Distant and Local Redistribution of Oral Bacteria

Associated with Periodontitis

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THESIS

Submitted as a partial fulfillment of the requirements
for the degree of Master of Science in Oral Sciences
in the Graduate College of the
University of Illinois at Chicago, 2012

Chicago, Illinois

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ACKNOWLEDGEMENTS

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LIST OF ABBREVIATIONS

DNA	Deoxyribonucleic acid
NHANES	National Health and Nutrition Examination Survey
PCR	Polymerase Chain Reaction
RNA	Ribonucleic Acid
rRNA	Ribosomal Ribonucleic Acid
SDS	Sodium Dodecyl Sulfate
tRNa	Transfer Ribonucleic Acid

SUMMARY

It is documented that periodontitis is commonly associated with changes in localization and proliferation of a variety of oral bacteria. However, it is not clear if there are changes in the oral bacteria localized to oral mucosa while chronic infections such as periodontal diseases are present. A demonstration of this relationship could give us insight into the connection between inflammation and the bacterial species in the oral cavity.

Recent laboratory findings show oral bacteria such as *Streptococcus* species will alter their adherence capacity and bind to oral keratinocytes, especially when tobacco or alcohol are present. Tobacco has also been associated with enhanced incidence and severity of periodontal diseases.

Multigenomic identification was used to evaluate 12 subjects and 10 controls for the types of oral bacteria present on the tongue in comparison to gingiva from a periodontitis site (e.g., site of attachment loss) and from a non-periodontitis site (e.g., no evidence of attachment loss).

The data showed that in patients with periodontitis, an average of 52.4 +/- 18.7 different bacterium species were identified on the tongue and an average of 15.9 +/- 4.8 of these were *Streptococcus* species**.** The control subjects had an average of 23.1 +/- 9.4 bacterium species on the tongue and an average of 11.1 +/- 2.99 of these were *Streptococcus* species. Gingival samples showed a similar trend. However, 57% of the bacteria at a 60% level of identification frequency were non-*Streptococcus* species, while control non-periodontitis subjects exhibited a 33% identification frequency of non-

SUMMARY (continued)

Streptococcus species. Current tobacco users also showed a few novel species found on the tongue that were not present in non-smokers.

This study demonstrates a trend of bacteria distribution changes among individuals with active bone loss associated with periodontitis. We suggest this bacterial distribution modification is anticipated to effect integrity of tongue mucosa.

I. INTRODUCTION

A.   **<u>Background</u>**

Like many other communities, the microbes living in the oral cavity are a diverse group that has been well studied because it is very assessable. The ecosystem of microorganisms in the human oral cavity is referred to as the oral microflora, oral microbiota, or oral microbiome (24). More than 600 species have been recognized, and as new techniques for identification become available, more will surely be found (1). Dental research has examined many of the bacteria found on tooth surfaces or in the gingival sulcus, but less attention has been given to the oral mucosa, an area that harbors many organisms. More research is needed to examine the microorganisms on the mucosa and the changes that occur when there are shifts to the oral environment. It is our expectation that insights into changes in microflora distribution will provide a better understanding of specific mucosa surface resistance to infection and changes in tissue integrity.

Recent evidence has shown that certain bacteria, including *Streptococcus* species will adhere to oral keratinocytes in the presence of chemicals such as ethyl alcohol or tobacco smoke. The mechanism of survival of Streptococcus in these areas is through a change in physiology with expression of a heparan-binding protein designated histone-like protein A (HlpA). This protein is considered to be a virulence factor (2-5). This initial finding has lead to further interest in determining the affect of outside influences on the oral bacteria.

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To attain this goal we must accurately identify the bacterial species present in biofilm on selected mucosa surfaces. This study was conducted in conjunction with Dr. Bruce Pasten, who is a leading investigator in The National Institutes of Health Human Microbiome Project (HMP). Therefore this project parallels the goal of the HMP to examine microbial genomes on tissues such as the oral mucosa (52).

B.   **Purpose of Study**

This study sought to evaluate the microbial biofilm on several oral mucosa surfaces in relation to an inflammatory response in response to a patient's oral flora. Specifically, we examined periodontitis, a chronic, multifactorial disease that is associated with continuous chronic inflammation. Moreover, patients with and without periodontitis were included to determine if chronic inflammation in the gingival sulcus was associated with an increase in bacteria outside of the sulcus; for example, the tongue. Tobacco use has been previously determined to increase severity and risk for periodontal diseases, but it is unclear as to its effects upon distribution change among oral microbiome

II. LITERATURE REVIEW

A. **<u>Framing the Relationship</u>**

The composition of microflora in the oral environment is dependent on many factors including host physiology and chemistry. This composition is constantly evolving in a survival of the fittest-type manner and varies across surfaces. As of late, much attention has been paid to the bacterial ability to form colonies on oral surfaces such as gingival epithelium or tooth structure. The concept of the biofilm describes bacterial colonies adhering to each other and the surface on which they are growing through a matrix. In this type of situation, microbes have the advantage of interaction through quorum sensing which can increase their survival potential (6-8).

It is unclear how bacteria affect the oral environment in which they live and how they are affected by the changes in their surroundings. The types of bacteria are not only altered by host factors, but also external factors such as chemicals, diet, and agents released from habits such as tobacco and alcohol. These types of influences can cause mutations in microorganisms and oral cells, affecting the interaction between microbes and epithelial cells, and both genotype and ultimately the phenotype of microbes and epithelial cells (9-11).

B. **<u>Survival and Success of Microorganisms</u>**

The ability of a microorganism to survive is judged by testing the fitness of the organism. This test takes into account the genetic variations of the organism that would allow for adaptation to changes in the oral environment. The ultimate goal and a high

fitness level would be an organism that could attach and colonize a surface and be assessable for identification. A bacteria's persistent presence in an environment is indicative of a high level of fitness. However, it is difficult to accurately ascertain the level of fitness due to the sampling methods available. Multiple samplings of a particular environment would help to determine the fitness of species and also to determine when the species peaks in their ability to adapt (12,13).

In the oral cavity like other biological systems, the concept of robustness or the ability to maintain stability regardless of internal and external stimuli is important. The bacteria's ability to mutate allows for continued adherence and survival and ultimately identification. Mutations can be spontaneous or inducible, and although mutations occur directly due to a change in the environment, they also naturally occur due to exposure to various substances, which alter growth and are sometimes favorable to survival enhancement. Salivary characteristics, diet, habits, and chemicals can all affect mutations in both microbes and oral cells because they influence not only interactions but also survival between the two entities. The association between environmental influence and mutation becomes evident and significant when the oral cavity is exposed to carcinogens such as tobacco product derived poly-cyclic aromatic hydrocarbons (PAH) and tobacco specific nitrosamines (TSNA) or alcohol derived acetaldehyde (AA). To achieve survival bacteria form biofilms that undergo constant remodeling. If too great an insult occurs, some common microbiota may lose their ecologic niche due to overgrowth of opportunistic infections that can damage oral surfaces. Robustness of the common microbes keeps this overgrowth to a minimum (14,15).

In both oral keratinocytes and *Streptococcus* species, alcohol can be metablolized by alcohol dehydrogenase (ADH), of which there are several variants. These genes produce acetaldehyde and then acetate by aldehyde dehydrogenase (ALDH). *Streptococcus* species can metabolize alcohol, and then attach to oral keratinocytes to generate large colonies. The levels of both alcohol and acetaldehyde in the oral cavity vary depending on exposure, concentration, salivary flow, and of course the metabolic activity of bacteria and the host keratinocytes, which comprise the mucosa (16-20).

C.   **The Effect of the Oral Environment on Survival of Microorganisms**

Saliva is another important factor that can affect the survival of organisms and contains many proteins such as mucins, lactoferrin, histatins, glycosaminoglycans, polysaccharides and immunoglobulins. Some of these proteins, such as proline-rich proteins can facilitate attachment of bacteria to teeth. Components such as glycosaminoglycans and histone binding proteins can aid in adherence to oral keratinocytes. Other salivary proteins such as amylases, cystatins, and mucin proteins can also interact with microbes. Proteins like amylases play a regulatory role in bacterial adhesion to mucosal surfaces, while mucins have been shown to be antibacterial. Saliva as protection of oral surfaces is evidenced by an increase in bacterial insult and a loss of lubrication (21-23).

The fore-mentioned characteristics of the oral biologic environment demonstrate the need for further specific analysis for changes in oral microbiome associated with the use of tobacco products and presence of continual host inflammatory responses observed in oral periodontum sites.

D.   **<u>Studies in Oral Microflora</u>**

A recent study found that there are 1,179 taxa present in the oral cavity: 24% were named (e.g., 280 bacterial species from the oral cavity isolated in cultures and named), 8% cultivated but unnamed, and 68% uncultivated (24). Zaura et al. attempted to identify the core species in the mouth by sampling several niches in the oral cavity (dental surfaces, cheek, hard palate, tongue, saliva) of 3 patients using 16s rRNA. They found 88-104 higher taxa, the most common being *firmicutes (Streptococcus, Veillonellacaeae, Granulicatella), proteobacteria (Neisseria, Haemophilus), actinobacteria (Corynebacterium, Rothia, Acitnomyces), bacterioidetes (Prevotella, Capnocytophaga, Porphymonas)* and *fusobacteria (Fusobacterium).* The most variation in species was found in dental samples, while the cheek showed the most uniformity. The 3 individuals shared 1660 of the 6315 unique sequences found, which also accounted for 66% of the reads, and these were identified as the core micobiome (25). According to Dewhirst, et.al. the Human Oral Microbiome (HOM) database, includes 619 taxa in 13 phyla: *Actinomyces, Bacteriodes, Chlamydiae, Chloroflexi, Euryarchaeota, Firmicutes, Fusobacteria, Proteobacteria, Spirochaetes,* SR1*, Synergistetes, Tenericutes,* and TM7 (24).

Similarly, other studies have shown that bacteria vary by area. Data from 5 healthy subjects revealed 141 predominant species. Over 60% of these species have not yet been cultivated. Some species such as *Streptococcus mitis* and *Granulicatella adiacens* were detected in almost all oral sites, where others were more site-specific. For example, *Rothia dentocariosa, Actinomyces species, Streptococcus sanguis, Streptococcus gordonii*, and *Abiotrophia defectiva* preferentially colonized teeth, while *Streptococcus salivarius* was found mostly on the dorsum of the tongue. *Streptococcus sanguinis* and *Streptococcus australis*

seemed to have a predilection for soft tissue, while *Simonsiella muelleri* colonized the hard palate.

Aas et. al. examined five subjects, with an age range of 23 to 55 with no sign of oral mucosal disease and healthy periodontal tissues. They defined bacterial species or phytoypes from tongue dorsum, lateral sides of tongue, buccal epithelium, hard palate, soft palate, supragingival plaque of tooth surfaces, subgingival plaque, maxillary anterior vestibule and tonsils. Species from all sites belonged to the genera: *Gamella*, *Granullicatella*, *Streptococcus*, and *Veillonella*. These investigators concluded that normal healthy oral mucosa had a varied bacterial flora, which was site specific (26). Moreover, oral flora associated with periodontitis such as *Porphymonas gingivalis, Tannerella forsythia*, and *Treponema denticola* were not detected in healthy mouths. Flora usually found in dental caries or deep dental cavities were also not found, including *Streptococcus mutans, Lactobacillus* species*, Bifidobacterium* species, and *Atopobim* species (26).

A comparable study by Mager et al. also found that bacteria varied depending on location in the oral cavity. The authors took brush-biopsies from 8 surfaces in the mouths of 225 systemically healthy patients. 44 of these subjects also gave plaque samples. They found that *Veillonella parvula* and *Prevotella melaninogenica* were higher in saliva and on lateral and dorsal surfaces of the tongue. *S. mitis* and *Streptococcus oralis* were in lower proportions in these areas. Supra and subgingival plaque differed from other areas because they had a greater proportion of *Actinomyces* species (27).

There may also be a genetic component to the bacteria found in the oral cavity. Moore et al. found that there were more common bacteria between identical twins than fraternal twins or unrelated children (28). It seems that there is truly a large group of bacteria that are found in

most people, however there is also a great deal of variation. In addition, there are many unidentified bacteria in the oral cavity.

Much of the research on oral microflora has been limited to the community residing on the teeth. The microflora in this area have been described as a biofilm because of their ability to act as a unit and change as a result of therapeutic intervention or changes in the host immunity (29). The three distinct steps of colonization on the tooth surface are the formation of the pellicle (comprised of salivary constitiuents such as albumin, glycoproteins, proline-rich proteins, mucins and cell debris), attachment of primary bacterial colonizers such as Streptococcus species, and finally colonization of mid and late colonizers by cell-to-cell interactions (30).

E.   **Periodontitis and Oral Microflora**

The oral microflora is not static, but can change and evolve. Diseases such as periodontal disease can cause a change in the flora as demonstrated by the classic 1965 study of experimental gingivitis, Loe et al. showing that the plaque biofilm changed from gram-positive cocci to gram-negative motile rods and spirochetes as gingivitis developed (31). Listgarten noted similar differences in the morphology of organisms between healthy patients and those with periodontitis (32). Although the microbes of plaque change in disease, there is also evidence that there is no difference between bacteria from a healthy site versus a diseased site in a patient with periodontitis. Moore et al. found that cultures from the healthy and diseased sulci of patients with periodontitis did not differ significantly in microbial species. However, there was a difference between species in patients with periodontitis compared to healthy patients (33).

Periodontitis is characterized by inflammation leading to the breakdown of attachment to

teeth due to bacterial insult. In 1999, the prevalence of periodontal disease was estimated in the NHANES III study to be present in 35% of adults aged 30-90 years in the United States (34). However, after revisiting the methods of the study, it was decided that the partial examination of the mouth used in the NHANES III lead to an underestimation of disease. It is hypothesized that in United States of America there is about 50% of adults aged 30 or greater with periodontitis (35,36).

The link between periodontitis and systemic health has become increasingly apparent. The best-established connection is between periodontitis and diabetes. In fact, periodontitis has been called the 6th complication of diabetes (37). The increased blood glucose found in diabetes leads to advanced glycation end-products (AGEs) that can lead to complications such as nephropathy and neuropathy. These AGEs increase the inflammatory response to bacteria and can also have an effect on vascular permeability leading to a greater breakdown of collagen. Conversely, the inflammation present in periodontitis can have an effect on diabetes and studies have shown that patients with active disease have less control of their blood sugar (38).

A possible connection between periodontitis and cardiovascular disease has also been investigated. Although the evidence is not concrete, there has been studies that show that active periodontitis causes an increase in c-reactive protein, an inflammatory protein that is a good predictor of cardiovascular disease (39). However, the evidence does not suggest a causal relationship, but merely an association between the two diseases. Similarly, some evidence suggests that there may be an association between periodontitis and problems in pregnancy such as low birth weight and preterm birth. The connection is hypothesized to be due to a bacteremia or increase in inflammation (40).

The connection to these systemic issues and the need for effective treatment has lead to an interest in determining the bacteria present in periodontal disease. Socransky and Haffajee found that the following bacteria were associated with periodontitis: *Porphymonas gingivalis, Prevotella intermedia, Treponema denticola, Tannerella forsythensis, Campylobacter rectus, Selenomonas* species, *Aggregatibacter actinomycetemcomitans, Eubacterium timidum, Fusobacterium nucleatum*, and *Peptostreptococcus micros* (41). However, due to the large number of oral flora, it is difficult to isolate the putative pathogens of periodontitis. In a study by Kumar et al., authors used 16S rRNA primers to identify new bacteria associated with chronic periodontitis in plaque. Several new species from the Deferribacteres phylum, the Bacteroidetes phylum, OP11 phylum, TM7 phylum in addition to the *Eubacterium saphenum, Porphymonas endodontalis, Prevotella denticola*, and *Cryptobacterium curtum* species were found (42). Studies have shown that the amount and type of bacteria vary depending on treatment, and that periodontal therapy can decrease the amount of bacteria in the sulcus and maintain the lowered level over an extended period of time (43). Historically the literature discussing bacteria associated with periodontitis has examined samples taken from the sulci of diseased or healthy sites in patients with periodontitis.

More recently, with the growing interest in determining the microbiology of the oral cavity, scientists have also examined the effect of periodontitis on the oral microflora in general. In one study, the authors used culture and phase microscopy to show that there was an association between periodontal breakdown and the presence of black-pigmented bacteria such as *Prevotella intermedia* and motile organisms on the tongue. They concluded that the tongue could serve as a habitat for periodontal pathogens (44). Similarly, Dahlen et al.

examined the tongues of diseased and non-diseased subjects and found that both had all 7 of the putative periodontal pathogens tested for, including *Porphymonas gingivalis, Prevotella intermedia, Campylobacter rectus*, and *Actinobacillus actinomycetemcomitans*. However, *Porphymonas gingivalis* was found more frequently on the tongue samples of periodontally diseased individuals (45). On the other hand, another study found that *A.actinomycetemcomitans* could be detected on the tongue dorsum, buccal mucosa and in saliva. There was no difference in the detection rate in periodontally healthy or diseased subjects, but there were more of the bacteria on soft tissue and saliva than in plaque (46). Majer et al. found that there was no statistical difference between bacteria (40 test species) that were examined by DNA-DNA hybridization on periodontally involved versus healthy patients. There was also no difference between smokers versus non-smokers. However, there was a trend of more bacteria on soft tissues in smokers and periodontally involved patients (47).

The effect of periodontal therapy on the bacteria in the sulcus was demonstrated by Haffajee et al, but this same effect was not demonstrated on the remaining microflora of the oral cavity (43). In one study, the effect of scaling and root planning and periodontal surgery on levels of *A. actinomycetemcomitans, P. gingivalis*, and *P. intermedia* on oral mucous membranes was examined using immunofluorescence. The authors found that although periodontal pathogens decreased in subgingival areas, they did not decrease on mucous membranes (48). In the studies cited, the authors examined the oral cavity for a few select bacteria. There has been very little research investigating the types of bacteria that are found on oral surfaces in patients with and without periodontal disease. The aim of our study was to

determine if patients with periodontitis had a change in the amount and distribution of bacteria on the oral epithelium.

F. **Periodontal pathogens found on oral soft tissue surfaces**

Previous studies have shown that microorganisms that appear to have a preferential accumulation in periodontal disease sites will also adhere and form biofilms on normal healthy mucosa. This assumes that are selective advantages for various microbes to locate to various mucosa sites. Although, extra-periodontal sites localization may be partially a result of over abundance of bacteria at the periodontal sites. These identified microorganisms included: *Fusobacterium,*species; *P. intermedia*, various spirochetes, and *A. actinomycetemcomitans*. A problem with identification of microorganisms that are initially identified as related to periodontal disease sites is whether the identification is selecting transient microbes as a result of saliva and other oral fluids (44-46).

G. **Tobacco smoke effects upon bacterial adherence to mucosa**

A relationship between tobacco product use and damage to oral mucosa has been documented (53-55). Although it is unclear as to the change in distribution for microorganisms associated with tobacco use, Mager et. al., showed that there were higher proportions of *P. nigrescens*, Fuseobacterium and Actinomyces species in smokers but they were not significantly elevated. However, 81% of 47 smokers (38 of 47 individuals) had periodontal diseases compared to 58% of nonsmokers (107 of 182 individuals) (47).

III. METHODS

A.   **Population**

A cross-sectional pilot study was conducted among patients with and without periodontitis from the University of Illinois College of Dentistry Periodontics Clinic in Chicago, Illinois. Severity of periodontal disease as it relates to loss of alveolar bone was carefully evaluated using ridge standard criteria (see below). Written consent was obtained from each subject after explaining the objective of the study. The present study was part of a project approved by the institutional review board. The population came from patients and staff in the Periodontics Clinic. The subjects were divided into two groups: subjects with chronic periodontitis (N = 12) and subjects with health or mild to moderate gingivitis (N = 10). There were 10 females and 12 males in the study. The mean age of the patients was 49 and the mean age of the controls was 35.

Exclusion criteria included patients who were 21 years or younger, edentulous, immunocompromised, and those with a history of cancer. There was no exclusion of patients due to presence of oral lesions but no individual presented with an oral pathologic condition other than periodontal disease.

B.   **Clinical Monitoring**

All subjects provided their medical, dental, and social history including smoking and alcohol use. The extraoral exam included a head and neck exam with screening of lumps, bumps, ulcers or lesions and lymphadenopathy. Intraoral exam included an oral cancer screening, number of diseased, missing, or filled teeth and full mouth periodontal charting. Periodontal charting was carried out by one examiner using a Marquis probe

(3,6,9,12mm markings) at 6 sites per tooth (mesiobuccal, buccal, distobucal

distolingual, lingual and mesiolingual). Probing depth (in millimeters), free gingival

margin (in millimieters, above or below the cemento-enamel junction), bleeding on

probing (present or not), furcation involvement (using the Glickman classification), and

mobility (using the Miller classification) were examined (49). Clinical attachment loss

was calculated and periodontal charting and full mouth radiographs reviewed. The

diagnosis for the type of periodontitis was determined using the Armitage Classification

(49).

C.   **Collection of Samples**

Prior to the study, 2 10-second oral brush cytology test samples were sent to the

Forsyth laboratory in Cambridge, Massachusetts to confirm that the brush cytology

would yield adequate information. The 10-second brushing was adequate for bacterial

identification. During the study, duplicate samples were sent to Forsyth laboratory at

different times to confirm that bacterial identification was the same for both and to detect

error if present. The 10-second soft tissue brush cytology samples were obtained by

gently passing the brush over the tissue. Samples were taken from 3 sites per patient:

one site with active periodontitis (probing depth >4mm), one inactive periodontitis site

(probing depth < or = 3mm), and one site on the lateral border of the tongue. Control

patients did not have active disease sites present, but 3 samples were also taken.

D.   **Microbiological Assessment**

The bacterial samples were immediately treated according to the MasterPure TM

Gram-Positive DNA Purification Kit to prepare them for analysis. The MasterPure Kit

uses lysozyme to lyse the cell wall, RNase for degradation of RNA, and Proteinase K for protein degradation. The samples were stored at -20°C until use.

The specific methodology to ascertain identification through 16S rRNA genes by PCR and purification of PCR reactions; cloning procedures; 16S rRNA gene sequencing and data analysis of unrecognized inserts were previously identified. Furthermore, the complete 16S RNA gene sequences of clones and clusters representing phylotypes can be electronically retrieved from EMBL GenBank and DDBJ nucleotide sequences databases under accession numbers (26,24).

The treatment of the DNA sent to the Forsyth lab in Cambridge, Massachusetts follows. To start, two separate PCR reactions were initiated with the samples: forward primer 5´-CCA GAG TTT GAT YMT GGC-3´ with reverse primer 5´-GAA GGA GGT GWT CCA RCC GCA -3´, and forward primer 5´-GAC TAG AGT TTG ATY MTG GC-3´ with reverse primer 5´-GYT ACC TTG TTA CGA CTT-3´. Deoxytriphosphates and Platinum High Fidelity *Taq* polymerase (Invitrogen, San Diego, CA) were also added. The samples were preheated at 94°C for 2 minutes, followed by 32 cycles of amplification under the following conditions: 94°C for 30 seconds, 55°C for 30 seconds, and 68°C for 1.5 minutes, with an additional 1 second for each cycle. A final 10 minute elongation step at 68°C was added.

The labeled nucleotide Cy3-dCTP (GE Healthcare Biosciences, Pittsburgh, PA) was incorporated during a second, nested PCR reaction using the forward primer 5´-GAG TTT GAT YMT GGC TCA G-3´ and the reverse primer 5´-GYT ACC TTG TTA CGA CTT-3´. The same cycling program as above was used to complete the PCR. The Cy3-labeled amplicons were purified using a supplementary protocol with the QIAquick

PCR Purification Kit (Qiagen). In this protocol, an extra wash was performed using a 35% guanidine hydrochloride aqueous solution to aid in the removal of excess Cy-dye.

16S rRNA-based oligonucleotide reverse capture probes were custom synthesized with a 5´-(C6)-amine modified base, eight spacer thymidines and 18 to 20 nucleotides of target sequence, and printed (Michigan State University Research Technology Support Facility) on 25 × 76 mm aldehyde-coated glass slides. Sixty µM oligos were plated in v-bottom 384 well plates in a 2:1 solution with 2X spotting buffer giving a final spot concentration of 30 µM. By printing an OmniGrid Arrayer (GeneMachines) was used at 55% humidity. Post-processing of printed slides included immobilization by baking.

Immediately before use, the slides were blocked to reduce non-reactive primary alcohols, to remove unreacted aldehyde groups and to minimize fluorescent background after hybridization. This was carried out using the Little Dipper Microarray Processor (SciGene, Sunnyvale, CA). The slides were washed to remove un-bound DNA molecules and buffer substances.

A hybridization solution was prepared using the purified labeled DNA, yeast tRNA, H_2O and SDS. The solution was mixed and spun briefly in a centrifuge, then denatured at 100°C for 5 minutes. Ten microliters of denatured hybridization cocktail was then carefully injected under its respective cover slip using a pipette. The hybridization chamber was sealed, and hybridization was incubated overnight at 55°C. After hybridization the arrays were removed from the hybridization oven and washed at room temperature using the Little Dipper Microarray Processor. The slides were then spun dry in the attached centrifuge on the Little Dipper Microarray Processor and stored

in a dark container until scanned as below. Each sample was hybridized in replicate and evaluated after one single hybridization (50). The identification of bacteria was completed using the Human Microbial Identification Microarray core, a molecular analysis based on 16s rRNA sequencing to identify over 600 species in the oral cavity. The samples taken were examined for 422 bacteria.

Identification of Microbial Cluster: Using RNA probe sequences to classify and identify bacterial taxonomy and amplify phenotypic criteria is the basis for the Human Oral Microbiome Database (HOMD) grouping of genus or species. The cluster grouping, especially for genus of microbes such as Streptococcus species is a product of clones that did not meet the criteria to the HOMD, Ribosomal Database Project (RDP) or Greengenes that is grouped into a novel cluster group defined by a 98% identity with a 95% coverage criteria. Clustering is performed by sorting identified clones by length of sequence. A novel taxon is identifiable in this manner and stated to be a reference sequence and each succeeding clone sequence is compared by Basic Local Alignment Search Tool (BLASTN) to the reference sequence to determine additions to the taxon set (24,56).

E.   **Data Analysis**

The data from the 12 periodontitis subjects and 10 controls was returned by the Forsyth laboratory in an excel spreadsheet listing the sample number, type of bacteria, and intensity of bacteria (scored from 0-5). Number and type of bacteria, mean, and standard deviation were calculated to obtain an identification frequency for each bacteria in relation to the total population of bacterial identified. This was performed for each sample based on the 422 bacteria for which the samples screened.   Mean and

standard deviation of bacterium species in the periodontitis and control groups were

calculated for each sample site, and then compared.

IV. RESULTS

12 subjects with periodontitis and 10 controls (9 Caucasian, 4 Hispanic, 4 African American, 2 Arabic, 1 Asian) who displayed gingival health or gingivitis were included the in the study. 3 sample numbers describe each subject: one for the diseased gingival sample, one for the healthy gingival sample, and one for the lateral tongue sample. The periodontitis subject samples are numbered 1 through 51, while the control subject samples are numbered 52 through 82. **Table I** describes the characteristics of both the periodontitis and control subjects including age, sex, race, smoking and alcohol status. There were 10 females and 12 males and there were a greater number of subjects that used alcohol and formerly smoked (7 individuals) in the periodontitis group with only one current smoker. In periodontitis patients, 8.3% were current smokers, 58.3% former smokers, and 33.3% non-smokers. In periodontitis patients, 33.3% used alcohol and 66.6% did not use alcohol. In controls subjects, 10% were current smokers and 90% were non-smokers. 70% of controls used alcohol and 30% did not use alcohol.

TABLE I

SUBJECT DESCRIPTIVES

Subject	Age (years)	Sex	Race	Smoking status	Alcohol use
1,2,3	52	1	2	1	2
4,5,6	59	1	4	3	2
7,8,9	27	1	2	3	2
10,11,12	29	1	1	2	2
22,24,26	77	2	1	2	2
23,25,27	77	2	1	2	2
34,35,36	51	2	3	2	2
37,38,39	46	1	3	3	1
40,41,42	57	2	2	2	1
43,44,45	46	2	1	2	1
46,47,48	35	2	2	2	1
49,50,51	37	1	3	3	2
52,53,54	30	2	1	3	1
55,56,57	31	2	4	3	1
58,59,60	23	1	2	3	1
61,62,63	36	2	4	3	2
64,65,66	25	2	5	3	2
68,69,70	28	1	1	3	1
71,72,73	62	2	1	3	1
74,75,76	28	1	1	3	1
77,78,79	36	1	3	1	1
80,81,82	53	2	1	3	2

Legend:
Sex: 1 = female, 2 = male
Race: 1 = Caucasian, 2 = Hispanic, 3 = African American, 4 = Arabic, 5 = Asian
Smoking status: 1 = current smoker, 2 = former smoker, 3 = non (never) smoker
Alcohol use: 1 = Uses alcohol, 2 = No alcohol

Table II describes the periodontitis group in more detail. The diagnosis of periodontitis, based on the Armitage Classification, is given and the stage of therapy is indicated. Patients included in the study were at various stages in treatment, from pre-scaling and root planing to post-surgery.

TABLE II

DESCRIPTIVE INFORMATION ON SUBJECTS WITH PERIODONTITIS

Subject (by sample number)	Initial Diagnosis	Treatment completed
1,2,3	2	1
4,5,6	2	1
7,8,9	3	3
10,11,12	2	2
22,24,26	2	2
23,25,27	2	2
34,35,36	3	2
37,38,39	2	3
40,41,42	2	3
43,44,45	5	2
46,47,48	2	1
49,50,51	2	1

Legend:
Initial Diagnosis:
    1 = Generalized mild chronic periodontitis
    2 = Generalized moderate chronic periodontitis
    3 = Generalized severe chronic periodontitis
    4 = Localized mild chronic periodontitis
    5 = Localized moderate chronic periodontitis
    6 = Localized severe chronic periodontitis
Treatment Completed:
    1 = Pre-treatment
    2 = Scaling and root planning
    3 = Surgical therapy

In periodontitis subjects, 75% had generalized moderate chronic periodontitis, 16.6% had generalized severe chronic periodontitis, and 8.3% had localized moderate chronic periodontitis. 33.3% of periodontitis were pre-treatment, 41.6% were post-scaling and root planing (all were treatment planned for surgery), and 25% were post-surgery and in periodontal maintenance.

The diseased samples taken from periodontitis patients varied by the probing depths present. **Table III** describes the location of diseased-site samples from periodontitis patients, including probing depth, bleeding on probing, and radiographic bone loss.

TABLE III

SUMMARY OF CHARACTERISTICS OF DISEASED SAMPLES TAKEN FROM
PATIENTS WITH PERIODONTITIS

Subject	Site of Diseased Sample	Probing depth	Radiographic Bone loss present	Bleeding present
1,2,3	Between #2,3	7mm	yes	no
4,5,6	Between #2,3	5mm	yes	yes
7,8,9	Between #3,4	5mm	yes	yes
10,11,12	Between #13,14	7mm	yes	yes
22,24,26	Between #14,15	6mm	yes	no
23,25,27	Between #2,3	6mm	yes	no
34,35,36	Between #3,4	6mm	yes	no
37,38,39	Between #3,4	5mm	yes	yes
40,41,42	Between #5,6	6mm	yes	no
43,44,45	Between #2,3	6mm	yes	no
46,47,48	Between #2,3	7mm	yes	no
49,50,51	Between #14,15	6mm	yes	yes

Table IV shows the average number of bacterium species and Streptococci present in both control and periodontitis patients, and is divided by site. The diseased samples in control subjects had an average of 16.6 $\pm$ 4.1 total bacterium species while diseased samples in periodontitis patients had an average of 46.1 $\pm$ 15.3 total bacterium species (P<.0001; t = 4.9629, df = 20, standard error of difference = 5.964). There was a similar difference between control and periodontitis patients for healthy and tongue samples. The *Streptococcus* bacteria also varied between control and periodontitis patients (P<.0010; t = 3.8391 df = 20; standard error of difference = 1.693).

TABLE IV

NUMBER OF BACTERIUM SPECIES FOUND BY SITE FOR BOTH CONTROL AND PERIODONTITIS SUBJECTS

Subjects	Diseased sample	Healthy Sample	Tongue Sample	Total Bacteria	*Streptococcus* Bacteria
Control	yes			16.6 +/- 4.1	8.5 +/- 1.9
Control		yes		15.2 +/- 6.2	7.5 +/- 3.2
Control			yes	23.1 +/- 9.4	11.1 +/- 2.99
Periodontitis	yes			46.1 +/- 15.3	15.2 +/- 3.4
Periodontitis		yes		44.8 +/- 15.6	15.1 +/- 3.7
Periodontitis			yes	52.4 +/- 18.7	15.9 +/- 4.8

Figure 1 pictorially demonstrates the average number of bacterium species present in control and periodontitis patients, divided by site. Samples from periodontitis patients' diseased, healthy, and tongue sites all have a higher average number of bacterium species than samples from control patients.
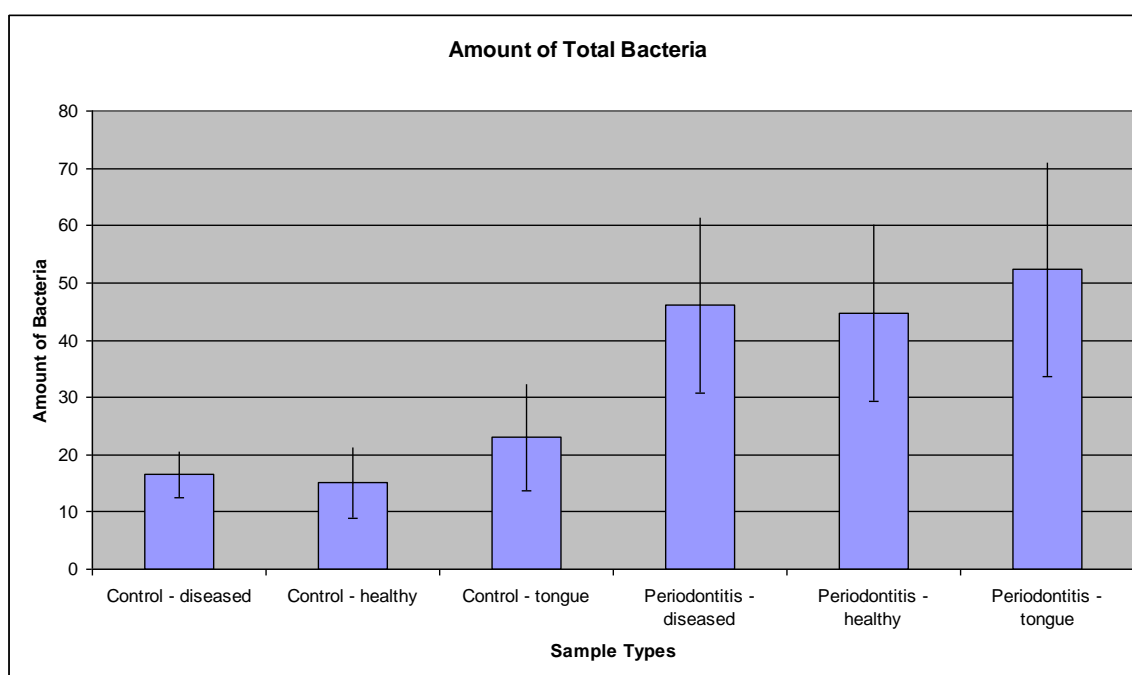

Figure 1. Average Amount of Bacterium Species Present in All Sample Types

Figure 2 describes the average amount of types of *Streptococcus* bacteria found in control and healthy patients. A greater average number of types of *Streptococcus* bacteria were found in patients with periodontitis when compared to control subjects as determined by the statistical analysis (see above).
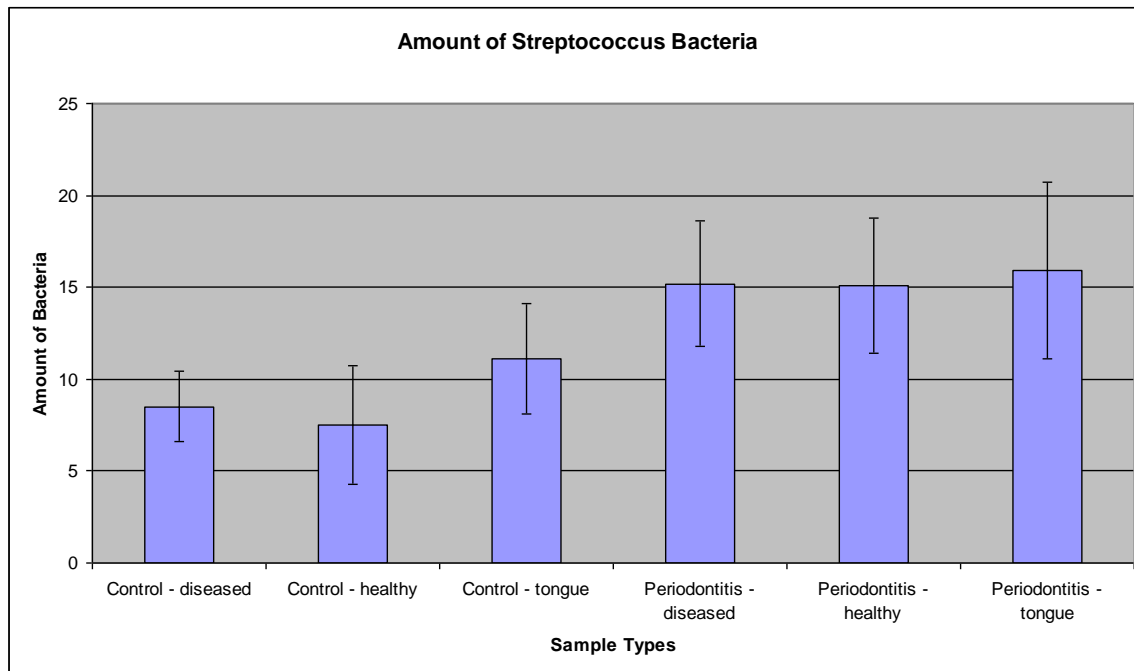
Figure 2. Average Number of Types of *Stretptococcus* Bacteria Present in All Sample Types

The types of bacteria found in healthy control patients compared to patients with periodontitis varied, but also had some identification similarities. **Figure 3.** Discloses a comparison between periodontitis and control subjects for bacterial species at a low identification frequency (0-50%). A difference in genus, species, and number among subjects with periodontitis were found (e.g., perodontitiis subjects had 98 species of bacteria identified in comparison to 76 species of bacteria identified for control subjects) with a predominance of *Streptococcus* species. The increased numbers of bacteria among subjects with periodontitis also extended to those bacteria identified at a 10 to 50% of the population with an additional number of 57 species of bacteria.
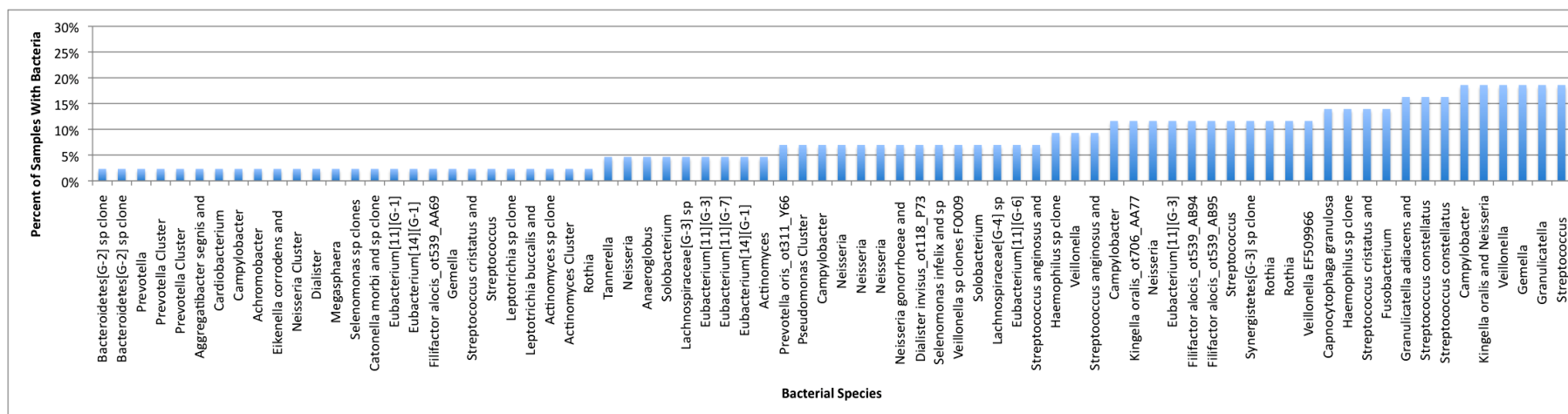
A.
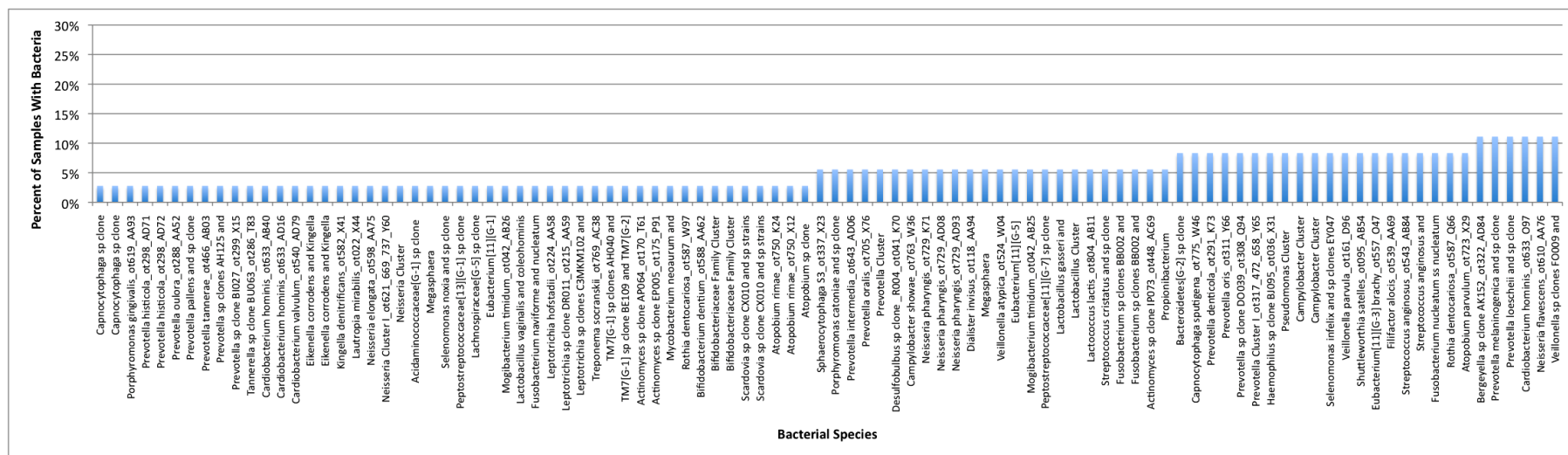
B.

Figure 3. Comparison of Periodontitis to Control Subjects Bacterial Species at a Low Identification Frequency (0-50%)
Key: A= Periodontitis and B= Non Periodontitis Healthy Subjects.

However among these bacteria, 85.9% were non-*Streptococcus* species (**Figure 4**.). **Figure 5** depicts bacteria at a frequency range of identification of 20-100% for periodontitis subjects (A) and controls (B). At this frequency, periodontitis subjects had 72 different species of bacteria while 46 were found among controls. In this population, 38.8% were non-*Streptococcus* species in the periodontitis group (68% *Streptococcus* species) while similarly 39.1% were non-*Streptococcus* species among control subjects.

At a 60% identification frequency a comparison between A, periodontitis (e.g., 19 different bacteria) and B, healthy controls (e.g., 8 different bacteria) showed a *Streptococcus* species predominance among group A (periodontitis patients). 57% of species in periodontitis patients are non-*Streptococcal* compared to 33% in group B, controls (**Figure 6**.).
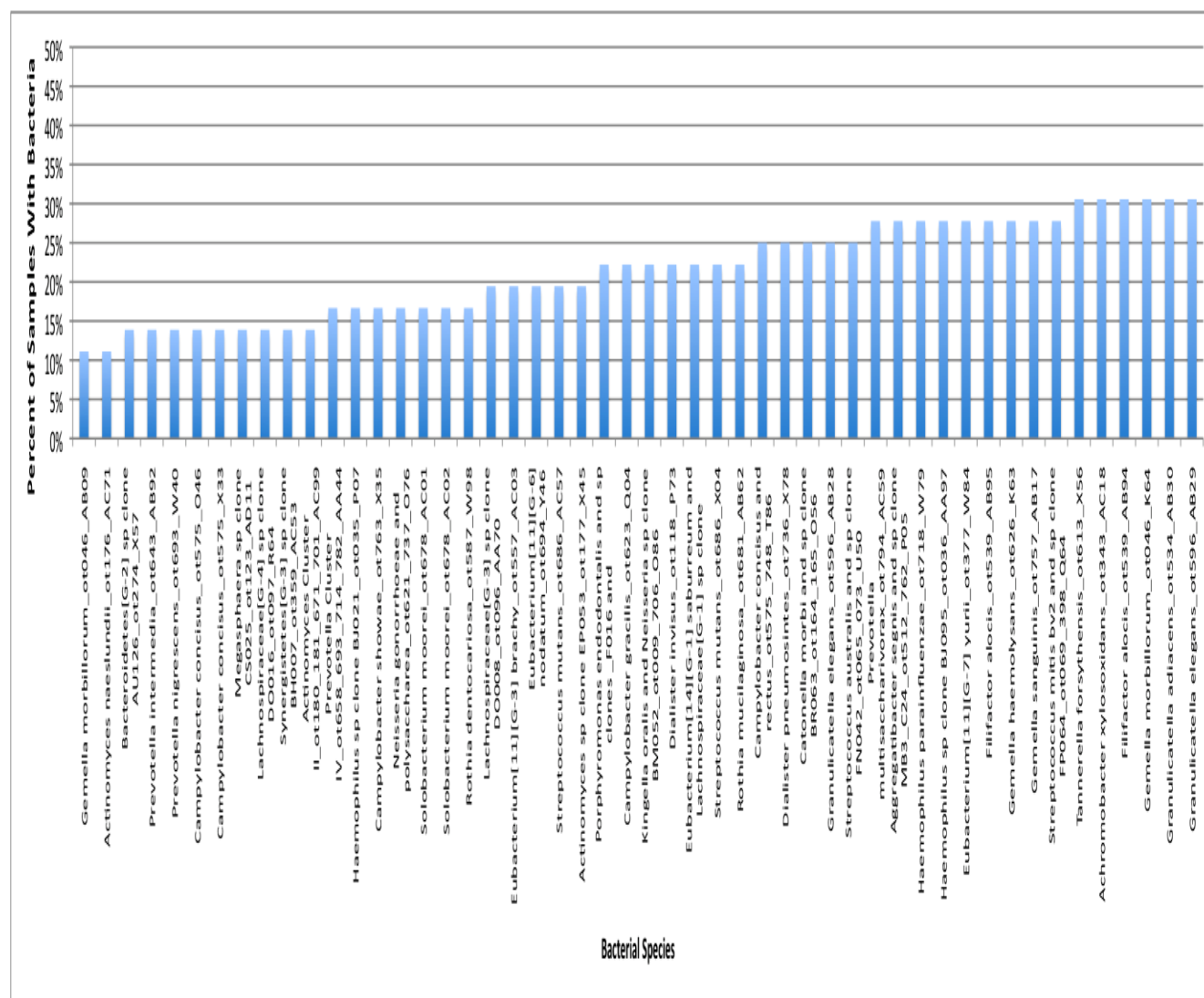
Figure 4. Periodontitis Subject Identification Frequency Continued (10-50%)
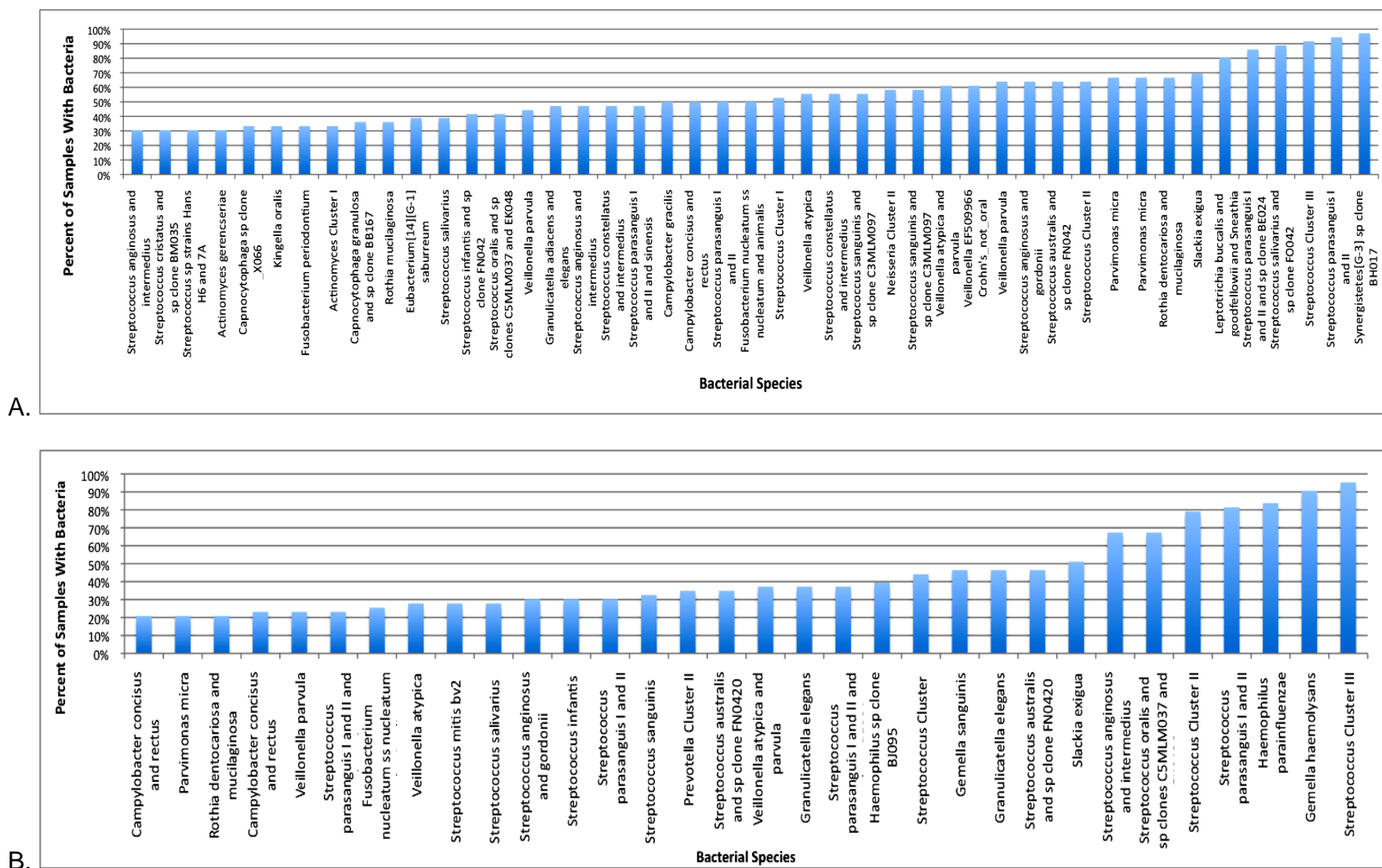
Figure 5. Further Comparison of Periodontitis and Control Subjects at High Identification Frequency (20-100%)
Key: A= Periodontitis and B= Non Periodontitis Healthy Subjects.

A.
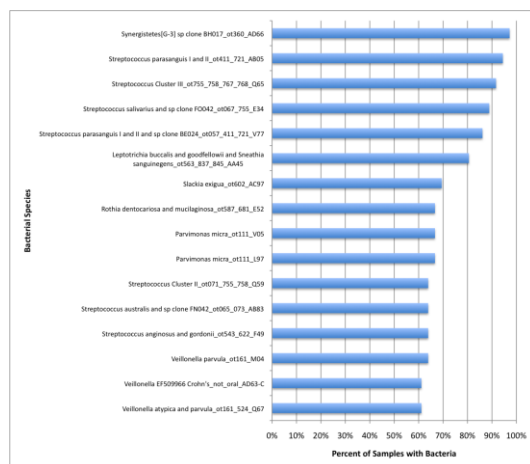
B.
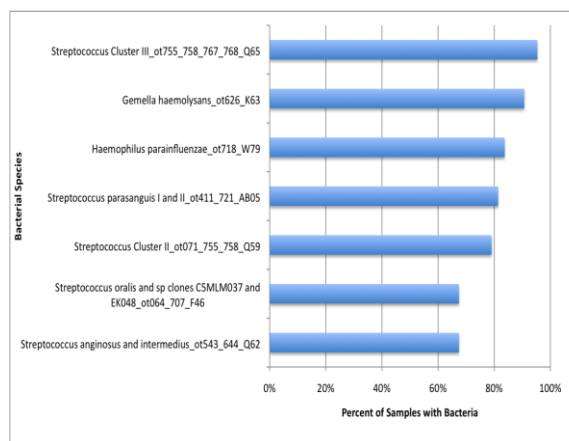
Figure 6. Bacteria at 60% Identification Frequency
Key: A= Periodontitis and B= Non Periodontitis Healthy Subjects.

One novel bacteria was present in current tobacco smoke users without peridontitis while several others were found in patients with periodontitis with current or former tobacco smoke use (**Table V**). No novel bacteria were found on the gingival sites, all were found on the tongue.

TABLE V

NOVEL BACTERIA FOUND IN TOBACCO SMOKE USERS

| Periodontitis Smokers	Periodontitis Former Smokers	Control Smokers
Eubacterium[11][G-1] infirmum_ot105_Y45	Capnocytophaga sp clone DS022	Campylobacter showae_ot763_X35
Leptotrichia sp clone DR011_ot215_AA59	Porphyromonas gingivalis_ot619_AA93	
	Prevotella histicola_ot298_AD71	
	Prevotella histicola_ot298_AD72	
	Prevotella pallens and sp clone DR022_ot310_714_R67	
	Tannerella sp clone BU063_ot286_T83	
	Cardiobacterium hominis_ot633_AB40	
	Cardiobacterium hominis_ot633_AD16	
	Cardiobacterium valvulum_ot540_AD79	
	Kingella denitrificans_ot582_X41	
	Neisseria elongata_ot598_AA75	
	Neisseria Cluster I_ot621_669_737_Y60	
	Acidaminococcaceae[G-1] sp clone C3MLM071 and sp strains FYA47 and GAA14_ot155_AB58	
	Megasphaera micronuciformis_ot122_X28	
	Selenomonas noxia and sp clone EL028_ot130_140_AC04	
	Peptostreptococcaceae[13][G-1] sp clone DA014_ot113_P72	
	Lachnospiraceae[G-5] sp clone BB124_ot080_AA65	
	Mogibacterium timidum_ot042_AB26	
	Leptotrichia hofstadii_ot224_AA58	
	Treponema socranskii_ot769_AC38	
	TM7[G-1] sp clones AH040 and BS003_ot346_349_W85	
	Mycobacterium neoaurum and tuberculosis_ot692_822_AD28	
	Atopobium sp clone C3MLM018_ot416_X30	

There was no significant differences in regards to tobacco smoke history for number of bacteria identified between periodontitis subjects and this pattern persisted for individuals that were controls with no periodontitis present in relation to tobacco smoke history. The average number of bacterium species is compared in **Table VI**.

However, current smokers that had periodontitis in comparison to current smokers with no periodontitis did show a significant increase in number of bacterium species identified (e.g., 46.3 among periodontitis subjects in comparison to 14.6 bacteria among healthy controls, respectively; P<.0001, t = 17.4068; df = 20; standard error of difference = 1.821).

TABLE VI
RELATIVE NUMBER OF BACTERIUM SPECIES IDENTIFIED AMONG CURRENT SMOKERS COMPARED TO NON-SMOKERS

	Healthy Control Subjects			Periodontitis Subjects		
	Non-smokers	Former smokers	Smokers	Non-smokers	Former smokers	Smokers
Average Number of Bacterium Species	24.4 +/- 12.1	N/A	14.6 +/- 2.9	51.3 +/- 15.0	46.4 +/- 18.5	46.6 +/- 6.7

Bacteria were also examined between subjects who were at different stages of treatment. **Table VII d**escribes the average number of bacterium species present in patients who were pre-treatment, post initial therapy (scaling and root planning, but planned for surgery), or post-surgery (osseous or flap surgery). The average number of bacterium species was the highest in post-surgical patients, followed by pre-treatment patients and finally post-scaling and root planning patients.

TABLE VII
AVERAGE NUMBER OF BACTERIUM SPECIES PRESENT IN PATIENTS AT
VARIOUS STAGES OF PERIODONTAL THERAPY

	Pre-treatment	Post-scaling and root planing	Post-surgery
Average Number of Bacteria	31.2 +/- 18.2	26.2 +/- 16.5	47 +/- 13.2

Figure 7 demonstrates the difference in number of bacterium species present

between phases of treatment pictorially.

Figure 7. Average number of bacterium species in subjects at various stages of
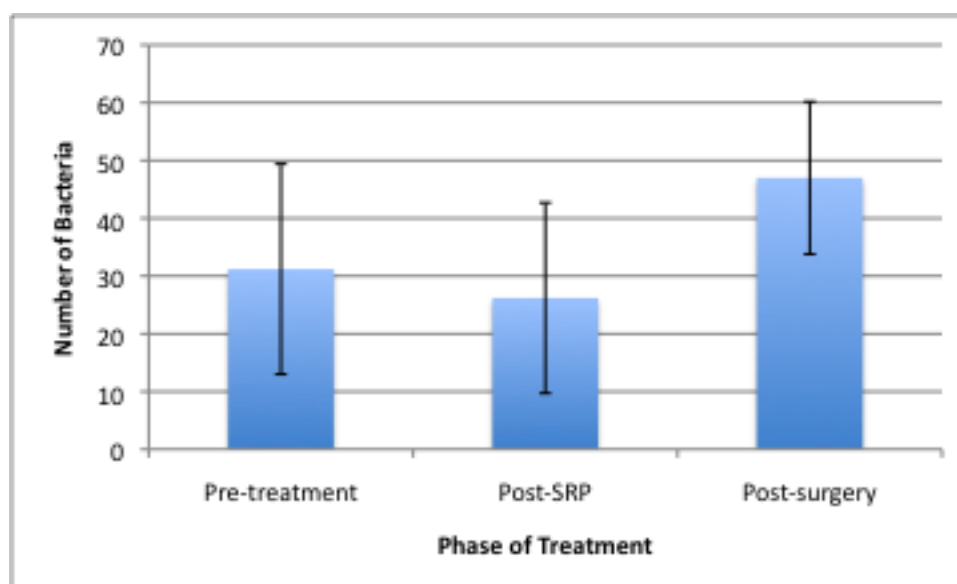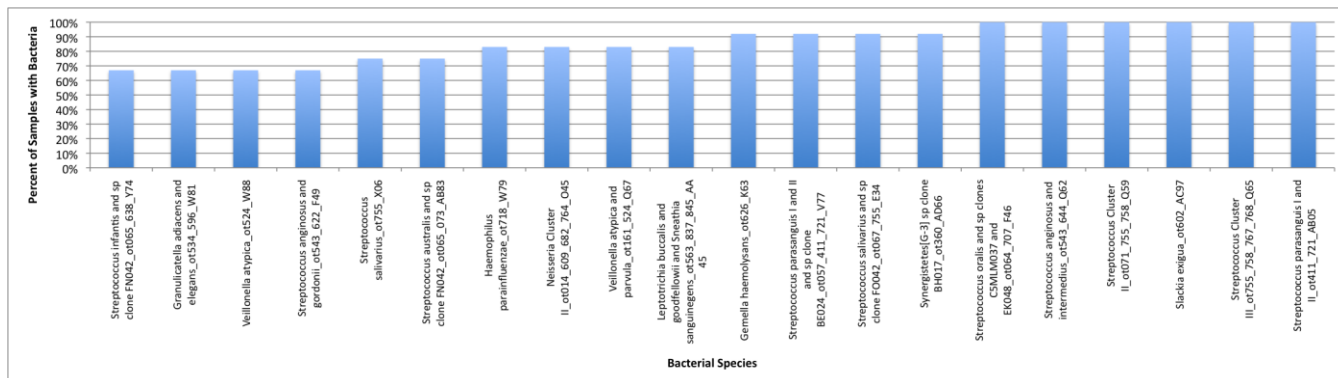periodontal therapy

The most frequently found bacteria (greater than 60% of samples) were also examined by phase of treatment. **Figure 8** illustrates the bacteria found in all phases of periodontal therapy.
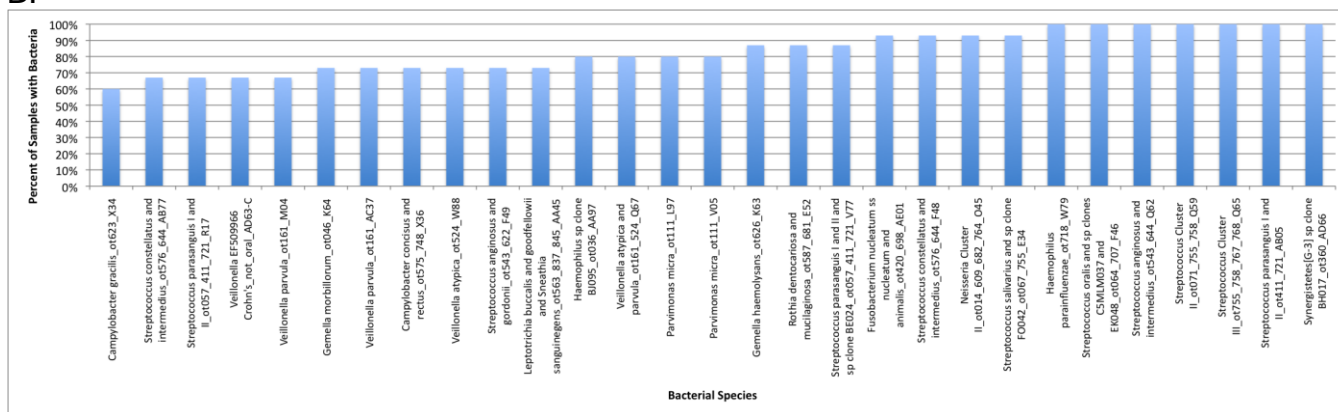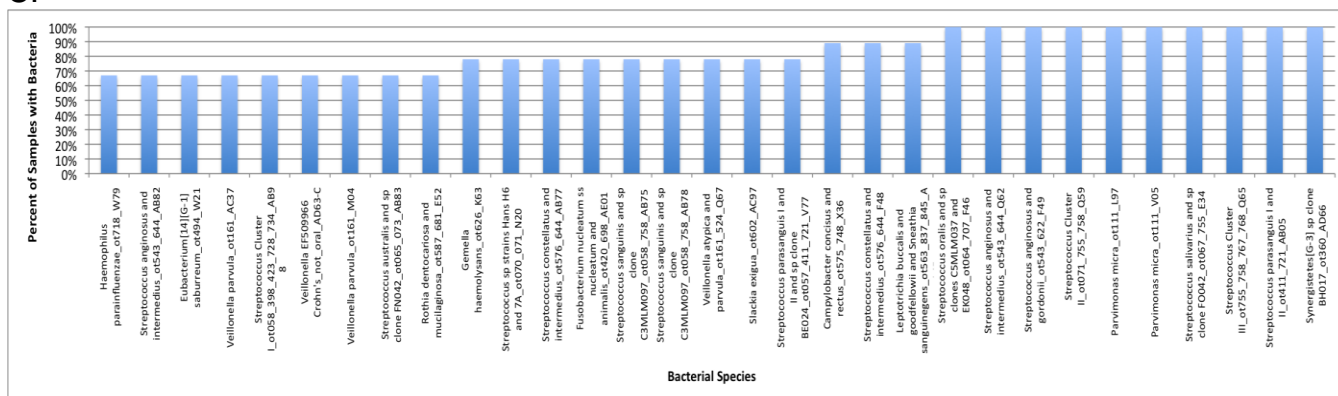
Figure 8. Bacteria at >60% Frequency in Patients with Periodontitis
Key: A = Pre-treatment, B = Post-scaling and root planing, C = Post-surgery

The number of species found in >60% frequency varied by phase of treatment. There were 20 species found in this freuqency for pre-treatment patients, 29 for post-scaling and root planing patients, and 31 for patients who were post-surgery.

There wer a total of 150 bacterial species found in patients who were pre-treatment, while there were 149 species found in patients who had completed scaling and root planing, and 118 bacterial species in post-surgical patients.

V. DISCUSSION

A.   **<u>Explanation of Main Findings</u>**

The primary finding of this project was that patients with periodontitis have a greater amount of bacterium species on the mucosal surface than healthy controls. Although bacterial presence in periodontitis has been an area well researched, few studies have investigated areas beyond the sulcus. Even fewer studies have examined the amount of species that were included in this experiment. Of the 422 bacteria that were included in the examination using oral brush cytology and Human Oral Microbe Identification Microarray (HOMIM), 193 were found in the mouths of the population studied. The most commonly found bacteria in healthy controls (present in over 80 percent of samples) were *Streptococcus Cluster III, Gemella haemolysans, Haemophilus parainfluenzae,* and *Streptococcus parasanguis I* and *II*. Patients with periodontitis had a slightly different set of most common bacteria including: *Synergistetes[G-3] sp clone BH017, Streptococcus parasanguis I* and *II, Streptococcus Cluster III, Streptococcus salivarius* and *sp clone FO042, Streptococcus parasanguis I* and *II* and *sp clone BE024,* and *Leptotrichia buccalis* and *goodfellowii* and *Sneathia sanguinegens*. Figure 3 and Figure 4 demonstrate the most common bacteria by percent in healthy control patients and patients with periodontitis, respectively.

Figure 1 and Figure 2 illustrated the drastic difference between healthy control patients and those with periodontitis. Although the standard deviation is substantial, there still remains and obvious difference between the groups. This difference could

possibly be explained by the oral hygiene of the patient or it may have more to do with the patients overall susceptibility to bacterial colonization.

Interestingly, phase of treatment did not seem to have an effect on number of bacteria. This is consistent with a similar study that noted that after periodontal therapy bacteria decreased in the sulcus, but not on mucous membranes (48). In this study, the average number of bacterium species did not decrease with further treatment. This may place less value on the influence of oral hygiene on the microflora, because all of the patients in the study were on regular periodontal maintenance and were seen every 3 months for prophylaxis. This may point to an outside factor such as host response that is responsible for amount of bacterium species.

A difference in species was seen between subjects that smoked versus those that were non-smokers. Smoking has been found to have a profound effect on the status of periodontitis. In fact, smoking has been shown to increase risk for periodontitis, with an odds ratio of 2.05 for light smokers and 4.75 for heavy smokers (51). Although few subjects were examined in this study, there was a few novel species found in smokers that were not present in non-smokers, which differs from the findings of Mager et al (47). However, this could be due to the fact that this study evaluated over 422 bacteria compared to the 40 species that were examined in the previous article. Matching control and periodontitis patients in relation to smoking would have improved the study.

B. **Limitations**

The main limitation of this study was the sample size. There were 12 patients with periodontitis and 10 healthy subjects included in this initial study. Due to these

small numbers, it is difficult to accurately define differences between groups and also to generalize the results to the population. However, as a pilot study, this investigation did provide some insight into the differences in number of bacterium species between patients.

In addition, the patients with periodontitis were at all at different stages of treatment. Although a few authors have shown that treatment of periodontitis does not cause a change in bacteria on mucous membranes, these studies examined only a few bacteria, and treatment may have been an influence in this study (43,48). It would be more interesting to take bacterial samples of the same patients at different stages of treatment over time to determine the effect of treatment on the bacterial flora around the oral cavity, but that was beyond the scope of this study.

Another limitation of the study was the possible inaccuracies in our method for data analysis. For example, a few of the samples were repeatedly submitted o the laboratory for analysis. One sample yielded very different results in number and type of species. However, most repeated samples yielded slightly different results each time with a slight variation in species. This indicates that there may have been some inaccuracies in the number and species of bacteria that were indicated.

C.   **Clinical Implications**

This study was interesting because it accurately defined the periodontal status of patients and phase of treatment, while examining a large number of bacteria. It provides a starting point for future research in changes in flora of the oral cavity. We know that changes in oral flora are due to changes in the environment, and that the opposite is also sometimes true. However, the extent to which the overall flora of the oral cavity

affects the rest of the mouth has yet to be deeply investigated. This type of study begins

to identify the differences in bacterial flora in health, periodontitis, smokers, and non-

smokers.

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