HOST BACTERIAL INTERACTIONS DURING EARLY
PLAQUE FORMATION IN CURRENT AND NEVER SMOKERS

A Thesis

Presented in Partial Fulfillment of the requirements for
the Degree Master of Science
in the Graduate School of The Ohio State University

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The Ohio State University
2010

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OBJECTIVE: To longitudinally examine patterns in supragingival and subgingival bacterial acquisition and colonization as well as host response in health and gingivitis in current and never smokers.

MATERIALS AND METHODS: 15 current and 15 non-smokers over 18 years of age with no history of systemic disease, current or planned pregnancy, recent or prophylactic antibiotic use were recruited. Following baseline evaluation and prophylaxis, stents were fabricated to protect 3 adjacent teeth in 2 quadrants during brushing. Clinical data, gingival crevicular fluid, supragingival and subgingival plaque samples were collected at day 0, 1, 2, 4, 7, 14 and 21. At each visit the patient was scaled, polished and flossed to ensure uninterrupted plaque formation for the next visit. 16S cloning and sequencing was utilized for bacterial identification and enumeration. A multiplexed bead-based assay was utilized to identify levels of 27 immune mediators in the crevicular fluid. Within-subject and between-subject comparisons were made using Wilcoxon signed rank and Kruskal-Wallis tests respectively.

RESULTS: Both current and never smokers developed gingivitis over the 21 days of plaque accumulation. No significant difference was noted in plaque index...
(Rustogi), or gingival index (Loe & Silness) at any time point. A Shannon-Weiner diversity index revealed increased diversity in never smokers with no significant alterations in current smoker diversity in both supragingival and subgingival biofilms as the onset of gingivitis occurred. Current smokers had potentially pathogenic bacteria present (Fusobacterium), even in health. A Bray-Curtis similarity index revealed a significant shift in subgingival bacteria at day 14 of plaque accumulation in the similarity of the never smoker biofilm to health. No such shift was seen subgingivally in current smokers. An inflammation score was obtained and it revealed that current smokers exist in a hyper-inflamed state when compared to never smokers.

CONCLUSIONS: Smoking alters both the profile of the bacterial biofilm present in health as well as the host-response to the bacterial insult that results in gingivitis.
Dedicated to:

Rachel, Vance, & Scarlet
ACKNOWLEDGEMENTS

I wish to thank my advisor Purnima Kumar for all of her support and guidance over the past three years. I certainly would not have been able to complete this thesis without her patience and knowledge.

I thank our program director Dimitris Tatakos for his guidance and support in the clinical aspects of my data collection, as well as Shaun, Janel, Irma, and all of the other residents at Ohio State for all of their assistance over the past 3 years.

I thank Dr. Vinayak Joshi and everyone else in the lab for their assistance in both the lab and clinical aspects. I would not have been able to complete the project in a timely manner without their advice and support.

I would like to thank Rachel whose continuous patience and support over the past 8 years of our marriage has been unparalleled. I also thank Vance and Scarlet, who have taught me to enjoy my life so much more in the short time they have been in it. Thank you to the rest of my family as well for always being there when I needed an outlet from periodontal activities.

This research was supported by a grant from Philips Oral Healthcare to Dr. Purnima Kumar.
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Kesavalu, L., Sathishkumar, S., Vasudevan, B., Matthews, C., Dawson, D.,
Ebersole, J. Polymicrobial Periodontal Disease: A Rat Model of Polymicrobial
2007;75(4):1704-12

FIELDS OF STUDY

Major Field: Dentistry
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CHAPTER 1

INTRODUCTION:

Periodontal disease is a condition that occurs as the result of an imbalance between the amount and type of bacteria present in plaque, as well as the host response to this bacterial colonization\textsuperscript{1-3}. Periodontitis is an endemic condition in the United States, with 35\% of Americans over the age of 30 years old having some form of the disease\textsuperscript{4}. There are many ideas and theories as to the exact cause of the condition, as well as to what can modify or prevent the disease. There have been several risk factors identified as directly causing an increase in periodontitis, with smoking being one of the most well documented risk factors. As much as 41.9\% of periodontitis in the United States can be directly attributed to the habit\textsuperscript{5}. Disease risk seems to be proportionate to the amount as well as the duration of tobacco use\textsuperscript{5-10}. Smoking also appears to increase the risk of disease in a dose-dependent manner; therefore, smoking presents a significant increase in risk of disease following even small amounts of tobacco exposure. Patients who have a history of smoking have been shown to have more severe periodontal disease with greater amounts of attachment loss, deeper pocket depths, and increased bone
destruction when compared to non-smokers\textsuperscript{11-16}. However smoking also has been shown to mask bleeding on probing, which is the hallmark sign of periodontal disease progression\textsuperscript{17}. This falsely creates a clinical appearance of health, when disease can actually be actively progressing. While many mechanisms for smoking causing this increased prevalence of disease have been suggested\textsuperscript{1-3}, no particular explanation has been proven. It is known that the primary etiologic agent of periodontal disease is the bacteria present in the dental plaque; however it is not well known how smoking modifies this community of bacteria from initial colonization during health as the biofilm progresses toward disease. It is possible that current smokers have potentially pathogenic bacteria present in their oral biofilms at a much earlier time period in colonization, setting the stage for future disease activity.

Bacteria throughout the body exist in complex ecosystems that rely on one another for survival. These complex biofilms can exist in both a health or disease associated environment and provide unique survival advantages to the bacteria present. The bacteria in these biofilms are able to communicate through a phenomenon known as quorum sensing\textsuperscript{18}. This provides bacteria the ability to alter their DNA profile slightly to increase their chance of survival. Bacteria in biofilms are also protected from mechanical trauma as well as host-defenses. Biofilms are colonized in a specific order, with early colonizers arriving initially followed by bacteria that attach directly to them. The bacteria present in a healthy
biofilm provide defense to disease-associated bacteria by a mechanism known as niche saturation or niche occupation. Niche saturation is the concept that an area presently occupied by a health associated bacteria cannot be occupied at the same time by a disease associated bacteria. Bacteria within these biofilms are able to quickly re-establish themselves on the tooth surface following debridement. Therefore identifying the composition of this biofilm in health is very important. If the health associated biofilm contains pathogens it becomes a question not of if periodontal disease will set in, but rather when.

It is becoming more apparent systemically that smoking alters this biofilm balance and provides for more preferential colonization by pathogenic bacteria. One of the potential mechanisms of this is by altering the body’s iron levels allowing iron loving pathogenic bacteria to populate. Smoking has also been shown to increase the ability of bacteria to adhere to epithelial cells as well as decrease the oxygen present in the subgingival environment. This alteration in available oxygen has a significant effect on the population of the community present. Recent evidence also suggests that smoking significantly reduces the levels of protective bacteria in other areas of the body such as the nasopharynx, vagina, ear, and gut which creates a sort of biological vacuum that allows for colonization of this niche by potential pathogens. Smoking has also been associated with increased rates of infections such as breast abscesses, ear infections, and candidiasis. Studies have also shown pathogenic subgingival
recolonization in smokers following therapy much more frequently than in non-smokers\textsuperscript{40}. Due to the increasing evidence of the effect of smoking on biofilms throughout the body, it seems logical that smoking will have similar effects on the subgingival ecosystem, and therefore will alter the microbial profile of this niche from initial colonization.

Healthy biofilms tend to be dominated by coccoid cells while periodontitis associated biofilms were associated with motile rods, curved rods, and spirochetes\textsuperscript{41}. The main bacterial genera reported in health are mostly gram positive, and consist of predominately \textit{Gemella, Granulicatella, Streptococcus, Actinomyces} and \textit{Veillonella} species\textsuperscript{42,43}, with the Gram negative \textit{Fusobacterium, Bacteroides}, and \textit{Haemophilus} species as well as facultative and anaerobic gram positive \textit{Actinomyces} species being implicated in periodontal disease\textsuperscript{43}. It has been shown, however, that the bacterial profiles in disease are significantly different from patient to patient, and region to region\textsuperscript{44}. Therefore, this seems to underscore the idea that bacterial presence is necessary but not sufficient for disease progression since no single combination appears to be what tips the scale towards disease.

The use of microscopy, cultivation, and molecular methods to examine the composition and evolution of the dental plaque biofilm has been occurring for over 70 years\textsuperscript{45-50}. When using microscopy and cultivation to monitor the changes in the biofilm from days 14-21, a significant shift in composition is
evident\textsuperscript{51}. 16S cloning and sequencing has recently been used to examine the composition of day old plaque\textsuperscript{49}. This data shows that numerous bacteria that were previously uncultivated or unknown are present in significant quantities. However the biofilm is still highly transient and evolving at this point and can become highly complex within the first week\textsuperscript{52}. Therefore, evaluation of day old plaque provides no information about secondary colonizers as the progression towards gingivitis and potential disease occurs. Bacteria such as \textit{Fusobacterium} act as important bridging bacteria between early and later colonizers and they are often present in more developed biofilms\textsuperscript{53} following disease onset.

Bacterial identification can be very difficult. Historically researchers have been limited by microscopy and cultivation based techniques\textsuperscript{41, 43, 46, 54, 55} which provided us with our belief that gram negative bacteria are the leading culprits in periodontitis\textsuperscript{56-63}. While these techniques provided an ample amount of bacterial data they were limited to bacteria that were viable at the time of cultivation, and more importantly they were limited to bacteria that were cultivatable\textsuperscript{64}, leaving a potentially large portion of the population unidentified. These techniques are also limited in their ability to accurately identify bacteria since they rely heavily on phenotypic characteristics for identification such as morphology, response to stains, etc\textsuperscript{65}. More definitive targeted molecular approaches such as PCR have failed to link any specific bacteria with smoking and periodontitis\textsuperscript{10, 40, 66-72}. This may be due to the fact that the bacteria present are unknown and therefore
unsuspected in targeted approaches. Modern open ended DNA based techniques such as 16S RNA cloning and sequencing\textsuperscript{73-77} provide us the ability to identify both viable and non-viable bacteria, as well as to identify bacteria that were previously uncultivated or unsuspected, thus eliminating the selective bias of cultivation. Recent studies have shown an incredibly diverse biofilm present subgingivally with up to 300 species present, and up to 60\% of these bacteria previously uncultivated\textsuperscript{42, 78-83}. The 16S sequence also provides a unique and accurate method of bacterial identification, eliminating the need for, and potential errors associated with phenotypic identification. Therefore using this open-ended molecular approach in smokers provides us with the ability to detect bacteria that are unsuspected since they may not be present in non-smokers.

Ultimately a susceptible host is required for the onset of periodontitis. It has been hypothesized that patients experience disease as the outcome of an exaggerated response to the previously mentioned bacterial trigger\textsuperscript{1, 84-87}. It is important for us to better understand the response by a host to the initial formation of a biofilm in order to determine when and where alterations in this response occur that may trigger the onset of disease. The microbial content of the pocket has been shown to be a determinant in gingival tissue gene expression in the area. These alterations in gene expression can also result in an altered host response\textsuperscript{88}. Studies have also shown that nicotine levels can alter the cytokine levels in the host\textsuperscript{89}. Some authors found that the immune response is actually muted in current
smokers. Therefore it is important to evaluate the variations in this response between current and never smokers.

**Hypothesis:**

The working hypothesis for this project was:

*Current smokers have altered bacterial colonization patterns and host-immune response in health and during progression to gingivitis when compared to never smokers.*

**Specific Aims:**

To address the above hypothesis, we developed the following specific aims:

1. To identify the bacterial profile of supragingival plaque during initial formation in periodontally healthy smokers utilizing an open ended approach.

2. To identify the bacterial profile of subgingival plaque during initial formation in periodontally healthy smokers utilizing an open ended approach.

3. To examine the levels of 27 selected immune mediators in gingival crevicular fluid during early plaque formation in periodontally healthy current and never smokers.

4. Evaluate the correlation of microbial profile to the immune mediators identified in aim 3 during early plaque colonization.
Significance:

1. Identifying any modifications to the bacterial community and colonization patterns in current smokers may provide better understanding of the mechanism of action of smoking on periodontal diseases.

2. Open ended molecular analysis of the bacteria present in the plaque will allow us to identify previously uncultivated or unsuspected bacteria present.

3. Better understanding the host-response to bacterial presence and the variations of this response in smokers will allow us to better maintain health and prevent disease.
CHAPTER 2
MATERIALS AND METHODS:

Patient Selection:

15 current smokers and 15 never smokers over 18 years of age with no history of systemic disease, current or planned pregnancy, or recent or necessary prophylactic antibiotic use were recruited for the study. Each patient received a baseline evaluation and prophylaxis. Patients were required to be free of periodontal disease and have at least 3 adjacent posterior teeth in opposing quadrants that were free of decay or interproximal restorations.

Clinical Examination:

All measurements were made by 2 calibrated examiners. At the initial evaluation appointment, following a full mouth probing and prophylaxis, impressions were made of the patient in occlusion using a posterior sextant triple-tray and polyvinyl siloxaine putty. 0.40 mm acrylic stents were then fabricated
using a vacuum matrix and trimmed to protect the teeth and gingival margins in the experimental sextants during brushing.

The patients returned approximately 1 week following initial evaluation for verification of a return to gingival health, delivery of the stent, and entry into the study. The patients were instructed to use a manual toothbrush and place the protective stents over their teeth whenever they brushed. They were also instructed to avoid using mouthwash, floss, or any other dental hygiene aids in the experimental area.

The patients returned at 1, 2, 4, 7, 14, and 21 days of plaque accumulation. At each visit, following sample collection, the teeth were scaled, polished, and flossed to ensure uninterrupted plaque formation for the next appointment. At baseline and each of these time intervals the following data was collected:

1. Plaque index (PI) according to Rustogi\textsuperscript{90}.
2. Gingival index (GI) according to Loe & Sillness\textsuperscript{91}.
3. Gingival crevicular fluid (GCF)
4. Tongue scrapings
5. Supragingival plaque
6. Subgingival plaque
7. Saliva

Following 21 days of plaque accumulation, the patients were given a Sonicare FlexCare R910 toothbrush and correct oral hygiene methods were
emphasized. The patients were then released without receiving a prophylaxis and were instructed to return to a normal hygiene routine using the new Sonicare brush in place of their previously used brush. The patients then returned 14 days later to have a follow-up set of samples taken. At this point the patients received a final prophylaxis and were released from the study.

For the purposes of this master’s thesis, only data through 21 days of plaque accumulation will be reported and discussed.

**Microbiological Sampling:**

Supragingival plaque samples were collected by supragingival scraping of interproximal plaque utilizing a 204S scaler. Care was taken not to enter the sulcus during collection. The plaque was then transferred to sterile endodontic paper points (Dentsply, Tulsa, Oklahoma) and immediately frozen.

The subgingival plaque samples were collected by inserting sterile endodontic paper points into the sulcus for a period of 10 seconds. A 204S scaler was then utilized subgingivally to collect any remaining plaque. This plaque was then transferred to the paper points and immediately frozen.

Gingival crevicular fluid was collected by inserting sterile periostrips into the interproximal sulcus for 30 seconds. These strips were then measured clinically with a Periotron 2000 and immediately frozen.
**Bacterial Identification:**

Bacterial DNA was isolated from each sample by placing the paper points in a 1.5 mL sterilized collection tube with 200 μL of PBS then agitated for 20-30 minutes. The paper points were then removed and placed into a 0.5 mL collection tube with a hole punctured in the base and this tube is returned to the 1.5 mL tube to be centrifuged for 3 minutes to allow the fluid from the paper points to return to the PBS solution. The paper points were then discarded.

180 μL of tissue lysis buffer and 40μL of Proteinase K were added to the PBS/Bacteria solution, and agitated for 15 seconds to mix the solutions. The collection tube was then incubated at 56°C in a hot water bath for at least 2 hours.

Following incubation 200 μL of cell lysis buffer was added to the collection tube, agitated for 15 seconds, and incubated at 70°C in a dry bath for 10 minutes.

Once this was completed, 200 μL of 100% Ethanol was added to the tube, agitated for 15 seconds, the solution was then transferred to a QIAamp Spin Column nested inside of a 2.0 mL collection tube and centrifuged for 1 minute. The filtrate was then discarded and 500 μL of wash buffer was placed into the spin column and centrifuged for 1 minute, filtrate was discarded, 500 μL of wash buffer was again added to the spin column and centrifuged for 3 minutes.

Once completed the collection tube was discarded, and the spin column was placed in a new sterile 2.0 mL collection tube. 50 μL of elution buffer
(trisHCl + EDTA) was added to the column, incubated at room temperature for 5 minutes and centrifuged for 1 minute, the sample was then moved to a sterile 0.2 mL collection tube. The 16S rRNA genes were then amplified by PCR using broad range primers.

The PCR amplicons were mixed with 250 μL of PBI in a spin column and centrifuged at 8000 rpm for 1 minute. The filtrate was then discarded and 750 μL of wash buffer was added to the column, centrifuged at 8000 rpm for 1 minute, filtrate was discarded, and the column was then centrifuged for an additional 3 minutes at 8000 rpm. Following this the tube with filtrate was discarded, and the column was placed into a new 1.5 mL tube and 30 μL of elution buffer was added to the column and allowed to incubate at room temperature for 5 minutes and centrifuged at 8000 rpm for 1 minute. The column was then discarded.

4 μL of this solution was then combined with 1 μL of MgCl₂ solution and 1 μL of plasmid vector and allowed to incubate at room temperature for 5 minutes. The ligated products were then inserted into E.coli cells. 3μL of the ligated mixture was added to the E.coli and shocked in a 42° C hot water bath for 30 seconds and returned to ice. 300 μL of growth Medium was added to the E.coli and the mixture was agitated at 37° C for 1 hour.

60 μL of the solution was then plated onto ampicillin containing agar plates that previously had been plated with 40 μL of X-Gal, and allowed to grow in an incubated aerobic chamber overnight. Colonies were then screened for
transformants utilizing ampicillin resistance and a blue/white colony screening. The transformed colonies grew as white, not blue colonies, and they were then selected and amplified again using the same PCR protocol previously described.

The presence of amplicons of 1500 basepairs was confirmed by gel electrophoresis of the PCR amplicons, and were sequenced at a high throughput sequencing facility at the Ohio State University. These sequences were then compared to GenBank for identification (18,000 total clones, over 27 million base pairs), and underwent final classification via a phylogenic analysis. Novel bacteria were classified utilizing a phylogenic tree.

**Immune Mediator Identification:**

The levels of 27 selected cytokines and chemokines were measured as indicators of the host immune response to the evolving biofilm. Periostrips were thawed on ice and GCF was eluted by adding 200 μl of buffer containing 50 mM Tris/HCl with 5 mM CaCl₂, 0.2 NaCl, pH 7.6 containing 1 mg/L antipain, 1 mg/L aprotinin, 1 mg/L leupeptin, 125 mg N-ethylaleimide and 50 mg Zwittergent 3-12 and vortexed at 15-minute intervals over one hour. Periostrips were removed and the eluted fluid used in a multiplexed bead-based assay designed to quantitate multiple cytokines (Bio-rad laboratories, Inc, Hercules, CA). 50 μl of GCF fluid and standards were incubated with 27 distinct sets of fluorescently dyed beads (conjugated with cytokine-specific monoclonal antibodies) in a 96-well filter
plate. The samples were sequentially incubated with 25 μl of biotinylated detection antibody and 50 μl of Streptavidin-phycoerythrin. The level of each cytokine was measured on a Bioplex 200 flow cytometry detection system, which simultaneously measured the internal fluorescence of the different colored beads as well as the fluorescent signal from the detection antibody. We validated the results of this assay by measuring the levels of IL-2 in all samples with ELISA. The relative level of concentration of each immune mediator was then obtained by normalizing the amount of mediator present for the amount of fluid collected clinically.

Data Analysis:

Bacteria:

The quantitative levels of each bacterial species was computed for each patient at each time period sampled. These bacteria were then classified by genera to allow for an overall prevalence (total present at each time period) of a given genus, as well as a percent prevalence (total of genera present/total number of all genera present x 100). This data allowed us to evaluate the composition both at a species and genera level.

A Shannon-Weiner diversity index and the Bray-Curtis similarity index were computed to quantify the amount of change in population of bacterial genera between two sampling appointments.
Immune Mediators:

The 27 immune mediators evaluated were divided into either pro-inflammatory, anti-inflammatory, or chemokine in nature. A score for each pro-inflammatory mediator, anti-inflammatory mediator, or chemokine was obtained by dividing the amount of the given immune mediator in the sample by the maximum amount of the mediator that was present in any sample at any time point, thus giving a relative concentration of the cytokine at that time compared to the maximum seen in any sample. This score was then adjusted for the volume of gingival crevicular fluid present (score divided by volume present). All mediator scores were then averaged into a single sample value for each time period sampled to provide composite pro-inflammatory, anti-inflammatory, and chemokine score.

Statistical Analysis

Within-subject and between-subject comparisons were made using Wilcoxon signed rank and Kruskal-Wallis tests respectively.
CHAPTER 3

RESULTS:

15 current and 15 never smokers were recruited for this study. The never smoker patient population consisted of 8 males and 7 females with an average age of 21 years and no pack year tobacco exposure which was confirmed with salivary cotinine levels of 0 ng/ml. The current smoker patient population consisted of 12 males and 3 females with an average age of 20 years and a reported average pack year tobacco exposure of 1.47 ±0.68 which was confirmed with salivary cotinine levels of 28 ng/ml. Table 1 shows the demographic details of the study population.

Figure 1 shows the change in clinical indices over 21 days of plaque accumulation for both groups. Figure 1A shows the plaque index (Rustogi) for current and never smokers. The mean plaque index on day 1 for never smokers was 0.29 ± 0.32 and 0.4 ± 0.46 for current smokers. On day 21 the plaque score for never smokers was 2.35 ± 1.54 and 3.45 ± 0.84 for current smokers. The differences between current and never smokers at all time points were not
statistically significant (p>0.05, 2-sample t-test). Figure 1B shows the gingival index (Loe and Silness). The mean gingival index at day 1 was 0.09 ± 0.20 for never smokers and 0.43 ± 0.59 for current smokers. On day 21 of plaque accumulation, never smokers had a gingival index of 1.88 ± 1.04 and current smokers had a gingival index of 2.05 ± 0.38. However, these differences were not statistically significant at any time point (p>0.05, 2-sample t-test). The shift from clinical health to disease occurred on day 7 in current smokers and day 14 in never smokers.

Figure 2 shows the Shannon Weiner diversity index for the bacterial populations in current and never smokers. Figure 2A shows the diversity index subgingivally for current and never smokers. Subgingivally, never smokers had a significant decrease in biofilm diversity on days 2 and 4 of plaque accumulation (p<0.05, 2-sample t-test) when compared to the population present in health (1 day of plaque accumulation). The biofilm then displayed an increased diversity on day 7 of plaque accumulation and thereafter. Current smokers never had a statistically significant alteration in their subgingival bacterial diversity (p>0.05, 2-sample t-test). Figure 2B shows the diversity index supragingivally for current and never smokers. Supragingivally, there was a statistically significant increase in the diversity of the bacterial population in never smokers at 14 and 21 days of plaque accumulation when compared to health (p<0.05, 2-sample t-test). There were no statistically significant differences in the diversity of the biofilms present
supragingivally in current smokers at any time point when compared to health
(p>0.05, 2-sample t-test). There were also no significant intergroup differences
(p>0.05, 2-sample t-test).

Figure 3 shows the percent prevalence of the bacterial genera in the
subgingival biofilm in both current and never smokers. Figure 3A shows the
biofilm composition in health (1 day of plaque accumulation) for both current and
never smokers. *Streptococcus, Veillonella, Neisseria, Delenomonas,* and
*Dialister* account for 75% of the microbial community in never smokers, while
*Streptococcus, Veillonella, Selenomonas, Campylobacter, Dialister,* and
*Corynebacterium* account for 75% of the microbial community in current
smokers. Current smokers have an increased amount of genera present in the
biofilm as well as several potentially pathogenic bacteria such as *Fusobacterium*
and *Lactobacillus*. Never smokers had significantly higher *Neisseria* and
*Actinomyces* (p<0.05, Kuskal Wallis analysis) while current smokers had
significantly higher levels of *Hemophilus* and *Abiotrophia* (p<0.05, Kuskal Wallis
analysis). Figure 3B shows the subgingival biofilm in current and never smokers
following the onset of gingivitis (21 days of plaque accumulation). The overall
subgingival bacterial profile in both groups appears much more diverse than in
health with many more genera present. *Selenomonas, Dialister, Streptococcus,
Veillonella, Campylobacter, Eubacterium,* and *Neisseria* accounted for 75% of
the bacteria profile in never smokers. *Selenomonas Dialister, Streptococcus,
Eubacterium, Veillonella, Treponema, Peptostreptococcus, and Campylobacter accounted for 75% of the bacterial profile present in current smokers. Significantly higher prevalence of Veillonella, Neisseria, Atopobium, and Campylobacter (p<0.05, Kuskal Wallis analysis) were seen in never smokers while current smokers had significantly higher prevalence of Actinomyces, Megasphaera, Mogibacterium, Hemophilus, and Abiotrophia (p<0.05, Kuskal Wallis analysis). Centipeda, Cardiobacterium, Fusobacterium, and Deferribacteres were acquired in never smokers, and Centipeda, Mogibacterium, Filifactor, and Porphyromonas were acquired in current smokers following the onset of gingivitis.

Figure 4 shows the percent prevalence of the bacterial genera in the supragingival biofilm in both current and never smokers. Figure 4A shows the supragingival biofilm composition in health (1 day of plaque accumulation) for both current and never smokers. The bacterial profile of the never smokers is much more diverse with Streptococcus, Veillonella, Hemophilus, Neisseria, and Abiotrophia accounting for 75% of the bacterial population in never smokers and only Streptococcus and Veillonella accounting for 75% of the population in current smokers. Never smokers had significantly higher prevalence of Hemophilus, Neisseria, Corynebacterium, Actinomyces, Rothia, Campylobacter, and Capnocytophaga (p<0.05, Kuskal Wallis analysis) while current smokers only had significantly higher prevalence of Kingella (p<0.05, Kuskal Wallis analysis).
analysis). Figure 4B shows the supragingival biofilm composition following the onset of gingivitis (21 days of plaque accumulation). Both the current and never smoker bacterial profiles are much more diverse following the onset of gingivitis with *Selenomonas, Streptococcus, Veillonella, Eubacterium, Campylobacter, Capnocytophaga, Neisseria, Dialister, and Hemophilus* accounting for 75% of the population in never smokers and *Selenomonas, Streptococcus, Veillonella, Eubacterium, Dialister, and Peptostreptococcus* accounting for 75% of the population in current smokers. Never smokers had significantly higher *Hemophilus, Neisseria, Corynebacterium, Actinomyces, Propionibacterium, Eikenella, Campylobacter, Leptotrichia, Prevotella, Atopobium, Capnocytophaga,* and *Lautropia* with current smokers only having significantly more prevalent *Streptococcus*. Never smokers acquired *Treponema, Centipeda, Prevotella, Lachnospira, Leptotrichia, and Megasphaera* following the onset of gingivitis, while current smokers acquired *Actinomyces, Rothia, Capnocytophaga Cardiobacterium, Atopobium, Centipedia,* and *Deferrribacteres,*

Figure 5 shows the Bray Curtis similarity index for subgingival plaque in current and never smokers. Figure 5A shows the similarity of the bacterial profile at a given interval of plaque accumulation to health (1 day plaque accumulation) in never smokers and Figure 5B shows the same information about the subgingival bacterial profile in current smokers. The microbial profile in never smokers demonstrated a significant shift in similarity on day 14 (p<0.05,
Repeated Measures ANOVA) with a mean similarity from a healthy biofilm shifting from 0.60 on day 7 to 0.43 on day 14. The current smokers bacterial profile never showed a significant shift in similarity as the profile progressed from health (day 1) to gingivitis (21 days of plaque accumulation.) (p>0.05, Repeated Measure ANOVA)

Figure 6 shows the Bray Curtis similarity index for supragingival plaque in current and never smokers. Figure 6A shows the similarity of the bacterial profile at a given interval of plaque accumulation to health (1 day plaque accumulation) in never smokers and Figure 6B shows the same information about the supragingival bacterial profile in current smokers. The microbial profile in never smokers did not display a significant shift in similarity as the biofilm progressed from health (day 1) to gingivitis (21 days of plaque accumulation) (p>0.05, Repeated Measures ANOVA). The current smokers bacterial profile demonstrated a significant shift in similarity to a health associated biofilm at day 7 (p<0.05, Repeated Measures ANOVA). The mean Bray Curtis similarity index shifted from 0.63 on day 4 to 0.46 at 7 days of plaque formation.

Figure 7 shows the relative cytokine scores for current and never smokers. Figure 7A shows the anti-inflammatory cytokine score for current and never smokers. Current smokers are shown to have significantly increased anti-inflammatory response on day 21 when compared to never smokers (p<0.05, 2-sample t test). Figure 7B shows the pro-inflammatory cytokine score for current
and never smokers. Current smokers are shown to have significantly increased pro-inflammatory response on days 1, 4, 14, and 21 when compared to never smokers (p<0.05, 2-sample t test).

Figure 8 shows relative cytokine scores for current and never smokers. Figure 8A shows the pro-inflammatory:anti-inflammatory cytokine ratio for current and never smokers. Both current and never smokers have a larger anti-inflammatory response than pro-inflammatory response (ratio <1) with these responses becoming more equal as the inflammation progressed. Current smokers had a significantly lower ratio on every day sampled when compared to never smokers (p<0.05). Figure 8B shows the chemokine score for current and never smokers. Current smokers have a trend for increased levels of chemokines present, however, there are no statistically significant differences between groups (p<0.05).
### TABLE 1: Demographic Data

<table>
<thead>
<tr>
<th></th>
<th>Never Smokers</th>
<th>Current Smokers</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Mean Age (Years)</strong></td>
<td>21</td>
<td>20</td>
</tr>
<tr>
<td><strong>Gender</strong></td>
<td>8 Males</td>
<td>12 Males</td>
</tr>
<tr>
<td></td>
<td>7 Females</td>
<td>3 Females</td>
</tr>
<tr>
<td><strong>Pack Year Tobacco Use</strong></td>
<td>0</td>
<td>1.46 ± 0.68</td>
</tr>
<tr>
<td><strong>Salivary Cotinine (ng/ml)</strong></td>
<td>0</td>
<td>28</td>
</tr>
</tbody>
</table>
Figure 1: Clinical indices in current and never smokers. A: Plaque Index (Rostogi). B: Gingival Index (Loe & Silness). Standard deviations are indicated. No significant differences were noted at any time point. (p>0.05, 2-sample t-test)
Figure 2: Diversity index for supragingival and subgingival bacterial populations in current and never smokers. A: Subgingival diversity index. B: Supragingival diversity index. Significant differences from baseline are indicated (*p<0.05, Repeated Measures ANOVA).
Figure 3: Percent population of bacterial genera subgingivally in health and gingivitis in current and never smokers. A: Health (1 day of plaque accumulation). B: Gingivitis (21 days of plaque accumulation). Significant intergroup differences are indicated (*p<0.05, Kruskal Wallis Analysis).
Figure 4: Percent population of bacterial genera supragingivally in health and gingivitis in current and never smokers. A: Health (1 day of plaque accumulation). B: Gingivitis (21 days of plaque accumulation). Significant intergroup differences are indicated (*p<0.05, Kruskal Wallis Analysis).
Figure 5: Subgingival Bray Curtis similarity index for current and never smokers. A: Never Smokers. B: Current Smokers. Significant differences between appointments and standard deviations are indicated. (*p<0.05, Repeated Measures ANOVA)
Figure 6: Supragingival Bray Curtis similarity index for current and never smokers. A: Never Smokers. B: Current Smokers. Significant differences between appointments and standard deviations are indicated. (*p<0.05, Repeated Measures ANOVA)
Figure 7: Cytokine scores for current and never smokers. A: Anti-Inflammatory Score. B: Pro-Inflammatory Score. Standard deviations between groups are indicated (*p<0.05, $p<0.01, 2$-Sample t-test).
Figure 8: Cytokine scores for current and never smokers. A: Pro-Inflammatory:Anti-Inflammatory Score Ratio. B: Chemokine Score. Significant differences between groups are indicated (*p<0.05, $p<0.01, #p<0.001, 2-Sample t-test).
CHAPTER 4

DISCUSSION:

The present investigation examined the compositional changes that occurred within the biofilm of 15 periodontally healthy current and 15 periodontally healthy never smokers. The study design allowed for comparisons of clinical indices of gingival health, supragingival and subgingival bacteria levels, and levels of immune mediators in gingival crevicular fluid following 1, 2, 4, 7, 14, and 21 days of uninterrupted plaque accumulation. These results provided an insight into the changes occurring within the biofilm during the progression from health to gingivitis, and the effect of smoking on these changes.

The Rustogi plaque index was utilized to record plaque levels throughout the study. The index was first introduced in 1992 as a modification of the original Modified Navy Plaque Index. The modification allows for more accurate assessment of both the interproximal contact area, as well as along the gingival margin of the teeth and provides an increased amount of classification categories allowing for a more definitive classification. The Rustogi Modified Navy Plaque
Index is commonly used in research studies, and compares well with other commonly used indices such as the Quigley Hein plaque index. During the course of our study both the current and never smoker groups underwent a similar increase in the amount of plaque present clinically. These results agree with data published by Bergstrom, Giannopoulou, and Salvi and conflict with results published by Feldman. There were no statistical differences between groups at any time point, therefore, both current and never smokers undergo similar changes to the amount of plaque present clinically when all methods of oral hygiene are removed. This suggests that smoking status has no effect on the quantitative accumulation of bacteria supragingivally. It has been reported by several groups including Ramberg, Daly, and Quirynen that there can be an alteration in the amount of marginal plaque formation in areas where gingival inflammation has previously occurred. This finding could be a potential limitation of the current study since there was no washout interval following each period of plaque accumulation, however both the current and never smokers underwent the exact same clinical protocol. Therefore, any alterations in plaque formation would have occurred in both study groups, and according to our data most likely occurred equally if at all.

The Loe and Silness gingival index was utilized in this study to evaluate the gingival status at each sampling appointment. The Loe and Silness gingival index is the most common index used in research to monitor gingival health, and
compares favorably to other methods of evaluating gingival status. Both the current and never smokers underwent a change in gingival health status from health to gingivitis over the 21 day period which is consistent with the study published by Loe in 1965, and confirmed by numerous others in recent years. Both groups experienced a mean Loe and Silness gingival index greater than 1 following 14 days of plaque accumulation, indicating the clinical transition to gingivitis. The smokers experienced an earlier clinical onset of gingivitis (day 7), which is in contrast with data reported by Bergstrom, Danielsen, and Salvi among others. However this difference was not statistically significant, and could potentially be explained by alterations in pre-study oral hygiene. Earlier studies have also evaluated smokers that had a greater pack year history, and therefore may not correlate well with our patient population who had a mean pack year history of 1.46. Although both examiners were calibrated several times during the duration of the study, McLanahan has reported the difficulties associated with calibrating multiple examiners to assess gingival index. They reported that even well calibrated examiners had a wide degree of variability in results.

16S cloning and sequencing was used to identify the bacteria present. This method targets the 16S gene which is a mosaic of highly conserved and variable regions giving each bacteria their own unique fingerprint. Since 16S cloning and sequencing does not rely on cultivation it provides information on
non-viable as well as bacteria not previously cultivated\(^49, 73-77\). The unique bacterial signatures are then compared to the database Genbank for final identification. The changes in bacterial composition are evident utilizing cultivation and light based techniques as healthy biofilms tend to be dominated by coccoid cells while the biofilms present following the onset of disease are dominated by motile rods, curved rods, and spirochetes with the ratio of motile to non-motile cells being 1:49 in healthy sites and 1:1 in periodontitis sites\(^41,42,45,52,53\). However, phenotypic identification is not an easy task, and greatly limits the results available. These studies have provided us much of the current foundation concerning the bacterial populations in health and disease\(^56-63\).

The Shannon Weiner diversity index was used to estimate the diversity within the community during each stage of plaque accumulation. The Shannon Weiner index provides a proportional diversity through evaluation of both the richness (amount of a particular species present) as well as the evenness (relative proportion of species present) of the bacterial population present in the biofilm\(^107\). The index is commonly used in ecologic research to estimate the diversity of the microbial communities present\(^107,108\). The largest limitation to the value being provided is based upon the resolution of the data being used within the index. Sequencing data tends to provide the highest diversity values since the Shannon Weiner index places larger weight on rare elements of the population than other diversity indices\(^108\). Both current and never smokers displayed an increase in the
diversity of their biofilms supragingivally as well as subgingivally which is consistent with the results reported by Quirynen\textsuperscript{52}, who found a complex biofilm present in less than 1 week using DNA-DNA checkerboard approach. Using 16S cloning and sequencing, Aas\textsuperscript{42} found 34-72 predominant species per site in periodontally healthy individuals, and in 2001 Paster reported an estimate of over 400 different species present in the biofilm\textsuperscript{79}. Both of these studies reported identifying 40-60\% novel bacteria with the utilization of 16S cloning and sequencing, underscoring the importance for open-ended analysis of the bacterial biofilm.

When the populations of the current and never smoker bacterial biofilms are compared we find that health in both groups was dominated supragingivally and subgingivally by gram positive species, specifically by the genera \textit{Selenomonas, Streptococcus, and Veillonella}. These findings are consistent with the results reported by Listgarten\textsuperscript{41} and Aas\textsuperscript{42}. Never smokers had significantly higher levels of the \textit{Neisseria} and \textit{Actinomyces} subgingivally, which are both well known health associated genera\textsuperscript{41}. The smokers had more genera of bacteria present subgingivally resulting in more diversity in the biofilm, with several subgingival bacterial genera present that were not present in health at any level in never smokers, including \textit{Fusobacterium}. Presence of potentially pathogenic bacteria in health is in agreement with the data reported by Zambon who found that smokers were at an increased risk of infection with \textit{B. forsythus}\textsuperscript{69}. This early
shift and presence of potentially pathogenic bacteria in health may be a large contributing factor to the increased risk of periodontal disease seen in current smokers.\textsuperscript{5, 6, 14}

The Bray Curtis similarity index provides a quantitative number to compare the similarity of the bacteria present at one given time point to another. There are numerous indices available to ascertain this information, and each index provides distinct benefits. Although each index has benefits, they also may provide vastly different results, and therefore comparisons between indices must be made with care.\textsuperscript{109} The Bray-Curtis index has been compared against numerous other similarity indices, and has been found to be an accurate reflection of true similarity.\textsuperscript{110} The information obtained from the Bray-Curtis similarity index is an important measure of the bacterial population change as patients progress from health to gingivitis. The results indicate that due to bacterial acquisition as the biofilm matures, the composition no longer resembles the population present in health. These variations in the bacterial population with the acquisition of new genera as the biofilm progresses from health to gingivitis agree with findings presented by Moore,\textsuperscript{111} Saglie,\textsuperscript{51} and Weiger.\textsuperscript{112} We note a significant change in the similarity of the bacterial profile from health at 14 days subgingivally in never smokers indicating that these disease associated bacteria have been acquired and are beginning to colonize the biofilm while current smokers never undergo this significant shift, indicating that many of the gingivitis
associated bacterial genera are present even in health. Conversely we note a significant shift in the similarity of the current smoker supragingival bacterial population to health at 7 days which is consistent with our increased gingival index also reported. Therefore it is evident that while the amount of plaque quantitatively forming supragingivally is similar in current and never smokers, there seems to be a marked shift in the population present subgingivally in never smokers as new bacterial genera are acquired, and supragingivally in current smokers that coincides with an increase in seen clinical soft tissue parameters.

An inflammatory and chemokine score was developed based upon the methods presented by Bauer\textsuperscript{113, 114}. They found that by grouping chemokine data into an overall chemokine score, the results displayed similar trends and allowed for stronger associations to be made. They also found that the chemokine scores were more strongly associated with systemic lupus erythematosus disease activity than were individual chemokine levels\textsuperscript{113}. A similar method of obtaining the score was used, and this provided a quantitative value to the pro-inflammatory, anti-inflammatory, and chemokine status as a whole at each plaque accumulation interval. The results indicate that current smokers exist in a state of hyper-immune response. Based upon the scores, current smokers had elevated immune activity of both a pro-inflammatory nature as well as an anti-inflammatory nature. These findings do not agree with several articles that link an immunosuppressive response to nicotine exposure\textsuperscript{115, 116}. However, these studies were in animals,
and had serum cotinine levels of 219±40 ng/ml, therefore the results may not correlate well to our patients with a mean salivary cotinine level of 28 ng/ml. While the data on the effect of smoking on inflammatory mediators is mixed, with Bostrom \textsuperscript{117, 118} and Sher\textsuperscript{119} finding no association between smoking and alterations in inflammatory mediators, and Giannopoulou\textsuperscript{94} and Moi\textsuperscript{120} have reported increases in mediators present in the GCF. The presence of increased mediators in agreement with Giannopoulou\textsuperscript{94} and Moi\textsuperscript{120} as well as data reported by Wannamethee\textsuperscript{23} and Helmersson\textsuperscript{24} of increased pro-inflammatory status systemically in smokers. This state of continued immune hyper-activity may be indicative of the highly diverse biofilm that is present even in health in current smokers, and may be a causative factor in future disease progression. When comparing a ratio of pro-inflammatory:anti-inflammatory state, it is evident that never smokers have a much more balanced immune response (indicated by a ratio closer to 1) which is likely a result of the immune tolerance developed by exposure to a health associated biofilm.
CHAPTER 5

SUMMARY & CONCLUSIONS:

The results of the present study show that over 21 days of experimental gingivitis both current and never smokers had a similar amount of plaque accumulation accompanied by a similar clinical shift toward gingivitis. While current smokers displayed clinical gingivitis at an earlier time point, there were no significant differences between groups in plaque index or gingival index at any plaque accumulation interval.

The Shannon-Weiner diversity index revealed an increase in population diversity as the biofilm matured toward gingivitis both supragingivally and subgingivally in current and never smokers.

The subgingival health associated biofilm present in current smokers is much more diverse than the biofilm present in never smokers, and contains potential pathogenic bacteria (Fusobacterium) that are not present in never smokers until the onset of gingivitis has occurred.

The Bray-Curtis similarity index revealed a significant shift in similarity to a healthy biofilm composition on day 14 in never smokers. Current smokers
never underwent this population shift, indicating that the biofilm present in health was similar to the biofilm present in disease.

The inflammatory scores obtained indicate that current smokers exist in a state of hyper-immune activity, in both a pro-inflammatory and anti-inflammatory manor.

Overall, smokers may be more primed for the onset of future disease due to the presence of potentially pathogenic even in the health associated biofilm, as well as due to the imbalance seen in the inflammatory response when compared to never smokers.
REFERENCES


