Bu10= bacterial universal primer 16s, BuITS= bacterial universal primer ITS, LBITS= *Lactobacillaceae*, StrepITS= *Streptococcus*, Lachno= *Lachnospiraceae/incertae sedis/clostridum Iva/Roseburia*, and Fuso= *Fusobacterium*

Crest mouthwash also reduced the prevalence of a number of specific species (Fig. 22). BuITS detected six species/melts that were significantly lower in Crest mouthwash versus control samples. They were BuITS-80.6 (22 in controls, 7 in Crest mouthwash, P = 0.0091), BuITS-86.4 (24 in controls, 5 in Crest mouthwash, P = 0.0006), BuITS-87.8 (26 in controls, 11 in Crest mouthwash, P = 0.0091), BuITS-88 (44 in controls, 22 in Crest mouthwash, P = < 0.0001), BuITS-88.2 (41 in controls, 23 in Crest mouthwash, P = 0.0012), and BuITS-88.4 (37 in controls, 14 in Crest mouthwash, P = < 0.0001). Species Bu10-87.0 was more than two-fold less prevalent in Crest mouthwash (58 in none, 18 Crest mouthwash, P = 0.0019). *Fusobacterium* (Fuso-85.0, possibly *Fusobacterium periodonticum*) was also reduced in prevalence by at least two-fold (59 in controls, 26 after Crest mouthwash (P = 0.0014). Fuso-85.2 (possibly

Fusobacterium periodonticum) was reduced by at least 1.6 fold with 59 in controls, 31 in Crest mouthwash (P = 0.0069). *Lachnospiraceae/ incertae sedis/ clostridum IVa/ Roseburia* detected species/melts that were significantly lower than the control. Lachno-86.4 (possibly *Oribacterium sinus*), was two- to three-fold lower, 45 in none and 17 in Crest mouthwash (P = 0.0014). *Lactobacillaceae* detected three species/melts with significantly lower titers: LbITS-87.4, possibly *Abiotrophia defectiva* (39 in controls, 9 after Crest mouthwash (P = < 0.0001), LbITS-87.6 possibly *Abiotrophia defectiva*, 33 in controls, 19 in Crest mouthwash (P = 0.0134), and LbITS-87.8 possibly *Abiotrophia defectiva*, with 41 in controls, 20 in Crest mouthwash (P = < 0.0001).

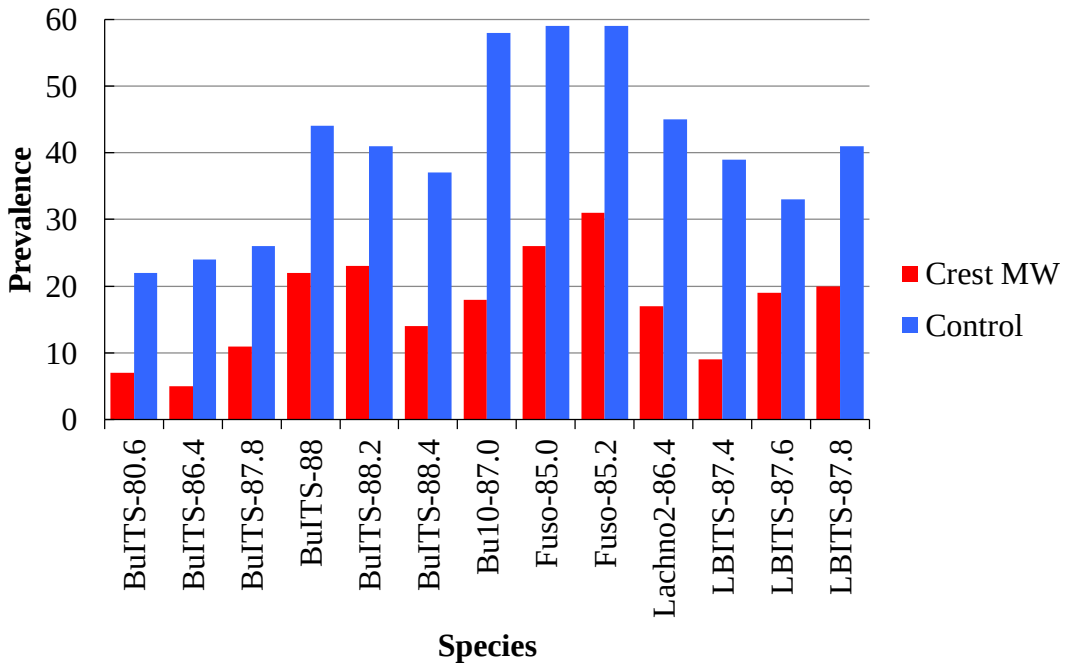

Figure 22. Prevalence of species significantly different for Crest mouthwash and control across volunteers.

3.5.4 Dominant species after Crest toothpaste

Crest toothpaste reduced average titers by a greater magnitude than the other routines, but by at most 4-fold. Four bacterial populations were reduced after the use of Crest toothpaste (Fig. 23). This included: bacterial 16s (1.30×10^6 in none, 7.21×10^5 in Crest toothpaste, P = 0.0009), *Lactobacillaceae* (1.03×10^4 in none, 4.05×10^3 in Crest toothpaste, P = < 0.0001), *Fusobacterium* (2.99×10^4 in none, 1.64×10^4 in Crest toothpaste, P = 0.0069), and *Lachnospiraceae/ incertae sedis/ clostridum IVa/ Roseburia* (7.69×10^2 in none, 3.53×10^2 in Crest toothpaste, P = 0.0138).

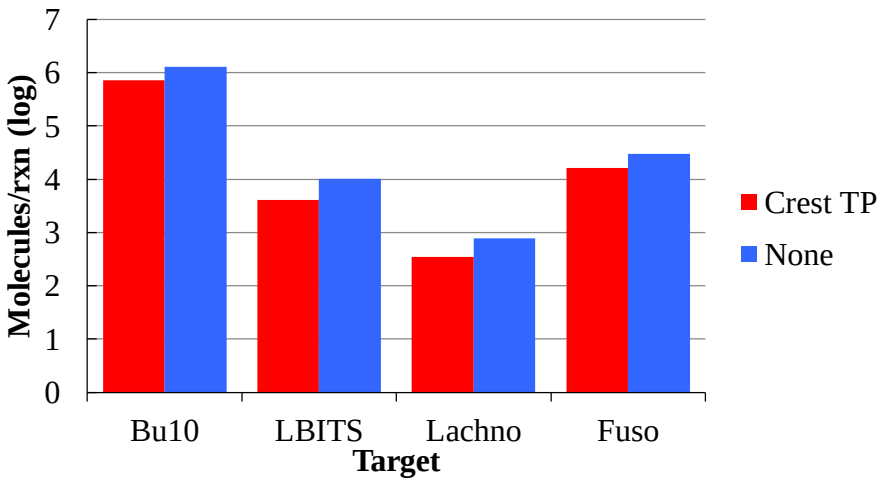

Figure 23. Average titers (log) significantly different for Crest toothpaste and none across all volunteers. The averages are based on titers from each primer per individual in each group. Bu10= bacterial universal primer 16s, LBITS= *Lactobacillaceae*, Lachno= *Lachnospiraceae/incertae sedis/clostridum Iva/Roseburia*, and Fuso= *Fusobacterium*

Significant species were reduced in several targets (Fig. 24). BuITS detected five melt temperatures that were significantly lower in Crest toothpaste versus control samples: BuITS-84.6 (17 in controls, 2 in Crest toothpaste, P = 0.0091), BuITS-86.4 (24 in controls, 8 in Crest

toothpaste, P = 0.0047), BuITS-88 (44 in controls, 21 in Crest toothpaste, P = < 0.0001), BuITS-88.2 (41 in controls, 19 in Crest toothpaste, P = < 0.0001), and BuITS-88.4 (37 in controls, 11 in Crest toothpaste, P = < 0.0001). Species Bu10-87.0 was more than two-fold less prevalent in Crest toothpaste (58 in none, 20 in Crest toothpaste, P = 0.003). *Fusobacterium* (Fuso- 85.0, possibly *Fusobacterium periodonticum*) was also reduced in prevalence by at least two-fold (59 in controls, 30 in Crest toothpaste (P = 0.0051). Fuso-85.2 (possibly *Fusobacterium periodonticum*) was reduced by at least 1.6 fold with 59 in controls, 32 in Crest toothpaste (P = 0.0094). *Lachnospiraceae/ incertae sedis/ clostridum IVa/ Roseburia* detected species/melts that were significantly lower than the control. The first, Lachno-86.4 (possibly *Oribacterium sinus*), was two- to three-fold lower, 45 in none and 14 in Crest toothpaste (P = 0.0004). *Lactobacillaceae* detected three species/melts with significantly lower titers: LbITS-87.4, possibly *Abiotrophia defectiva* (39 in controls, 20 after Crest toothpaste (P= 0.0004), LbITS-87.6 possibly *Abiotrophia defectiva*, 33 in controls, 15 in Crest toothpaste (P= 0.0009), and LbITS-87.8 possibly *Abiotrophia defectiva*, with 41 in controls, 14 in Crest toothpaste (P= < 0.0001). *Streptococcus* detected two species/melts that were significantly lower in a few of the nightly routines. Strep-87.6 was reduced ~2-fold (73 in controls, 36 after Crest mouthwash, P = 0.01, and Strep-88 was reduced more than two-fold (51 in controls, 8 after Crest toothpaste, P = 0.0027).

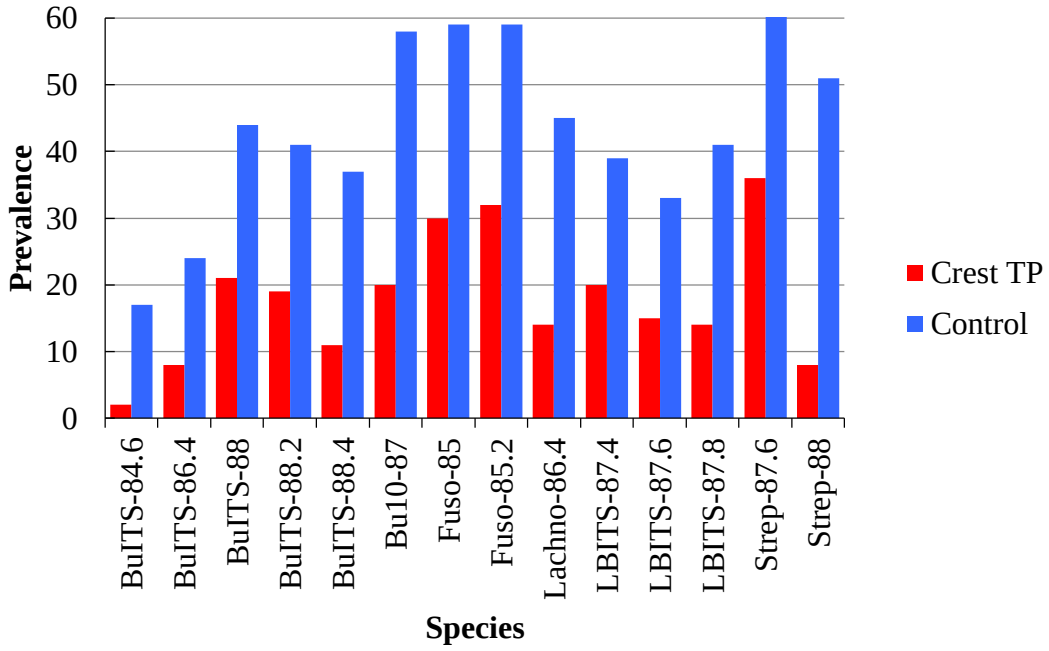

Figure 24. Prevalence of species significantly different for Crest toothpaste and control across volunteers.

3.5.5 Dominant species of Colgate toothpaste

Overall, Colgate toothpaste only reduced average bacterial titers in *Lactobacillaceae* (1.03×10^4 in none, 8.19×10^3 in Colgate toothpaste, $P = 0.01$; Fig. 25). Titers for each nightly routine per branch tested, and all of the figures showing the effects of each nightly routine on each volunteer per primer can be found in Appendix J.

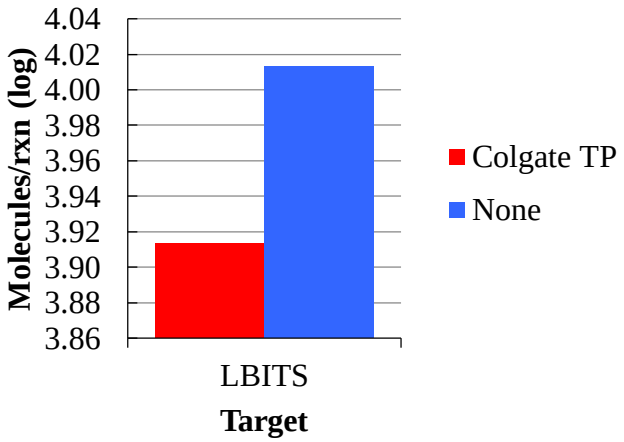

Figure 25. Average titers (log) significantly different for Colgate toothpaste and none across all volunteers. The averages are based on titers from each primer per individual in each group. LBITS= *Lactobacillaceae*. [Graph can be found in excel file: "Thesis-Paragraph-Tables1"]

Significant species were reduced in prevalence, in several target groups (Fig. 26). BuITS detected five species/melts that were significantly lower in Colgate toothpaste versus control samples. They were BuITS-80.6 (22 in controls, 8 in Colgate toothpaste, P = 0.0168), BuITS-86.4 (24 in controls, 9 in Colgate toothpaste, P = 0.0091), BuITS-88 (44 in controls, 23 in Colgate toothpaste, P = 0.0001), BuITS-88.2 (41 in controls, 17 in Colgate toothpaste, P = < 0.0001), and BuITS-88.4 (37 in controls, 20 in Colgate toothpaste, P = 0.0024). Species Bu10-87.0 was more than two-fold less prevalent in Colgate toothpaste (58 in controls, 29 in Colgate toothpaste, P = 0.010). *Fusobacterium* (Fuso- 85.0, possibly *Fusobacterium periodonticum*) was also reduced in prevalence by at least two-fold (59 in controls, 25 in Colgate toothpaste (P = 0.001). Fuso-85.2 (possibly *Fusobacterium periodonticum*) was reduced by at least 1.6 fold with 59 in controls, 36 in Colgate toothpaste (P = 0.0304). *Lachnospiraceae/ incertae sedis/ clostridum IVa/ Roseburia* detected species/melts that were significantly lower than the control. The first, Lachno-86.4 (possibly *Oribacterium sinus*), was two- to three-fold lower, 45 in none and 15 in Colgate toothpaste (P = 0.0006). The second melt/species, Lachno-86.6, was

significantly reduced only after Colgate toothpaste, by ~4-fold (38 in controls, 9 in Colgate toothpaste, P = 0.0009). *Lactobacillaceae* detected three species/melts with significantly lower titers: LbITS-87.4, possibly *Abiotrophia defectiva* (39 in controls, 20 after Colgate toothpaste (P = 0.0004), LbITS-87.6 possibly *Abiotrophia defectiva*, 33 in controls, 15 in Colgate toothpaste (P = 0.0009), and LbITS-87.8 possibly *Abiotrophia defectiva*, with 41 in controls, 17 in Colgate toothpaste (P = < 0.0001). *Streptococcus* detected two species/melts that were significantly lower in a few of the nightly routines. Strep-87.6 was reduced ~2-fold (73 in controls, 39 after Colgate toothpaste, P = 0.0188). Strep-88 was reduced more than two-fold (51 in controls, 20 after Colgate toothpaste, P = 0.0346).

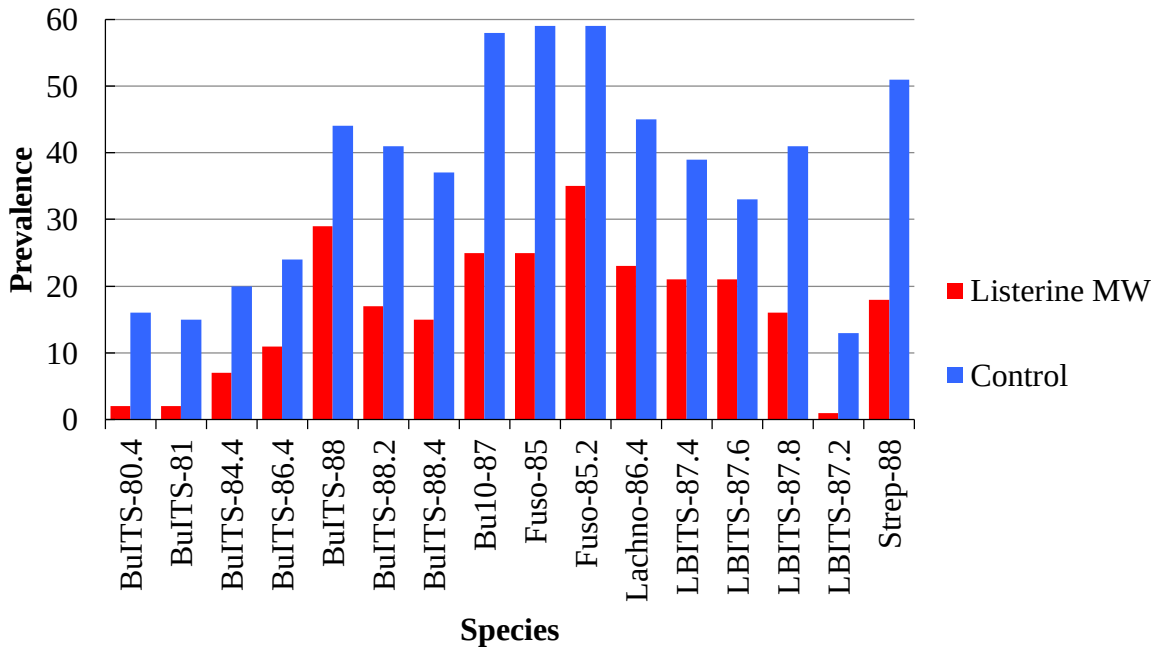

Figure 26. Prevalence of species significantly different for Colgate toothpaste and control across volunteers.

3.5.6 Fold titer decreases of differences in nightly routines

The above analyses focused on titers averaged over all patients to characterize trends that might be expected in general. However, the strongest impacts depended on the individual and were muddied by averaging. These widely varying reductions after nightly routines depend on the individual, the target, and the treatment are detailed in Appendix L; representative examples are shown in Figure 27. For example, five individuals show greater than 10 fold decreases *Lactobacillaceae* after Listerine mouthwash use (Fig. 27A). The volunteers were M01 (↓15.8), M06 (↓18.6), M10 (↓17.4), M13 (↓13.6) and M16 (↓10.5). Whereas, *Lachnospiraceae/ incertae sedis/ clostridum IVa/ Roseburia* (Fig. 27B) had four individuals with greater than 10 fold decreases after Crest mouthwash use, and of these four, three were different volunteers than seen in *Lactobacillaceae*. The three that were greater than 10 fold were: M02 (↓10.2), M06 (↓43.7), M11 (↓17782), M12 (↓55). M06 was seen to be reduced at least 15 fold with both nightly routines and targets.

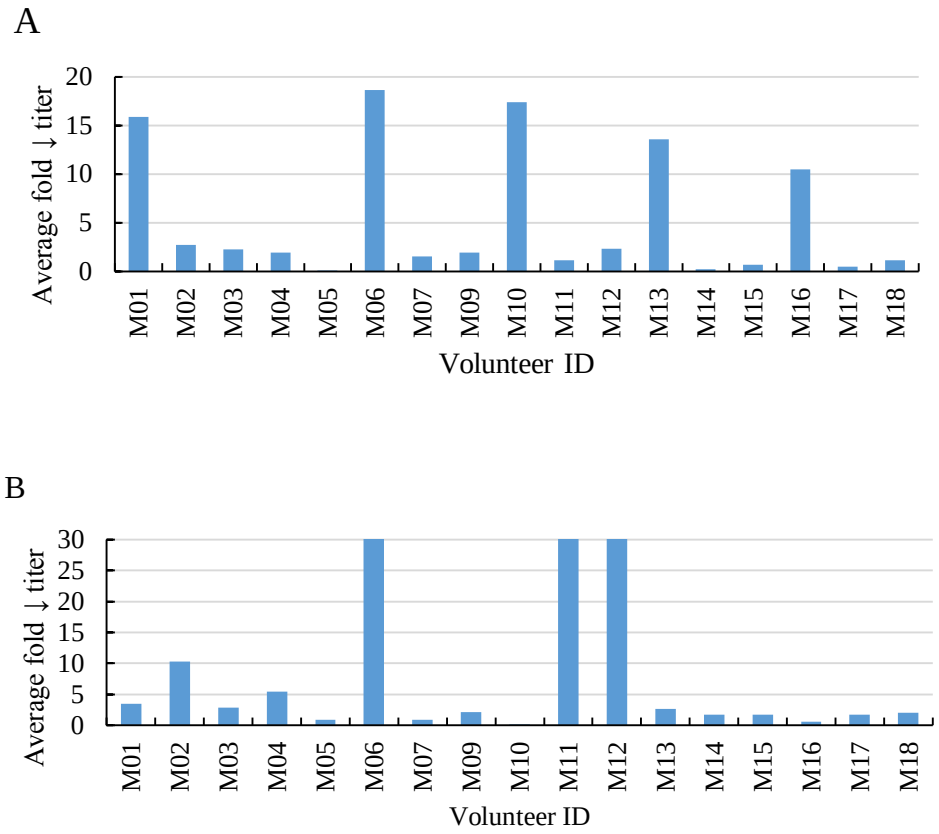

Figure 27. Average fold decrease in titers. (A) Average fold decrease in titers in Listerine mouthwash in *Lactobacillaceae*, (B) Average fold decrease in titers in Crest mouthwash in Lachno= *Lachnospiraceae/incertae sedis/clostridum Iva/Roseburia*.

A more useful analysis of this information determined the number of volunteers with greater than five-fold decreases in titer values (Fig. 28). Both Crest mouthwash and Crest toothpaste significantly reduced the populations of *Lachnospiraceae/incertae sedis/clostridum Iva/Roseburia*, in 5 individuals. Listerine mouthwash and Crest toothpaste decreased populations of *Lactobacillaceae* also in 5 individuals. Overall, Colgate toothpaste had the least amount of total patients with decreases greater than 5 fold in titers of targets. Whereas, Crest mouthwash and Crest toothpaste tied with the greatest number of volunteers with decreases greater than five

in target titers. With this knowledge at hand, it would seem that the most effective way to reduce all of the target titers tested would be to combine the use of Crest mouthwash and Crest toothpaste - where one fails, the other compensates.

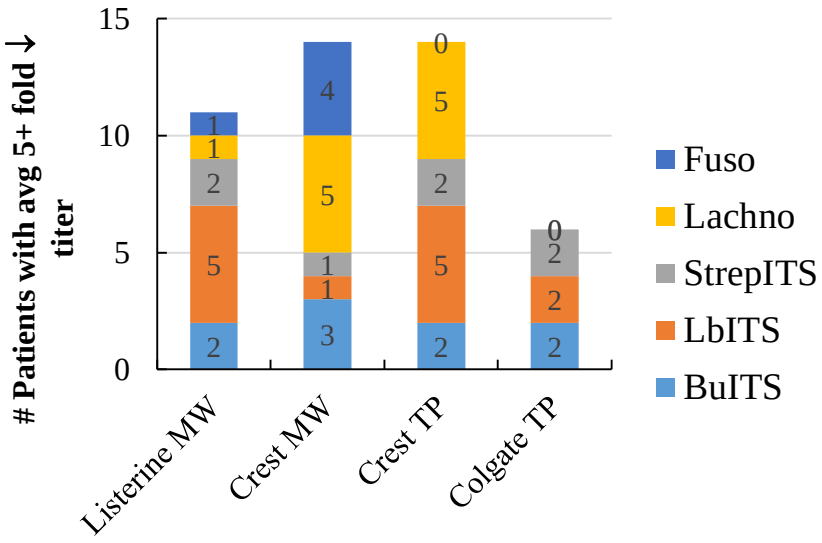

Figure 28. Prevalence of patients with 5+ average fold in titer difference. Fuso= Fusobacterium, LBITS= *Lactobacillaceae*, Lachno= *Lachnospiraceae/incertae sedis/clostridum Iva/Roseburia,* StrepITS= *Streptococcus*, and BuITS= bacterial universal primer ITS.

CHAPTER 4: CONCLUSIONS AND DISCUSSION

4.1. Overview

Most of the specific aims originally proposed for this thesis were accomplished. I secured an IRB which allowed me to collect saliva samples and patient information from 18 controls and 18 patients who self-reported xerostomia. I also collected 30 samples from 17 control patients, who practiced 5 different nightly oral hygiene routines before donating a saliva sample the next morning. I developed a storage buffer that stabilized DNA in these samples for at least 30 days, optimized a DNA extraction protocol and showed that it was more efficient at extracting amplifiable genomic DNA across almost all tested bacterial species, including *Streptococcus* species that are problematic with commercial extraction kits. I characterized and analyzed microbial compositions of these samples using qPCR with broad-spectrum and phylogenetic branch specific primers. I established a repository of 364 colony-purified oral bacterial species, along with DNA preps of each, and verified 110 of these by sequencing.

4.2 Key findings

1. Microbial DNA can be effectively stored in SSB buffer for at least 30 days, and efficiently extracted for qPCR with my modified FLB extraction protocol.
2. Oral bacterial species compositions identified by culture and qPCR were diverse and were largely consistent with those characterized by NGS in the literature.
3. Oral bacterial compositions were dominated by *Streptococcus* species and those in the *Veillonella* target groups, in both control and xerostomia groups.

4. A core group of bacteria seen in all patients included *Streptococus, Fungi, Fusobacterium, Actinobacteria, Bacteriodaceae/ uc Prevotella* and *Veillonella*. Groups that were seen more sporadically included *Lactobacilliaceae*, *Lachnospiraceae*, and *Betaproteobacteria.* Patients typically had at least 3 species at co-dominant titers, and these changed daily, often by an order of magnitude.
5. Surprisingly, total bacterial titers and overall compositions at the branch levels were not dramatically different in control versus xerostomia groups.
6. However, several subgroups of bacteria (*Lachnospiraceae* and *Bacteroidaceae*) were reduced by 5 to 20 fold on average among xerostomic patients.
7. However, there were many bacterial and fungal species that were more prevalent in patients with low saliva flow than those with high flow, and others bacterial species that were more prevalent in high flow. These differences were enhanced by considering only patients whose xerostomia was induced by medication.
8. Nightly oral routines did have an impact by reducing next-morning saliva bacterial compositions and titers, but this was highly variable between individuals and routines. Overall, Crest toothpaste and mouthwash routines reduced titers more in more individuals.

4.3 SSB/FLB as storage/lysis buffer

Microbial DNA can be effectively stored in SSB buffer for at least 30 days, and efficiently extracted for qPCR with my modified FLB extraction protocol. With all of the previous experiments on buffer/lysis saliva storage and extraction efficiency, it was in the study's best interest to use the saliva storage buffer SSB with lysis of FLB. The SSB has been

optimized for the saliva samples in this study, finding that a 4.0 mL kills cells in the saliva and prevents nucleic acid degradation for at least 30 days at room temperature. We also optimized sample DNA extraction, finding that microbes pelleted from the storage solution, then subjected to hot detergent–lysis, is as good or better across phyla than commercial DNA extraction kits.

4.4 Culture cell library

Oral bacterial species compositions identified by culture and qPCR were diverse and were largely consistent with those characterized by NGS in the literature. Of my 110 sequenced cultures I found 33 different species from 8 different Families. I was able to culture 3 of the 6 major phyla from HOMD (*Firmicute, Actinobacteria,* and *Proteobacteria)* [3]. All of my cultures were not identified by sequencing, because of lack of funds. Therefore, I cannot compare distinctions between xerostomia individuals and control saliva cultures. Also, I did not culture saliva on media that supported fungi growth and in-turn I cannot come to any conclusions on the fungal populations in my culture library. However, my selective sequencing did identify species consistent with the literature on oral microbiota, including: *Lactobacilli spp.* [17, 25], *Neisseria, Rothia, Gemella* [28], *Streptococcus, Neisseria,* and unclassified bacteria (*Uncl)* [2]. This validates the quality and analysis of my sequencing data. With further sequencing of the entirety of my library, I am confident that we would be able to either confirm or refute the culture differences in xerostomia or healthy individuals.

4.5 Xerostomia vs. Control Arm

Saliva flow tests showed significant reductions in averages of unstimulated flow, stimulated flow and saliva flows sum in self-reported xerostomia versus controls. However, there was a great deal of overlap between these two groups. This could be due to poor categorization based on ambiguous symptoms and self-reporting, in the first place. Individuals in the control group that matched volumes of those in the self-reported xerostomia group could have never noticed that they actually have low spit flow. In this case there could have been poor tools to put each individual in each group. There was no significant difference in the pH of these two groups.

Surprisingly, bacterial titers and overall compositions at the branch levels were not significantly different in control versus xerostomic groups. Oral bacterial compositions were dominated by *Streptococcus* species and those in the *Veillonella* target groups, in both control and xerostomia groups. These saliva compositions were also found to be part of the major core genera in healthy oral saliva in literature [2]. Literature also stated that *Streptococcus* was the most abundant groups that dominated nearly all of the oral mucosal sites [4], this was also found true in my volunteers' saliva. *Streptococus* and *Veillonella* [28] were also prevalent in Xerostomia patients. A core group of bacteria seen in all patients included *Streptococus, Fungi, Fusobacterium, Actinobacteria, Bacteriodaceae/ uc Prevotella* and *Veillonella.* Patients typically had at least 3 species at co-dominant titers, and these changed daily.

Comparing target levels with-in the groups revealed that there was an 18 fold reduction in *Bacteroidaceae* and a 5 fold reduction in *Lachnospiraceae* average titers in xerostomia. In fact, all other significant differences between the two groups (less than 5 fold difference) showed a

reduction in xerostomia. A logical explanation for this could be from the amount of saliva tested, xerostomia patients could have had a harder time getting saliva into the test tubes causing this "reduction" of cells. This also held true when comparing high flow to low flow and "medication-induced xerostomia" (MIX) to controls.

However, there were many specific bacterial and fungal species that were more prevalent in xerostomia patients than controls, and other bacterial species that were more prevalent in controls. Xerostomia patients were found to have a higher prevalence in species OidesITS-81.6, LBITS-90.2, BProITS-84, Fungi-84.4, Fungi-84.8, and Fungi-86.6. Some of these differences were enhanced when comparing groups of low flow to high flow, such as Fungi-84.4, Fungi-84.8, Fungi-86.6, LBITS-90.2. There were also higher prevalence seen when comparing MIX to controls in: BuITS-81.2, Fungi-86.4, BProITS-84.0, and LBITS-87.6. BuITS-84.6, LBITS-84.6 were only seen in MIX. These species being higher in prevalence or found only in our dry mouth patients could explain their negative oral hygiene. Fungi, for instance was found to be involved with oral mucosal disorders [15] and Lactobacillus has been found to be associated with dental caries [9]. The species OidesITS-88.6 was non-existent in any of the low flow patients, and BProITS-85.6 and OidesITS-90.0 were non-existent in the MIX patients. These species that are lacking in the dry mouth patients could be the bacterial that is stabilizing the healthy oral microbiome. Such as, *Bacteriodetes* have been abundant in healthy oral saliva [2]. Identification of these species through sequencing can give us a better understanding of their role.

4.6 Nightly Oral Hygiene Practice Arm

Nightly oral routines did have an impact by reducing next-morning saliva bacterial compositions and titers, but this was highly variable between individuals and routines. Average titers across all individuals and *Lactobacilli* and *Fusobacterium* targets titers were significantly reduced after Listerine mouthwash. Across all species showing differences, Listerine mouthwash was found to reduce prevalence. All average target titers and species after nightly use of Crest mouthwash was significantly reduced. The nightly use of Crest toothpaste showed 4-fold decreases in average titers of bacteria 16s, *Lactobacilli*, *Lachnospiraceae*, and *Fusobacterium*, as well as decreases in significant species. Colgate toothpaste resulted in significant decrease in *Lactobacilli* and was also significantly reduced in several species prevalence. The use of these nightly routines were found to significantly reduce average titers and certain species, as expected.

However, individually volunteers had widely varying reductions in bacterial titer populations following a specific nightly routine. In most cases only few volunteers had greater than 5-fold decreases in bacterial titers after use of a nightly routine. Across all the targets tested and nightly routines tested, there were only 6 instances in which Colgate toothpaste reduced titers by more than 5-fold, only 11 times after Listerine mouthwash. Crest mouthwash and toothpaste reduced titers more often, 14 occurrences each. Overall, Crest toothpaste and mouthwash routines reduced titers more in more individuals. The groups affected most varied between the two, suggesting that a combination of both treatments might be synergistic.

4.7. Study Limitations

1. Only saliva samples were taken, restricted by feasibility, so bacterial compositions in other oral niches, such as embedded in subgingival plaque, or tongue, were not collected and therefore might be more of a factor in xerostomia or in assessing nightly routines.
2. Data from the xerostomia versus control patients' needs more analysis at the individual level to supplement my analysis of averaged data.
3. Amplicons that were identified by melting temperatures as being more prevalent in one group or after a nightly routine, need to be identified to species by sequencing.
4. A number (155) of species that are potentially not in public databases were found by sequencing but need further analysis to confirm whether they truly represent novel species.
5. Funding restricted use of NGS.

4.8. Future studies

In addition to conducting experiments to address the limitations listed in Section 4.7, if I had another 6 months, these are the experiments I would pursue.

In the xerostomia arm, I would address the potential role of species that were more prevalent in patients with low saliva flow. Since total bacterial loads were not dramatically different, the poor oral hygiene status of the group (e.g. twice the incidence of cavities) may result from virulence traits of these species, such as elaboration of metalloproteinases or enhanced biofilm. Therefore, I would perform in vitro assays of relevant phenotypes, on pure cultures of species I identified in this study to be more prevalent in xerostomic samples.

In the oral hygiene arm, I would pursue the observation that specific individuals show much more dramatic reductions to a nightly oral routine than others. My hypothesis would be that this variation results from specific differences in starting bacterial compositions, at the species or even strain level. This effort would be enhanced by NGS analysis, which I could perform on pooled and barcoded DNA samples in my repository. I would also test whether pure cultures of species unique to high-responding patients were more sensitive to the mouthwashes or toothpastes than their cousins from non-responding patients, with standard microbroth dilution and viability assays.

The highly individualized responses shown after specific nightly hygiene practices suggest that a large-scale prospective study is warranted. Participants' responses to each routine would be characterized as I did, and then they would be assigned their optimal hygiene regimen, and tracked long-term with monthly sample monitoring. The issue I'd focus on, is whether consistent reduction in one or more specific bacterial groups, which would differ in each cluster of patients is strongly correlated with better oral hygiene. This I would quantify at the dentist, with cavity and gum health assays. I'd also include a comparison group that was randomized with respect to which nightly routine caused the largest reductions, to determine if individualized, non-random routine groups had better oral health outcomes.

APPENDIX A: QUESTIONNAIRE

Code ID:_____ Date:_____
Dental health questionnaire
Age:_____ Sex:_____ Race:_____ Ethnicity:_____
Have you read and signed the informed consent form for this study? YES ___ NO___
1. Are you currently in any pain? YES ___ NO___ Describe if yes_____
2. Have you been hospitalized in the last two years? YES ___ NO___ Reason if yes_____
3. Have you seen any physicians in the last two years? YES ___ NO___ Reason if yes_____
4. Have you been taking any medication in the past two years: YES ___ NO___ Describe if yes_____
5. Are you allergic to anything? YES ___ NO___ Describe if yes_____
6. Circle any condition you have had, or box if you currently have it:

AIDS/HIV	Asthma	Radiation treatment
Heart Disease/Failure	Cold Sores	Chemotherapy
Liver Disease	High blood pressure	Shortness of breath
Heart Attack	Seasonal allergies	Kidney problems
Hepatitis A, B, or C	Epilepsy/seizures	Psychiatric care
Diabetes	Sinus problems	

7. How often do you brush? Once daily ____ Twice daily ____ Three times daily___ Other____
8. What brand of toothpaste do you currently use?_____
9. Your brush is: Soft ___ Medium ___ Hard___
10. Your brush is: Manual____ Electric____ Brand_____
11. How often do you floss? Once daily ____ Twice daily ____ Three times daily___ Other____
12. How often do you use a fluoride mouthwash? Once daily ____ Twice daily ____ Three times daily___ Other____ Brand_____
13. How often do you see your dentist? Once a year____ Twice a year____ Other_____
14. When you visit your dentist, do you most often have:
a. No new cavities___ b. One new cavity___
c. Two new cavities___ d. Other___
15. How many total cavities have you had filled? _____
16. How many root canals have you had?_____
17. How many crowns have you had?_____
18. Do you wear dentures? YES____NO____ If yes, for how long have you had them?_____
19. Have you ever been treated for periodontal/gum disease? YES ___ NO___ Most recent date:_____
20. Have you had cosmetic dentistry, such as caps or veneers? YES____ NO____ List_____
21. Would you say your mouth is: comfortable?_____ moderately uncomfortable_____ very uncomfortable____ if so, describe:_____
22. Do you have or have you ever had any of the following, if so, what was the most recent incident:
- Bleeding, sore gums? YES ___ NO____ When?_____
- Loose teeth? YES ___ NO____ When?_____

- Frequent unpleasant taste/ bad breath? YES ___ NO____ When?_____
- Sensitivity to hot? YES ___ NO____ When?_____
- Sensitivity to cold? YES ___ NO____ When?_____
- Sensitivity to sweets? YES ___ NO____ When?_____
- Sensitivity to biting? YES ___ NO____ When?_____
- Burning tongue/lips? YES ___ NO____ When?_____
- Frequent dry mouth? YES ___ NO____ When?_____
- Frequent blisters on lips or mouth? YES ___ NO____ When?_____
- Swelling/lumps in mouth? YES ___ NO____ When?_____
- Ortho treatments (braces)? YES ___ NO____ When?_____
- Oral surgery? YES ___ NO____ When?_____
- Food impaction? YES ___ NO____ When?_____
- Problems with biting cheeks/lips? YES ___ NO____ When?_____
- Problems with clenching/grinding? YES ___ NO____ When?_____
- Problems with clicking/popping jaw? YES ___ NO____ When?_____
- Difficulty opening or closing jaw? YES ___ NO____ When?_____
- Shifting in bite? YES ___ NO____ When?_____

APPENDIX B: MEDIA

Media Name	Media Type	Media Full Name	Ingredients
Blood TSA	Agar	Trypticase Soy Agar + human blood	30 g TSA powder, 15 g agar powder, 1 L deionized water, 50 mL human blood
MLT Max	Agar	Mes-Lib-Thio-Casein-Starch	15 g Proteose Peptone No3, 4 g MES powder, 5 g Glucose, 5 g yeast extract, 5 g NaCl, 5 g Casein enzymatic hydrolysate, 1 g starch, 0.5 g Cysteine HCL, 1.25 mL Thioglycollate, 1 L deionized water
Rogosa Agar	Agar	Rogosa Agar	660 mL deionized water, 15 g Agar, 330 mL Rogosa stock
TSA	Agar	Trypticase Soy Agar	30 g TSA powder, 15 g agar powder, 1 L deionized water
YPD + AMP	Agar	Yeast extract/ Peptone/ Dextrose Agar	10 g Peptone, 5 g yeast extract, 20 g dextrose, 15 g Agar, 1 L deionized water, 1ml of a 10 mg/mL stock of Ampicillin added after autoclaving
LIB + Supplements	Broth	*Lactobacillus iners* Broth+Supplements (modified from ATCC medium 1685 NYC III)	0.5 g/L cysteine, 4 g HEPES, 15 g Proteose Peptone No.3 (BD 211693), 5 g NaCl, 875 mL deionized water, 5 g Glucose, 25 mL Fresh Yeast Extract (Gibco 360-8180), 100 mL Heat inactivated horse serum
MYEG	Broth	Milk/Yeast Extract/Glycerol	130 g dried milk, 5 g yeast extract, 10 g dextrose, 1 L deionized water, autoclave and cool to add 150 mL 100% sterile glycerol
FLBA	Buffer	Fungal Lysis Buffer-A	8 mL deionized water, 2 mL 10% TritonX114, 20 µL 0.5 M EDTA
FLBB	Buffer	Fungal Lysis Buffer-B	2.5 mL 2 M Tris pH 8.3, 0.2 mL 0.5 M EDTA, 0.41 mL conc. HCL, 22 mL deionized water
1 x PBS	Buffer	1 x Phosphate Buffered Saline	8 g 137 mM NaCl, 0.2 g 2.7 mM KCl, 1.44 g 10 mM Na_2PO_4, 0.24 g 1.8 mM KH_2PO_4 (Cold Spring Harbor Laboratory Press, NY)
1 x SB Buffer	Buffer	1 x Sodium Boric acid Buffer	~3700 ml deionized H2O (genetic), 100 mL 40 x SB, 80 uL Ethidium Bromide [52], Check pH to be ~8.0, make as 40X stock
SSB	Buffer	Saliva Storage Buffer	800 mL deionized water, 5.88 g sodium citrate, 700 g ammonium sulfate, 40 mL 0.5 M EDTA, adjust pH to 5.2 with sulfuric acid
1 x TE	Buffer	1 x TE	10 mM 83 Tris, 1 mM EDTA

2% Agarose LE Gel	Gel	2% Agarose LE Gel	250 mL deionized water, 5 g Agarose LE Powder, 6.25 mL 40 x SB Buffer, 5 uL Ethidium Bromide

APPENDIX C: ORAL CELL LIBRARY

Table can be found in:

https://drive.google.com/drive/folders/0BwlH15OQpZ4kfjVPV3RxSzd4VkNJSDN1TnFVZjhFaWY1d2pnUFdmdUd5czVTQ2g0XzVRaWs

APPENDIX D: QPCR PRIMERS

Table can be found:

https://drive.google.com/drive/folders/0BwlH15OQpZ4kfjVPV3RxSzd4VkNJSDN1TnFVZjhFaWY1d2pnUFdmdUd5czVTQ2g0XzVRaWs

APPENDIX E: SEQUENCING MATCHES

Table can be found:

https://drive.google.com/drive/folders/0BwlH15OQpZ4kfjVPV3RxSzd4VkNJSDN1TnFVZjhFaWY1d2pnUFdmdUd5czVTQ2g0XzVRaWs

APPENDIX F: QUESTIONNAIRE FOR XEROSTOMIA AND CONTROL PATIENTS

Table can be found:

https://drive.google.com/drive/folders/0BwlH15OQpZ4kfjVPV3RxSzd4VkNJSDN1TnFVZjhFaWY1d2pnUFdmdUd5czVTQ2g0XzVRaWs

APPENDIX G: XC TITERS

Please see:

https://drive.google.com/drive/folders/0BwlH15OQpZ4kfjVPV3RxSzd4VkNJSDN1TnFVZjhFaWY1d2pnUFdmdUd5czVTQ2g0XzVRaWs

APPENDIX H: XC DOMINANT SPECIES

Please see:

https://drive.google.com/drive/folders/0BwlH15OQpZ4kfjVPV3RxSzd4VkNJSDN1TnFVZjhFaWY1d2pnUFdmdUd5czVTQ2g0XzVRaWs

APPENDIX I: QUESTIONNAIRE FOR NIGHTLY ROUTINE VOLUNTEERS

Please see:

https://drive.google.com/drive/folders/0BwlH15OQpZ4kfjVPV3RxSzd4VkNJSDN1TnFVZjhFaWY1d2pnUFdmdUd5czVTQ2g0XzVRaWs

APPENDIX J: NIGHTLY ORAL ROUTINE TITERS

Please see:

https://drive.google.com/drive/folders/0BwlH15OQpZ4kfjVPV3RxSzd4VkNJSDN1TnFVZjhFaWY1d2pnUFdmdUd5czVTQ2g0XzVRaWs

APPENDIX K: NIGHTLY ORAL ROUTINE DOMINANT SPECIES

Please see:

https://drive.google.com/drive/folders/0BwlH15OQpZ4kfjVPV3RxSzd4VkNJSDN1TnFVZjhFaWY1d2pnUFdmdUd5czVTQ2g0XzVRaWs

APPENDIX L: REDUCTION BY NIGHTLY ROUTINE PER INDIVIDUAL

Please see:

https://drive.google.com/drive/folders/0BwlH15OQpZ4kfjVPV3RxSzd4VkNJSDN1TnFVZjhFaWY1d2pnUFdmdUd5czVTQ2g0XzVRaWs

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ABSTRACT

CHARACTERIZATION OF ORAL MICROBIOTA IN XEROSTOMIC VERSUS NON-XEROSTOMIC VOLUNTEERS AND IN DAILY SAMPLES FOLLOWING STANDARD ORAL HYGIENE PRACTICES

by

CHELSEA ANN MYERS

August 2015

Advisor: Professor Robert Akins

Major: Biochemistry and Molecular Biology

Degree: Master of Science

A thesis presented on the characterization of oral microbiota in xerostomic versus non-xerostomic volunteers and in daily samples following standard oral hygiene practices. Xerostomia is a difficult and burdensome disease that can be very difficult to diagnose. Understanding the oral microbiota between these diseased and healthy (non-xerostomic) can give us great insight on new treatments and/or prevention. Goals of the study included determining whether there substantial differences in oral microbial populations between the two groups, and whether varying nightly oral hygiene practices had an impact on next-morning oral microbiota titers or composition. Microbial loads were determined by qPCR using broad-spectrum primers. Microbial compositions were estimated based on melt curve analysis of amplicons that spanned the internal transcribed spacer between small and large ribosomal RNA genes, and by qPCR using phylogenetic branch-specific primers. The project succeeded in developing and optimizing

a storage media that allowed 30 day room temperature storage, and an DNA extraction method that outperformed commercial kits.

The xerostomia versus control study used three sequential daily saliva samples, collected from 18 xerostomia and from 18 healthy, control volunteers. Fungal populations and several potentially novel species were found to be more significantly prevalent in xerostomia patients as compared to healthy ($P = 0.001$). Surprisingly, total bacterial titers and overall compositions at the branch levels were not dramatically different in control versus xerostomia groups. 6. However, several subgroups of bacteria (Lachnospiraceae and Bacteroidaceae) were reduced by 5 to 20 fold on average, and specific species were less prevalent; among xerostomic patients, and none were elevated. These studies suggest fungal species may play a role in the poorer oral hygiene of xerostomic patients and that more detailed analysis using next generation sequencing is warranted.

Mouthwashes and toothpastes are composed of several different ingredients, many of which purported to have anti-caries or anti-gingivitis activities. However, the quantitative impact of these is not well studied. The objective to this part of the thesis was to examine the shift in populations after a specific oral hygiene practice repeated over 5 nights and assayed from saliva the next mornings. This essentially uses the mouth as in incubator for microbial regrowth. A total of 30 saliva samples were collected from 17 individuals the morning after a given nightly oral hygiene practice, including no routine, Listerine mouthwash only, Crest mouthwash only, Crest toothpaste only, and Colgate toothpaste only. These samples were analyzed using qPCR and sequencing. Total bacterial loads returned to approximately the same levels after the 4 routines

compared to no routine. Overall, Crest toothpaste and mouthwash routines reduced titers more in more individuals. However, individual routines did have an impact by reducing next-morning saliva bacterial compositions and titers of specific groups, but these reductions were highly specific to the individual and the routine. This suggests that we have highly individualized responses to common oral hygiene products, and that tailoring our choice of these to optimize specific bacterial group reductions could improve oral health.

AUTOBIOGRAPHICAL STATEMENT

My name is Chelsea Ann Myers. I was born in New Baltimore, Michigan. My parents' names are Harry and Melissa Myers. I have three sisters, Kayla, Heather and Carrine. I graduated from Anchor Bay High School and started my college career at Macomb Community College on a full-ride cross-country running scholarship. After completing two years at Macomb Community College, I transferred to the University of Michigan where my long-term academic goals were to go to dental school and become a dentist. While at the University of Michigan, I volunteered in a lab at the dental school. My involvement in the project earned me second and third other in two separate scientific research articles in the *Journal of Dental Research*. This accomplishment sparked my love of research in dentistry. I then continued my education at Wayne State University School of Medicine with my Masters of Science in Biochemistry and Molecular Biology; I was fortunate enough to study oral microbes in my masters. This masters has been a very good experience and now I will be attending Indiana University School of Dentistry for my Doctor of Dental Surgery (DDS) degree to pursue my dreams of becoming a dentist.