Investigating the Oral Microbiome in Health and Periodontal Disease

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science Periodontology Specialty
Faculty of Dentistry
University of Toronto

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Abstract

16S rRNA was used to determine the microbiome associated with health and chronic periodontitis (CP). We hypothesized that a comparison of plaque in health and disease will help identify CP-associated bacteria to develop novel diagnostics for CP. NGS was done against the V3 hypervariable region of the 16S rRNA gene and sequences were clustered based on 97% similarity. Taxonomic assignment and distance measures were used to assess bacterial composition. We identified disease indicators: Filifactor alocis, Synergistes, Tanerella forsythia and TM7 taxa in SupG and SubG sites. Health indicators included Rothia dentocariosa, TM7_7BB428, Selenomonas noxia, Fusobacteriales and Campylobacter. Surprisingly, ‘classic’ periodontal pathogens could be isolated from the tongue in CP patients, which may provide a novel sampling site for prognostic tests. We have identified known periodontal pathogens, including F. alocis, a strong indicator of CP, which could be included as a novel member in the Red complex of periodontal pathogens.
Acknowledgments

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This project would also not have been possible without the love, encouragement and support of my husband Chris and my little girl, Ariadne. Thank-you for being the loves of my life!

Finally, this thesis is dedicated to Maria and Konstantine Galimanas for always believing in me and for all their sacrifices to help me get to where I am today; with them, anything is possible. Thank-you!
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Preface

Dissertation format

This dissertation is presented in the “Publishable Style”. Chapter 1 serves as a thorough general introduction and literature review on periodontal disease and the role of bacteria as identified through different research techniques over the past 20 years. Chapter 2 involves the materials and methods used in carrying out the experimental design and Chapter 3 is a collection of the experimental data to be submitted for publication. Finally, Chapter 4 provides a discussion of the collected results.
## Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AAP</td>
<td>American Academy of Periodontology</td>
</tr>
<tr>
<td>AXIOME</td>
<td>Automation, extension &amp; integration of microbial ecology</td>
</tr>
<tr>
<td>BOP</td>
<td>Bleeding on probing</td>
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<td>CAL</td>
<td>Clinical attachment level</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<td>GM</td>
<td>Gingival margin</td>
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<td>HOMIM</td>
<td>Human Oral Microbiome Microarray</td>
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<td>IF</td>
<td>Immunofluorescence</td>
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<td>MRPP</td>
<td>Multiple-response Permutation Procedure</td>
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<td>NGS</td>
<td>Next-Generation Sequencing</td>
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<tr>
<td>OTUs</td>
<td>Operational Taxonomic Units</td>
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<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<tr>
<td>PCoA</td>
<td>Principle Co-ordinate Analysis</td>
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<tr>
<td>PD</td>
<td>Probing depth</td>
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<tr>
<td>QIIME</td>
<td>Quantitative Insights into Microbial Ecology</td>
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<tr>
<td>rRNA</td>
<td>Ribosomal ribonucleic acid</td>
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<td>Term</td>
<td>Definition</td>
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<tr>
<td>ss-DNA</td>
<td>Single stranded deoxyribonucleic acid</td>
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<td>SubG</td>
<td>Subgingival</td>
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<td>SupG</td>
<td>Supragingival</td>
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Periodontal Disease

Periodontitis is a chronic inflammatory disease-affecting tooth supporting structures including the alveolar bone, connective tissue attachment and gingiva. Several forms of the disease exist including chronic and aggressive, with chronic predominating in adults in their 4\textsuperscript{th} decade of life (6). Recent studies have shown that 47\% of the American population has periodontal disease, with the prevalence increasing to 64\% in those over 65 years of age (34,35,36). The current figures are considerably higher than the prevalence reported previously (35\%), 12\% of which was considered as moderate-to-severe disease (4). Indeed, the increasing prevalence of periodontal disease is concerning. The end outcome of periodontal disease is loss of the attachment apparatus and subsequent loss of teeth often leaving patients unable to eat and function properly. An estimated 85,000 visits to hospital emergency departments in the United States (in 2006), with cost of treatments over 33 million dollars are the result of periodontal disease-related morbidity (37).

The pathogenesis of periodontal disease is multifactorial and includes the host reaction to plaque biofilm on and around the teeth and other oral structures, factors related to the genetic makeup of the host and other epigenetic or environmental factors (e.g. smoking, socioeconomic status) (59,82,95,115,117,121). Host genetic factors associated with the disease include polymorphisms in the genes for IL1-beta-one and Fc\gammaR gene to name just two (86). Environmental factors such as smoking increase the odds of developing disease by up to 3-fold and the combination of smoking and gene polymorphisms have a synergistic effect on the development of periodontal disease, an odds ratio of 7.7 (86).
Löe et al., (1978) demonstrated the natural progression of this disease through a series of studies following a population of Sri Lankan tea workers over several years (78). This was a relatively uniform population that had little to no dental care and also had extremely poor oral hygiene. Overall, the presence and progression of disease was highly variable; the majority of the population was classified as “moderate progressing” (83%) while the minority were “non-progressing” (11%) or “rapidly progressing” (8%).

Since that time it has also become increasingly apparent that periodontal disease may be associated with several non-oral diseases including cardiovascular disease, diabetes as well as strokes and osteoporosis, although the evidence for the latter two is weaker than for the other disorders (11,38,41,87,89,95,105,108). It has also been reported that women with periodontal disease are at higher risk of having complicated pregnancies (16,28,96). While it is accepted that chronic periodontal disease shares many immuno-inflammatory features with other chronic diseases, it is not yet known whether periodontal disease is an actual risk factor for these comorbidities or more of a risk indicator. Regardless, patients with periodontal disease have higher levels of inflammatory markers in their serum that could theoretically lead to a hyper-inflammatory state also seen in the aforementioned diseases, and which could predispose patients to the development and/or exacerbation of these conditions (71,72,103). Alternatively, such ‘inflamed’ patients are those who are more likely to present with more than one condition with underlying pathophysiological conditions consistent with systemic inflammation. It has also been demonstrated that the severity of diabetes in patients with periodontitis can be improved following successful management of periodontitis (64,66). For example it has been shown in Pima Indians, a group of individuals who have a disproportionately high prevalence of periodontitis, that HbA1C can be decreased by up to 10% following treatment of periodontal disease (47). This was accomplished by the use of antibacterial agents such as Povidone-iodine.
and tetracycline antibiotics to complement mechanical removal (debridement) of plaque and calculus deposits on teeth. Similar findings have been reported by others (66). The association between periodontal disease and pregnancy complications such as pre-term low birth weight babies (LBWB) and pre-eclampsia has been claimed, but overall the evidence is weaker despite earlier findings reporting an odds ratio (OR) of 7.9 for LBWB in pregnant females with periodontitis (28,43,96). However, subsequent investigations could only demonstrate increases in OR in the range of 1.5, which was deemed to be insignificant (16,28).

Although there are several possible mechanisms that might explain the evident links between periodontal disease and non-oral diseases, it has been suggested that organisms implicated in periodontal disease, by way of their ability to disseminate systemically, could trigger or at least exacerbate atherogenesis (5,39,79). Moreover, periodontopathogens have been identified in atherosclerotic plaques, cerebral abscesses, pneumatic abscesses, and have also been associated with the development of bacterial endocarditis, although the mechanisms involved are not understood completely, they are thought to involve common immune-inflammatory mediators seen in both periodontal and the above mentioned systemic diseases (42,89,104,105).

**Diagnosis of Periodontal Disease**

Periodontal disease or health is diagnosed based on the presence and/or absence of several clinical measures as well as radiographic interpretation. The currently used American Academy of Periodontology (AAP) diagnostic guidelines were created in 1999 as a result of the consensus that earlier guidelines were age-specific and had significant overlap between categories (7). Also, categories like refractory periodontal disease and recurrent disease were removed from the classification system as any form of periodontal disease can be Refractory to treatment or recurrent. The current categories include Chronic and Aggressive, periodontitis as a
consequence of systemic disease as well as gingivitis. Early onset and Juvenile periodontitis were grouped into Aggressive periodontitis, again removing the age specification (6). In the new guidelines, patients are placed into Chronic or Aggressive categories and then further classified into localized or generalized disease based on involvement of 30% or more teeth. Further grouping into mild, moderate or severe is done based on clinical and radiographic measures. The ultimate marker of periodontal disease is tooth-loss. However, this process can take a long-time to occur with hopeless (according to McGuire classification) but maintained teeth being retained for longer periods of time (12,13,60,83). As a result, substitute markers such as clinical attachment loss (CAL) & probing depth (PD) are used in place of tooth loss to assess presence and severity of disease (84,85). Bleeding on probing (BOP) as well as furcation involvement, suppuration and mobility are also part of the diagnosis. Currently, there are no measures that signify “active” disease; repeat examinations with worsening conditions are often used to assess disease activity.

PD is measured from the gingival margin (GM) to the base of the gingival crevice. In general, 1-3mm is regarded as “healthy” with 4-5mm considered mild disease, 5-6mm moderate and >6mm as severe. Early studies looking at reproducibility of probing as well as the location of the periodontal probe relative to tissue inflammation showed probing to be reproducible within 1mm between and within examiners and that inflamed tissue did not affect PD as long as a constant marker on the teeth was used for measuring (2,20,46,63). However, tissue inflammation leads to swelling of the gingiva, and thus a stable position to anchor the probe is rarely achievable. Therefore, while PD is used as an indicator of disease, it can vary, and thus is not as reliable of a marker as CAL. CAL is measured from the cemento-enamel-junction (CEJ) to the bottom of the sulcus/top of the connective tissue attachment. If the CEJ is not available for restorative reasons, then a surrogate marker, such as a crown margin can be used.
Classification of periodontal disease based on CAL is as follows: 1-2mm (mild), 3-4mm (moderate) & 5-6mm (severe). Despite its association with inflamed & infected tissue, Lang et al., have shown that BOP is not a good marker of periodontal disease but absence of BOP is a good marker of periodontal health with a specificity of 98%. Overall, a diagnosis of periodontal disease is made from the collection of several surrogate markers of possible tooth loss, clinical measurements and bone loss around teeth (Figure 1a, 1b).

Figure 1a: Tooth and periodontium in health and disease. Blue arrow denotes bone loss, brown arrow SubG plaque/calculus and green arrow SupG plaque/calculus. Periodontal probing depth determined from free-gingival margin to base of pocket.

Modified from www.intelligentdental.com
The Role of Bacteria in the Development and Progression of Periodontal Disease

It is estimated that in the human body bacterial cells outnumber human cells by 10-fold and that bacteria contribute over 1,000,000 genes to the body while human (host) DNA provides approximately 25,000 genes (92,93). The influence of the majority of these bacteria on their host is un-known. However, bacterial association with periodontal diseases is clearly important and in fact, several organisms have been shown recurrently to be relevant to the disease process (22,25-27,39,42,50-58,67-70,76,92-94,98,101,111-114,118-120,125-126,128,129).
studies have shown that certain bacterial complexes associate with each other and potentiate periodontal diseases (112). To demonstrate this, DNA-DNA hybridization was used to test 185 healthy or periodontal diseased patients for the prevalence of 40 bacterial species using as well as for community ordination and association with 0-3mm, 4-6mm and >6mm probing depths. Microbiological profiles were assessed both at baseline and after unspecified periodontal treatment and in so doing, 5 different clusters of microbial organisms were identified that had >60% similarity and were classified as being “red”, “orange”, “yellow”, “green”, “blue” and “purple” complexes; the colours relating to the association of these clusters with varying degrees of the severity of periodontal disease parameters. Red and orange complex organisms associate with periodontal disease while blue, green and purple complex organisms are more “health” associated (Figure 1c). In this regard, it was shown, that in deeper sites that bled upon probing, *P. gingivalis* and other “red complex” species were highly prevalent (112). The “red complex” organisms typically presented with those from the “orange complex”. The reverse was not necessarily true, and thus the authors hypothesized that community ordination occurs whereby the communities build upon each other. In the healthy subjects, the less virulent “green” and “purple” complexes predominated whereas minimal amounts of “orange” and “red complex” organisms were seen. Earlier studies evaluated the probability of finding oral treponemes at sites that harbored *P. gingivalis* (101) showing that the likelihood of co-infection increased as PD increased. In 2000, Ximenez-Fyvie et al., showed that species present in disease were also present in the healthy state but in lower proportions (128). Also, SupG sites (above the gingival margin (GM)) harbored the same species as SubG sites (below GM), again in smaller proportions. The authors postulated that this may be a mechanism by which re-infection occurs and these “healthy” sites with “red” complex organisms may be at higher risk for progression compared to site lacking red complex organisms (129). In fact, earlier studies did show that the
progression of periodontal disease could be correlated to the presence of certain bacterial species (50-59, 118-120). In a Korean population, Choi et al., (2000) showed that healthy sites within patients who had advanced periodontal disease, there was a higher prevalence of *A. actinomycetemcomitans*, *Prevotella micros*, *Prevotella intermedia*, *P. gingivalis* and *Treponema* sp. compared to healthy patients perhaps confirming the impact of these bacterial species on the presence, progression and severity of periodontal disease (22).

**Figure 1c:** Bacterial complexes in health and periodontal disease. The red complex is most associated with disease and present in the deepest probing depths; red complex species are often found with orange complex species. Blue, green, purple-complex organisms are not typically associated with periodontal disease (Figure modified from Socransky et al., 1998).
Tanner et al., (1998) conducted a longitudinal study using microbial culture and DNA-probing to characterize bacteria found in samples of bacteria taken from the SubG regions in patients who were healthy, or had either gingivitis or active periodontitis (49). The comparisons were done within and not between subjects. Fifty-six healthy patients were monitored longitudinally for 18 months. Average pocket depth was 2mm and minimal loss of attachment was detected. Clinical measurements included CAL and PD, which were measured in duplicate. Other measures included levels of plaque, gingival-redness, BOP and SubG temperature on 6 sites per tooth (all but the third molars). CAL and PD were measured twice within two weeks to assess variation in probing measurements since such small differences in CAL were being measured. Clinical measurements were repeated at 3-month intervals for a period of 12-18 months. Sites were considered active if they showed CAL: 1) greater than that due to known variations in probing measures and 2) these changes had to be ≥ 1.5mm less than the measures made at baseline. Sites that demonstrated ≥1.5mm change in CAL were regarded as “initial” periodontal sites. Delineating the species present may identify those associated with initiation of disease initiation. Cluster analysis showed that 8/9 “active” patients with active disease had microbial profiles that were similar; higher Bacteroides forsythus (re-named Tannerella forsythia), Campylobacter rectus, Selemonas noxia, while lower levels of Pm and Capnocytophaga gingivalis were seen. The findings from this investigation suggest that T. forsythia, C. rectus and S. noxia are “candidates” organisms that might play an important role in the initiation of periodontal disease-related loss of tissue. These results were verified for T. forsythia by Tran et al., (2001) as well as van Winkelhoff et al., (2002). In the Tran study, 44 subjects with low prevalence and severity of periodontal disease were followed over 2-years, some had CAL, while others had gain or both CAL and gain of attachment (122). Patients who had persistently positive tests for T. forsythia had a 5.3 X increase in CAL compared to those in
whom *T. forsythia* was not detected continuously. In the Van Winkelhoff study, 116 patients with periodontitis were compared to 94 healthy patients. It was shown that both *P. gingivalis* and *T. forsythia* were associated with destructive disease. The OR for the detection of *P. gingivalis* in diseased versus non-diseased patients was 12.3 and for *T. forsythia* the OR was 10.4 (126).

**Microbial Testing**

While the clinical community understands that periodontal disease is of a microbial nature with modifying host factors, the use of routine microbial testing in clinical practice is lacking. In a systematic review of the literature, Listgarten (2003) attempted to find evidence showing that the identification of these bacteria can be valuable if the information gathered can affect diagnosis and/or treatment planning in a positive way (76). The literature was scanned for longitudinal studies with reference to outcome variables and bacterial identification. Longitudinal studies were lacking; the majority of studies could be described as being case-series and case-controlled investigations. Thirty one papers were retrieved by hand search and Pub-Med search; 13 of these focused on the use of microbial analysis to guide treatment of patients and differential response to treatment depending on microbial detection was shown in 11 publications. Unfortunately, since most studies did not include appropriate controls, it was not possible to determine with certainty whether the use of microbial analysis had any impact (positive or negative) on either diagnosis or treatment-outcome. Also, study designs were *heterogeneous* and so, data could not be pooled. The one publication that did include a control group reported that microbiologic testing actually did modify treatment plans and led to a reduction in the use of surgical treatment modalities following the use of appropriate antimicrobial agents as indicated by the results of microbial testing (Levy et al., 1993). Many
studies showed that in the absence of suspected periodontal pathogens, periodontal health could be expected. However the obverse was not found! In this regard, the presence of ‘indicator’ microbes did not always predict the presence of periodontal disease. While other studies showed that bacteria above a certain level were associated with worsened clinical parameters/recurrence of disease. Of all the periodontopathogens, *P. gingivalis* was associated with poorer response to treatment and increased recurrence of disease. Overall, it was concluded that there is a lack of RCT’s using microbial analysis limiting its value to diagnosis and treatment planning (76). Since there is only one study with an appropriate design, it is not possible to draw generalizable conclusions regarding the impact of microbial analysis on the diagnosis and management of periodontal diseases despite the *face validity* of this concept.

On the basis of information derived from the studies noted above, it is obvious that scientific research on the role bacteria play in periodontal disease has been done on a limited and select group of organisms that have been shown repeatedly to be associated with periodontal disease. However, as mentioned previously, bacterial cells outnumber human cells ten-fold; hence one cannot justify, in reality, focusing on a small subset of species while possibly ignoring potentially huge numbers of other microbes that could also contribute to disease (i.e. the bigger picture so to speak) (92,93). Given the vast array of microbes associated with the human body in general and the oral cavity in particular, it can be speculated that the aforementioned studies, which have linked certain microbial species with periodontal disease have overlooked other and perhaps many as of yet un-known species that are just as relevant and possibly even more-so, to the disease process than species that have previously been identified. Given this issue, the Human Oral Microbiome Project originated in 2007 in an attempt to delineate all species of bacteria present in the oral cavity (31). This project is only now possible with the advent of
newer molecular techniques and should provide a more complete representation of the complexity of bacterial species in the oral cavity (21,23,24,45,92,93,100,102).

Detection and Identification of Bacteria

Cell Culture

Historically, the so-called Gold Standard for identification and analysis of bacteria has relied on bacterial cell culture. However, there are limitations with this method. For one, only viable bacteria will grow, and stringent transport and storage conditions will be needed to identify those bacteria that are ‘cultivable’. Those that are not cultivable would of course remain unidentified. This is due in part to the fact that many species in the oral cavity are fastidious and require the presence of other organisms and/or very specific growth conditions. If those conditions, which might even be unknown and unexplored, are not provided, then the bacterial species that are the most fastidious will simply not grow in culture and will remain unidentified. Hence, bacterial identification using newer molecular methods will surpass such limitations associated with classical microbiological methods for identifying bacteria. However, this does not imply that cell culture will not be useful. For instance, cell culture is still essential to assess bacterial sensitivity to antibiotics and, ultimately, if possible, will be invaluable for verifying the presence of known species.
Immunofluorescence for Identification of Bacteria

Immunofluorescence (IF) methods are used to identify known species of bacteria, since this method relies on the use of antibodies generated against these organisms. In this regard, IF depends on the use of specific primary and secondary antibodies generated in animals, which function in recognizing and binding to known pathogens. IF may be more sensitive than culturing methods especially if the bacteria are present at less than threshold levels. IF does not allow for testing of antibiotic sensitivity but can be used to validate the presence or absence of species seen in culture studies. In a 1998 study by Tanner et al., a rapid-chair-side DNA probe test was compared to IF and culturing (119). IF was used to “reconcile” results from culturing and the PCR test thereby reducing false positives and negatives. Overall, the ability of each test to detect *T. forsythia* and *P. gingivalis* was similar; sensitivity and specificity increased with “reconciliation” using IF. Also, Noiri et al., (1997) showed that IF has the potential to “locate” the precise position of periodontal pathogens in pockets (94). Extracted teeth were exposed to IF and showed *P. gingivalis* predominant in deeper pockets and in close approximation to the epithelium. *Actinomyces viscosus* was seen in shallow, mid and deep pockets and were more densely packed and associated with plaque. IF is also useful in assessing host levels of periodontal pathogen specific antibodies (91).

Molecular Analysis of Bacteria

Molecular analysis for the identification of oral bacteria became popular in the 1990’s with closed-ended techniques such as whole genome probes and Polymerase Chain Reaction
(PCR) methods to identify specific bacteria. While useful in identifying the presence or absence of specific species, whole genome probes allow for cross-reactivity between species, and thus has sensitivity comparable to culture methods. Newer probes against oligonucleotides or species specific regions of the 16S rRNA gene are more specific. Nevertheless, probes provide an analysis of species that are known to be periodontal pathogens. Haffajee in 1992 presented a study using genomic probes against specific bacteria in localized vs. generalized disease (57). The conclusions of this study were that the probes provided a substantial but incomplete estimation of the microbial composition of SubG plaque. In a study comparing culture methods with species-specific probes, Van Steenbergen et al., showed high sensitivity and specificity using a mock community of species (P. intermedia, P. gingivalis and A. actinomycetemcomitans); these results did not hold true for clinical samples as the probes were less sensitive and less specific, potentially due to cross reactivity against other species (124). Further studies (Tsai et al., 2003) showed that a DNA probe kit was highly sensitive for T. forsythia but less sensitive and specific for P. gingivalis in comparison to culture techniques (123).

Newer methods using DNA-DNA hybridization demonstrated higher sensitivity and specificity against 40 known species as compared to DNA probe techniques (113). Cross reactivity of probes was minimal and differences between healthy and diseased patients as indicated by the presence of “red” and “orange” complexes in diseased, but not healthy patients were shown. Socransky et al., (2004) showed that this method detected $10^4$ species and that adjusting detection to $10^3$ bacteria can modify its sensitivity (113). PCR methods have also been used extensively for qualitative analysis of the presence of bacteria in test subjects. Meurman et al., (1997) showed that by using PCR methods, it was possible to detect T. forsythia in 52 of 58 patients whereas when using cultures, T. forsythia was found in only 22 of 58 patients (88). The
investigators concluded that PCR methods provide a more accurate representation of bacteria in periodontal pockets than does culture. This is likely due to a lower threshold of detection as well as higher specificity and sensitivity of PCR compared to culture. Basic PCR methods do have limitations in that they assess bacterial presence more or less qualitatively, and yet when carrying out investigations related to the pathogenicity of the organisms in question, it is also necessary to quantify bacterial load and this can be done using real-time (RT-) PCR. Indeed, it has been shown that (Boutaga et al., 2005) in comparison to data obtained with the use of RT-PCR data obtained from culturing suggested that the prevalence of various bacterial species was lower than that demonstrated with the use of molecular methods (17). Taken together, molecular methods developed in the 1990’s and early 2000’s focused on identifying (or verifying) the presence of known periodontal pathogens but due to their inherent limitations these methods could not be used to search for other as yet unidentified, but potentially clinically relevant (i.e. pathogenic) species. That is, these approaches were essentially closed-ended insofar as the identification of bacteria is concerned. It was not until the studies of Paster and Dewhirst that the research community understood the breadth of complexity of the bacterial populations harbored in the mouth, now referred to as the “oral microbiome”. Paster et al., 2001 used PCR amplification, cloning and sequencing of 16S rRNA segments to identify 347 species from 9 phyla present in subjects with and without periodontal disease (98). The authors reported that 68 species, previously unidentified, were present for a total of 415 species within the oral microbiome. The nine phyla included: Obsidian pool OB11, TM7, Deferribacteres, Spirochaetes, Fusobacteria, Actinobacteria, Firmicutes, Proteobacteria and Bacteroidetes. In analyzing bacterial samples from subjects with or without periodontal disease, the investigators demonstrated the presence of unique species that were associated with periodontal disease but were absent in health. Using these methods, known cultivable species including P. gingivalis and T. forsythia, were found
thereby substantiating the efficacy of the novel assay methods, which also provided data identifying other novel clones that made up a portion of the microbiome associated with periodontal disease. Consequently it was suggested that at least 10 of the newly identified phylotypes could be relevant to the disease process. In 2010 the Dewhirst group assessed 16S rRNA gene sequences of 36,043 clones and >1000 isolates for the purpose of identifying novel clones and also generating a 16S rRNA reference set for the human oral microbiome (31). A sequence similarity cutoff of 98.5% was used to define different phylotypes. From 1000 isolates, 619 taxa were identified and 65.5% of these have been cultured; the 6 major phyla accounted for 96% of these taxa while remaining phyla contained the other 4%. From clonal analysis, another 434 named and un-named taxa were identified in this study. Indeed, it is noteworthy that by 2010, there was an increase in the number of phyla from 9 that were described in 2001 to 13 as identified by 2010 and in fact it is estimated that >22 phyla exist in the oral microbiome. These studies showed that the current methods of studying bacterial populations and their relationships to periodontal disease were actually quite narrow in scope and that a much greater diversity of oral bacteria than what was ever expected actually exists. Consequently, it might be suggested that the research community is missing clinically relevant species by using closed-ended molecular techniques. Furthermore, in 2003, Kumar et al., used 16S rRNA analysis to show that in the diseased state, novel clones were just as prevalent as known pathogens (67). Also, in this study, Gram-positive species seemed to dominate the diseased state; the currently accepted notion of the pathogenesis of periodontal disease implicates Gram-negative bacteria as the consortium that leads to pathogenesis (71,98). The foregoing notwithstanding, even cloning and sequencing has its limitations in that it depends on the physical capacity to clone DNA fragments. This limitation is now being addressed with the advent of next-generation sequencing, as discussed below.
Next-Generation Sequencing and Bacterial Identification in Periodontal Disease

In a review of new molecular techniques to identify oral microbes, Pozhitkov et al., (2009) presented a diagram explaining the emerging direction of microbiological methods for bacterial identification (100). In this diagram, a clear representation of the progression from single to multiple to complete species identification in the oral cavity was illustrated; a feat made possible with the advent of ‘Next Generation Sequencing’ (NGS). NGS provides a new tool by which identification of novel phyla and species have become a practical means for further study of the oral microbiome (80,100,102,110). NGS became popular in the mid 2000’s with the advent of clone-independent sequencing methodologies and the realization that identification of microbes in a particular habitat (i.e. microbiome) could be completed in a short period of time. Several 1st, 2nd & 3rd generation NGS approaches now exist; The 1st generation approaches rely on DNA-amplification and are not as helpful for the identification of single molecules, whereas 2nd and 3rd generation methods are amplification-independent and can therefore detect single molecules (100). Unfortunately at this time, 3rd generation systems use nanopore technology and are not yet available to the scientific community at large (100). There are three 1st generation systems that function slightly differently from one another; Illumina-Solexa is one of those systems and relies on the following approach. In short, DNA is fragmented and adaptor sequences are added to each end. These fragments are immobilized on a microfluidic membrane with oligonucleotides matching the adapters. DNA templates are then copied via “bridge amplification” whereby the attached DNA fragment bridges over and hybridizes with a matching adaptor. Amplification yields a cluster of molecules with the same sequence; reverse strands are denatured and rinsed away leaving a single stranded (ss)-DNA strand that is then sequenced.
Polymerase and a mixture of unique fluorophore labeled nucleotides are added; one nucleotide attaches to its base pair at a time and a charge-coupled device camera captures the emitted light and deciphers the nucleotide associated. The reaction continues until the sequence is complete. This method allows for rapid sequencing of 16S rRNA genes from a multitude of bacterial samples (45). Thus far, Lazarevic et al., (2009) have used this method to identify salivary and oropharyngeal species from a small group of six patients (73). They analyzed 1,373,824 sequences identifying 135 genera; a greater depth of coverage was achieved by Illumina Sequencing than previously used molecular methods. Also, Ahn et al., (2011) compared the use of NGS with the available human oral microbiome microarray (HOMIM) and showed broader spectrum capacity of NGS (3). NGS was able to detect 77 genera whereas 49 were detected with HOMIM; 37 genera were commonly identified. In a study looking at differences in the oral microbiome of human subjects with and without gingivitis, the use of pyrosequencing led to the identification of 464 to 737 different operational taxonomic units (OTUs) per microbiome. Interestingly, different species were identified when comparing samples taken from subjects with and without gingivitis and this was seen only in samples derived from dental plaque but not from bacterial samples derived from saliva. Seventy genera were identified in this high-throughput study with 26 genera differing when comparing health and disease (61). In a recent publication by Griffen et al., (2012) 454-pyrosequencing was used to assess bacterial composition in subgingival samples derived from patients with periodontitis compared to healthy patients (44). In this study, the authors noted greater resolution, up to 2 orders of magnitude, was achieved with this technique compared to previously used methods including DNA-DNA hybridization. Patients with periodontitis had more diverse combinations of species but also had all of the health-associated species albeit at a lower frequency. Specifically, 123 species were found to be more prevalent in the disease state and 53 species were found to cluster within the healthy group.
Sixteen phyla, 106 genera and 596 species were identified with 81% of species matching to cultivated species.

Historically, bacteria linked to periodontal disease are typically considered as Gram negative, strict or facultative anaerobes. In this broad-spectrum study, a Gram positive organism, \textit{F. alocis}, clustered within the disease group inadvertently showing that previous techniques probing ‘known’ periodontal pathogens may have overlooked certain species not thought to associate with disease. Similarly, Liu \textit{et al.}, 2012 showed that Fusobacteria and Porphyromonas, 2 of several purported periodontal pathogens, were actually not very prevalent in samples derived from patients with disease to the extent that their levels were considered to be insignificant. The overall bacterial profile of the diseased group clustered together and had greater taxonomic diversity while the bacterial profile shown in healthy patients varied more between subjects with less taxonomic diversity; one healthy patient overlapped with the diseased group. Similarly, in a study looking at subgingival bacterial samples from healthy and periodontaly diseased patients, Abusleme \textit{et al.}, (2013) also showed increasing diversity in diseased compared to healthy sites (1). Diversity did not increase between bleeding and non-bleeding diseased sites but an increase in the total bacterial load was demonstrated in the SubG samples obtained from bleeding sites. Griffen \textit{et al.}, as well as Abusleme \textit{et al.}, both noted a clear distinction between healthy and diseased SubG sites when using distance metric analysis. The latter authors also attempted to convey the idea of a “core” microbiome of bacteria that is seen in health and disease as well as those that overlap. Most abundant periodontitis-associated species included several Treponemes and TM7 while \textit{T. forsythia, P. gingivalis}, several Peptostreptococcaceae, Synergistes, Eubacteria and \textit{F. alocis} rounded out the list of periodontal pathogens. Several Actinomyces as well as \textit{R. dentocariosa} were the most abundant health-associated species with a Porphyromonas species and \textit{Burkholderia cepacia} also on the list.
Overlapping species included several Fusobacteria, *Lautropia mirabilis*, *Corynebacterium matrichotii*, *Campylobacter gracilis* and others. Interestingly, *F. alocis* was again seen on the spectrum of periodontal disease organisms (1,44).
Rationale

The human oral microbiome is a highly complex group of bacteria, only some of which are associated with the disease state. Most current analyses of this bacterial community appreciate and yet underestimate the taxonomic diversity of the oral microbiome in both health and disease. In this study, we aimed to use an ultra-deep sequencing approach to investigate the microbial community structures associated with the human oral cavity in health and chronic periodontal disease. The overarching goal is to gather an as complete as possible taxonomic characterization of the microbiome found in healthy and diseased mouths thereby permitting the identification of health and disease indicator organisms, as well as the discovery of novel bacteria that could have a significant impact on the pathogenesis of periodontal disease relative to those already identified as disease indicators, to date. Results from this study can expand our understanding of the pathophysiological processes underlying periodontal disease, which could also lead to the development of novel and more effective strategies for treatment than exist currently.
Objectives

The objectives of this study were designed to answer the following questions:

1) Is there a unique oral microbial consortium associated with chronic periodontal disease (CP) relative to health?

2) Can we identify bacterial biomarkers abundantly and frequently present in subjects affected by CP, relative to those in health?

3) Can supragingival and tongue sites serve as suitable sampling sources to determine the presence and relative abundance of disease indicator bacteria?

4) Our long-term objective is to harness our knowledge of the oral microbial community composition in different sites of the mouth under health and CP for use in diagnostics, prognostics and development of novel therapeutic treatments. The use of tongue plaque for the monitoring of treatment success in CP is novel, convenient and minimally-invasive.
Hypotheses

1) CP is associated with a distinct plaque microbiome in SupG, SubG and tongue sites relative to that in periodontal health.

2) Bacterial communities present in tongue and SupG plaque harbor indicator organism’s characteristic of periodontal health or disease.

3) In addition to probing gingival pockets to obtain SubG plaque, SupG and tongue plaque can be utilized as relatively novel sources to detect indicator organisms present abundantly and frequently under periodontal disease.
Chapter 2
Materials and Methods

This cross-sectional clinical study of the oral microbiome included 24 patients recruited through the University of Toronto Graduate Periodontics and Undergraduate Clinics. The study was approved by the University of Toronto REB (#: 23872) and informed consent was obtained after answering all patient questions. Eleven healthy and 13 advanced chronic periodontitis patients, as assessed by clinical and radiographic examinations, consented to the study. Patients with systemic disease, antibiotic use and/or professional cleaning within the last 6-months were excluded from the study. Supragingival (SupG) and subgingival (SubG) plaque samples were gathered from interproximal sites between teeth 16/15, 15/14 as well as 26/25, 25/24 along with a midline scrape of the tongue. After collection of SupG plaque with Gracey curettes, the tooth was cleaned with sterile gauze and separate curettes were used to collect SubG samples. All SupG samples of a single patient were pooled as were the SubG and tongue samples resulting in three samples per patient. DNA was extracted using GenScript BacReady DNA extraction kit (Piscataway, NJ) and samples were sent for Illumina-Solexa Sequencing. Verification of DNA extraction protocol was completed as seen in Supplemental file 1.

The Illumina library against a 150 base-pair region of the V3 16S rRNA gene was generated using modified universal 341F and 518R primers (Table 2). Three PCR reactions were carried out for each sample using 50ml reaction volumes with each mixture comprising 25pmol of each primer, 200mM of each dNTP and 1.5nM MgCl2 and 1 U of Phusion Taq
Polymerase enzyme (New England Biolabs). The PCR protocol included denaturation at 95°C for 5 minutes and 20 cycles at 95°C for 1 minute, 50°C for 1 minute and 72°C for 1 minute. The reaction ended with an extension step at 72°C for 7 minutes in a DNA Engine thermocycler (Bio-Rad, Mississauga, Ontario, Canada). Two-percent agarose gels were used to separate products from primers and primer-dimers and to recover the PCR product with QIAquick gel extraction kit (Qiagen). Ten ng/μl of DNA was used for sequencing; sample concentration was verified by running DNA on gel and also by nanodrop analysis by NanoDrop (Thermo Scientific, Wilmington, DE, USA). Paired-end sequencing with indexing was done on the samples. The complete protocol is described in Bartram et al., 2011 (10).

**Data Analysis**

Sequence data was run in four separate AXIOME (v1.6) pipelines (SubG samples, SupG samples, tongue samples, and all samples pooled). All pipelines used the same configuration. For clustering, cd-hit-est (v4.5.4) was used with a 97% sequence identity threshold. Multiple sequence alignment was completed with PyNAST (v1.2) via QIIME (v1.6). Tree building for UNIFRAC distances was performed by FastTreeMP (v2.1.3) using default settings. Classification was completed using RDP (v2.2) via QIIME with a confidence cutoff of 0.8. A merged GreenGenes (October 2012 revision) and OSU CORE oral (February 9, 2012 revision) database was used to classify all sequences. OTU table generation was completed by QIIME (30).

UNIFRAC PCoA and alpha rarefaction curves were created through QIIME. Dufrene-Legendre indicator species analysis (32), Bray-Curtis PCoA, and MRPP analyses were created by the AXIOME pipeline. Alpha diversity plots were created by Phyloseq (v1.3.14).
**Table 1:** Primer sequences have a 3’ region representing the 341F and 518R segments of bacterial 16S rRNA as well as Illumina sequencing priming sites and indices on the reverse primer read. The forward primer also contains a high diversity area adjacent to the Illumina forward sequence segment to allow for improved clustering and identification in data analysis domains. Paired end sequencing provides quality control during the assembly process. (Bartram *et al.*, 2011)
Chapter 3
Results

Use of tongue plaque as a novel indicator of chronic periodontal destruction

1) Demographics, clinical attributes and sequencing

Fifteen females and 9 males ranging in age from 18-71 years were recruited for this study. The periodontally healthy group consisted of eight females and 3 males ranging in age from 18-56 years, whereas those with chronic periodontitis (CP) comprised of 7 females and 6 males aged 25-71. We diagnosed CP based on radiographic and clinical examination of the patient’s periodontium with probing depth (PD) and clinical attachment loss (CAL), as well as radiographic bone loss evaluated on every tooth. In this study, the diseased group comprised of patients with >30% sites with ≥5mm clinical attachment loss and probing pocket depths ≥6mm, whereas the healthy group had >30% sites with ≤2mm attachment loss and no pockets >3mm. No patients with systemic diseases, antibiotic use and dental cleanings within a 6-month timeframe were included in the study. We have summarized the demographics, clinical data and personal habits (smoking and alcohol consumption of participants) in Table 2.

To comprehensively characterize how bacterial communities change in periodontal health and in CP, we collected plaque samples from SupG, SubG and tongue sites from 24 individuals. For community profiling, we amplified plaque DNA with primers covering an approximate 150-bp segment of the V3 region of the 16S rRNA gene. DNA sequencing yielded ~5.8 million sequences, with a minimum of 27,187 sequences per sample (average of 57,441) and between-sample analyses subsampled to 27,187 sequences.

After quality filtering and assembly by PANDAseq, ~4.1 million sequences remained.
These sequences were collapsed into operational taxonomic units (OTUs) based on pairwise sequence identity cut-off value of 97%. OTUs with less than 3% difference in sequence similarity were arbitrarily assumed to form the same species, genus and family. Of the total sequences, 49,327 formed singletons, whereas 6,160 OTUs did not classify to phylum level, representing 66,466 sequences (~0.02% of the usable sequences). By binning sequences into unique OTUs, we detected a total of 19 phyla, 114 genera, and 303 species.

<table>
<thead>
<tr>
<th>Demographics, Clinical Parameters and habits</th>
<th>Healthy Group (n=11)</th>
<th>Diseased Group (n=13)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td>3 males; 8 females</td>
<td>6 males; 7 females</td>
</tr>
<tr>
<td>Probing depth (mm.) in &gt; 30% of sites</td>
<td>≤2mm</td>
<td>≥5mm</td>
</tr>
<tr>
<td>Attachment loss (mm.) in &gt;30% of sites</td>
<td>≤1mm</td>
<td>≥3mm</td>
</tr>
<tr>
<td>Periodontal diagnosis&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Periodontal health</td>
<td>Chronic Periodontal disease (CP)</td>
</tr>
<tr>
<td>Smokers&lt;sup&gt;+&lt;/sup&gt;</td>
<td>1</td>
<td>6</td>
</tr>
<tr>
<td>Drinkers&lt;sup&gt;+&lt;/sup&gt;</td>
<td>2</td>
<td>5</td>
</tr>
</tbody>
</table>

**Table 2:** A smoker<sup>+</sup> was defined as someone who had one or more cigarettes per day ≥3m, whereas a drinker was defined as an individual who consumed ≥56 drinks per year<sup>a</sup>. Periodontal diagnosis based on criteria as outlined in the 1999 American Academy of Periodontology Diagnostic Guidelines.
Advanced periodontal disease is not significantly correlated with changes in $\alpha$-diversity.

Recent studies that have comprehensively examined site-specific community diversity associated with individuals suffering from severe caries and periodontal disease have implicated significant changes in taxonomic diversity found in health versus disease (1,44). For instance Abusleme et al., and Griffen et al., had noted increased $\alpha$-diversity under periodontal disease relative to that in health. To determine whether community composition under CP was correlated with substantial alterations in OTU-level diversity from that in health, we used Phyloseq to detect $\alpha$-diversity in SupG, SubG and tongue sample sites between our healthy and diseased groups. Interestingly, our results showed that an increase or decrease in species diversity was not accompanied with the disease status in any of our sampling sites (Figure 2). Unpaired T-test was done on each alpha-diversity calculation showing that no statistical significant difference in diversity was seen between disease and health state ($S_{\text{obs}}$: $P=0.9316$; $S_{\text{chao1}}$: $P=0.6445$; $S_{\text{ACE}}$: $P=0.5969$; Shannon: $P=0.8596$; Simpson $P=0.2695$). It is likely that discrepancies in the sampling technique (curette versus paper-point methods), sampling location, or method of DNA extraction possibly explain differences in $\alpha$-diversity measures in these studies.

**Figure 2:** Shannon, Simpson, $S_{\text{chao1}}$, as well as other alpha diversity metrics were used to measure the alpha diversity between samples in healthy and diseased patients. No difference in
alpha diversity between healthy and diseased patients was observed denoting similar richness and evenness in community structures in these samples.

Tongue harbors a unique consortium, and periodontal destruction is associated with a distinct ecological shift relative to health

To assess bacterial community structure similarities within SupG, SubG and tongue sample sites in healthy and diseased patients, we used PCoA with Bray-Curtis dissimilarity measures. A multidimensional scaling analysis was then performed on the Bray-Curtis dissimilarity matrix and the clustering structure was highlighted based on sample site marked with different colors (green for SubG; blue for SupG and red for tongue). Figure 3 shows a clear separation between a cluster of tongue samples and a cluster of tooth samples, suggesting that the tongue community is relatively unique from that in SupG and SubG sites in our patient cohorts. On the other hand, no obvious clustering was apparent between SupG and SubG samples. Running the PCoA analysis using the UniFrac distance measure produced similar results and MRPP analysis of the weighted means of the UniFrac distances showed statistically significant differences between tongue and tooth plaque (MRPP Statistics: A=0.05431, T=-16.9, p<0.001) (data not shown).
Figure 3: Bray-Curtis dissimilarity measures were used to generate PCoA plots of SubG, SupG and Tongue samples. Tongue samples (red) clustered distinctly from tooth plaque (blue, green), whereas a clear separation between SupG and SubG was not apparent (MRPP Statistics: $A=0.05431$, $T=-16.9$, $p<0.001$).

Next, we asked whether the CP community composition in SupG, SubG and tongue sites was distinct with that present under health. Previously, Griffen et al., had reported clustering based on disease, although only SubG plaque was sampled in their study. Our PCoA results showed that SupG, SubG and tongue samples derived from CP patients tended to cluster (Fig 4 A, B, C), although in the case of SubG and tongue, 3 and 4 healthy samples overlapped with the diseased clusters, respectively (Fig 4A and 4C). Despite this overlay, the level of clustering observed between the healthy and CP samples suggest that a distinct community composition could be identified based on health and disease states of the periodontium, in accordance with reports from other groups (1,44). As for SupG sites, 5 samples from CP patients overlapped with the healthy cluster, suggesting a similar community composition between them (Fig 4B). UniFrac dissimilarity analysis was also conducted comparing health and CP at each sampled location; MRPP analysis showed significant separation between health and disease for SubG and Tongue samples only (SubG MRPP A: 0.014, T: -2.16, P=0.029; Tongue MRPP A: 0.016, T: -1.82, P=0.042; SupG MRPP A: 0.038, T: -0.48, P=0.267). Relative to SupG, the strongest clustering of health and disease samples was observed in the SubG and tongue site samples (Figure 4 A, C).
Figure 4: PCoA plots of SubG (A), SupG (B) and Tongue (C) samples showing mild clustering between health and disease. The strongest clustering between health and disease was seen in the SubG and tongue samples (A, C), which was supported with MRPP statistics.
Relative abundance of distinct taxa is affected by periodontal destruction in oral plaque communities

Using our CP and healthy samples, we compared the relative abundance of taxa to determine whether periodontal destruction was correlated with drastic changes in the abundance of phylotypes. To do this, we pooled SupG, SubG and tongue datasets for health versus CP, and sequence data was binned by classification to OTU levels. Relative abundance was calculated by dividing the number of sequences (in healthy or diseased sites) classified to a particular phylotype by the total number of sequences found for that site in the same group of healthy or diseased individuals. The most abundant taxa in CP belonged to phylum Firmicutes (Fig 5). To define an association with SupG, SubG and tongue locations, we also examined the relative abundance of taxa by sample sites. Compared with SupG, a significant difference in the relative Firmicutes abundance of over 20% in disease versus health was noted for tongue and SubG sites (Fig 5 B, C). In both locations, roughly, the Firmicutes’ members comprised nearly 40% of the healthy consortia, whereas a substantial number nearing 60% was noted under CP. These results suggested that the tongue and SubG sites were substantially affected by periodontal destruction showing drastic shifts in community composition. Among other phyla, CP patients also harbourred Synergistes and Spirochaetes in greater abundance (>1%) relative to those in healthy subjects (Fig 5). On the other hand, the health status was marked by an increased abundance in phyla belonging to Actinobacteria, Bacteroidetes and Proteobacteria among all sample sites (Fig 5); (>0.1% difference). Once again, the difference in relative abundance between health and disease for these phyla were profoundly affected in tongue and SubG locations relative to that in SupG sites (Fig 5 A, B);
 (>0.1% difference). It is noteworthy that the relative abundance of Fusobacteria was dramatically high (>0.1%) in the SubG consortia under health and SupG consortia under disease; however, this increase was not observed in tongue sites.
B  Relative Abundance, Phylum Level, Supra

Phyla

- Proteobacteria
- Actinobacteria
- TM7
- Unclassified Bacteria
- Synergistetes
- Spirochaetes
- Chloroflexi
- GN02
- Tenericutes
- OP11
- SR1
- Bacteroidetes
- Fusobacteria
- Firmicutes

Group

- Red: Diseased
- Green: Healthy
**Figure 5:** Relative abundance plots at the Phylum level for SubG (A), SupG (B) and Tongue (C) samples. Firmicutes were most abundant in all three locations of CP patients; Synergistetes and Spirochaetes more abundant under CP in SubG and SupG samples; Fusobacteria were abundant under CP in SupG samples and SubG healthy samples. Healthy SubG and SupG samples were dominated by TM7, Actinobacteria, Proteobacteria, Bacteroidetes and Fusobacteria, while tongue samples also harboured members of the SR1 phylum.
Closer examination of the CP-associated abundance profiles at a higher resolution revealed classic periodontal disease-associated OTUs in high abundance under disease; these included, *T. forsythia, F. alocis, P. intermedia,* and *Fusobacterium nucleatum.* In addition to these known species, we were able to identify a wide range of taxa that were detected in high abundance in CP patients (Fig 6). Unexpectedly, periodontal disease-associated species, *P. gingivalis* and *F. nucleatum* were found in abundance in healthy SubG samples. Along with tongue samples, the SubG sites in healthy individuals also harboured *R. dentocariosa* and TM7 7BB428 (member of the unclassified TM7 phylum) in high relative abundance compared with that in disease (Fig 6 A, B).
Relative Abundance, Species Level, Supra

Species

Group

Diseased

Healthy

% Abundance (within group)
Figure 6: Relative abundance plots at the species level for SupG (A), SubG (B) and Tongue (C) samples. Previously identified periodontal pathogens such as \textit{T. Forsytha} and \textit{Synergistes} OT362 & 363 were highly abundant in diseased samples as was a relatively new pathogen: \textit{F. alocis}. While \textit{Synergistes} have been linked to periodontal disease, little is
known about them, as they are fastidious and difficult to cultivate. On the other hand, several new studies look into *F. alocis* and its virulence potential. *R. dentocariosa* was abundant in health.

**Periodontal destruction and periodontal health are associated with characteristic indicator organisms**

To determine if organisms present in high abundance in our sample sites served as characteristic taxa of health or disease, we performed indicator analysis of binned sequences using Indval function of the R language’s ‘labdsv’ package. Indicator species were defined as the most characteristic found mostly in a particular group (e.g. health or disease) and present in the majority of sites belonging to that group. In addition to the species relative abundance, indicator species analysis takes into account the relative frequency of occurrence in the various groups of sites (Dufrene & Legendre 1997). Figure 8 shows the indicator associations with health and/or CP. At the species level, periodontal destruction at the SubG, SupG and tongue sites was associated with 16, 14 and 19 species whereas health associated indicators included 11, 7 and 7 species. In all sites, the majority of species were not found to be associated with either health or disease. In fact, this large number of health-un-associated species suggests that microbial composition in CP is a reflection of changes in the relative abundance and frequency of particular taxa that emerge from an ecological shift in the baseline community composition of healthy individuals.

Indicator analysis from pooled data for SupG, SubG and tongue sites revealed several indicator OTUs as strong representatives of disease and health states (Fig 7). Synergistes oral
taxon (OT) 363 and Synergistes OT 362 showed the highest indicator values for CP, whereas *Streptococcus sanguinis*, *T. forsythia*, *F. alocis*, *Dialister invisus*, *Streptococcus sp.* and TM7 401H12 also had high indicator indices for disease. *F. alocis* is a Gram-positive anaerobe determined to be prevalent in periodontal disease by Griffen *et al.*, as well as Schlafer *et al.*, 2010 (1,107). It is thought to be associated with progression of the disease and is an “uncharacteristic” species previously not “probed” by conventional methods. Interestingly, *F. alocis*, *T. forsythia*, as well as the unclassified Synergistes OT 363 and 362 were indicators of disease in all three sampled locations when the Indicator species cut-off was set at >0.46. While the level of indication was higher from SubG samples, presence in SupG and more importantly tongue samples also indicated the diseased state. On the other hand, *R. dentocariosa* was strongly associated with periodontal health, whereas others in this group included unclassified Fusobacterales, TM7 7BB428, *Campylobacter rectus*, Uncultured Lachnospiraceae, Oral Taxon 100, Unclassified Bacteroidetes, Aggregatibacter, *Capnocytophaga gingivalis*, *Corynebacterium matruchotii*, *Neisseria*, *Selenomonas noxia* 2 and *Selenomonas artemid*. 
A

Synergistes oral taxon 363
Desulfovibrio
unclassified Clostridiales MCE10 174
Streptococcus intermedius constellatus
Synergistes oral taxon 362
Filifactor alocis 1
Streptococcus 1
Peptostreptococcus
TM7 401H12 1
Tannerella forsythia
Streptococcus 2
Filifactor alocis 2
Filifactor alocis 3
Filifactor alocis 4
Streptococcus 3
Synergistes
TM7 401H12 2
Veillonellaceae oral taxon 135 145 148 155
Synergistes oral taxon 362 2
TM7
Streptococcus 4
Actinomyces cardiffensis
Choroflexus genomosp. P1
Filifactor alocis 5
Filifactor alocis 6
Unclassified Fusobacteriales 1
Prevotella leschekii 1
Campylobacter 1
Uncultured Lachnospiraceae oral taxon 100
Campylobacter 2
Kingella oralis
Porphyromonas
Unclassified Fusobacteriales 2
Corynebacterium matruchotii 1
Prevotella oris
Leptotrichia
Unclassified Fusobacteriaceae 1
Neisseria
corynebacterium matruchotii 2
Prevotella melaninogena
Unclassified Firmicutes
Unclassified TM7
Capnocytophaga leadbetteri
Unclassified Fusobacteriales 3
Tannerella BU063
Unclassified Bacteroidetes 1
Actinomyces georgiae
Prevotella leschekii 2
Campylobacter 3
Capnocytophaga spuligena 1
corynebacterium matruchotii 3
Neisseria flava mucosa pharyngis
Capnocytophaga spuligena 2
Campylobacter 3
Unclassified Bacteroides 1
Unclassified Bacteroides 2
Prevotella
Unclassified Fusobacteriales 4
corynebacterium matruchotii 4
Unclassified Fusobacteriaceae 2
Unclassified Bacteroidetes 3
Unclassified Gamma proteobacteria
Prevotella oral taxon 781
Prevotella leschekii 3
SR1 AF125207
Porphyromonas CW034

Classification

Abundance (sequences)

Indicator OTUs, Subg

Group

Diseased

Healthy
Figure 7: Indicator species of CP and health status at SubG (A), SupG (B) and tongue (C) sites.

Indicator value cut-off 0.6 was used showing *F. alocis* and *T. forsythia* as well as Synergistes OT 363 and 362 as strong indicators of disease in SupG and SubG samples. A cut-off of 0.46 would include *F. alocis* as an indicator species of CP in the tongue (not shown).
**Tongue harbors the highest number of indicator organisms characteristic to periodontal destruction, whereas indicators of health were highest in SubG sites**

Our results showed that the number of indicators at phylum, genus and species levels was more abundant under disease relative to that in periodontal health for SubG, SupG and tongue sites (Fig. 8). A perturbation in the ecology under CP is triggered by or leads to a significant increase or decrease of taxa whose frequency of detection can be relatively higher than that in health. This was especially apparent in tongue sites, where 19 indicator species were detected in disease compared with only 4 indicator species under health. A comparison of the numbers of health and disease indicators in all sampled sites revealed that tongue sites harbored the most number of indicator species. *Catonella morbi*, several Fusobacteria species, *T. forsythia*, *T. denticola*, Synergistes OT 363& 363 and *F. alocis* were all indicators of CP from tongue samples (Indicator value >0.46). While *F. alocis* had a lower indicator value in the tongue compared to SubG and SupG samples, it was still associated with the disease state. This finding has important implications in perhaps using the tongue as a sampling site for studies aimed at community profiling or to assess treatment success in persons suffering from periodontal destruction.
Figure 8: Pie diagrams showing the number of indicator species in health and/or disease, as well as those that were not associated with either status. The tongue had the most indicator species of disease compared to SubG and SupG locations.
Chapter 4
Discussion

The microbial composition in dental plaque remains relatively stable, despite regular exposure to subtle perturbations in the oral environment (49,54,67,68,69,81,97,111,112,131,132). This stability or “microbial homeostasis” in a biofilm is maintained by a dynamic balance of both synergistic and antagonistic microbial interactions (72); disintegration of this balance leads to initiation of infections such as periodontal disease and dental caries. In this study, we attempted to identify the consortia associated with CP and periodontal health by examining the plaque microbiome derived from SupG, SubG and tongue sites. Here we report a comprehensive cross-sectional “snap shot” of the microbial community composition in CP and health using high-throughput Illumina Sequencing. Our findings reinforce results from other studies in that periodontal disease is marked by an ecological shift that appears as a disruption of the microbial homeostasis under health (1,40,44,61,77). In addition to identification of known taxa previously associated with CP, here we also reveal previously unreported OTUs that are strongly correlated with periodontal health or CP.

A novel aspect of our work is the use of tongue plaque for a comprehensive OTU analysis underlying health versus chronic periodontal disease. The keratinized mucosal surface present on the tongue dorsum is a different colonization surface relative to that of tooth structures, which are comprised of highly mineralized tissues including enamel and cementum. The tooth surfaces are colonized by different consortia depending on the distinct anatomical site (120). Not surprisingly, in our PCoA dissimilarity matrices using
SupG, SubG and tongue plaque, tongue samples clearly separated from tooth plaque samples (Fig 2). Hence, this indicated a different community composition on the tongue dorsum as opposed to SupG and SubG sites. Further, using tongue plaque, we were able to detect CP-associated OTUs that included: *T. forsythia*, *T. denticola* and *F. alocis*, as well as two novel phylotypes designated Synergistes oral taxon (OT) 362 and Synergistes OT 363, which we confirmed as common indicators of disease (indicator cut-off $>0.46$). The overlap of indicator species under periodontal destruction between tooth plaque and tongue plaque meant that despite differences in colonization surfaces, some species indicative of the disease state were found abundantly across all our test sites in a majority of CP individuals. In our study, *R. dentocariosa* was highly abundant in individuals with periodontal health and in SupG and SubG sites; this organism was one of the best indicator species of health. However, despite its high abundance in healthy tongue samples, a smaller cut-off value was necessary to include *R. dentocariosa* as a tongue indicator species of health.

Previous work, including a renowned study by Socranky *et al.*, (1998) have shown that certain pathogenic bacterial complexes associate with each other and potentiate periodontal diseases (112). These complexes were designated by a color code that included “Red”, “Orange”, “Yellow”, “Green” and “Purple”, wherein the colors related to the association with varying degrees of the severity of periodontal disease parameters (112). In this regard, the Red complex encompassed *P. gingivalis*, *T. forsythus* and *T. denticola*, which were highly prevalent in deeper sites that bled upon probing (112). Moreover, these organisms were typically present with those from the Orange complex, although the reverse was not necessarily true. Prior to this, it was reported that that the probability of
finding oral treponemes at sites that harbored *P. gingivalis* was escalated as periodontal destruction was increased (101). Also, Ximenez-Fyvie *et al.*, showed that species present in disease were also present in the healthy state but in lower proportions (129), while the same group demonstrated that SupG sites harbored the same species as SubG sites below the gingival margin, again in smaller proportions (128). These studies postulated that this may be conducive to re-infection and individuals with “healthy” sites with abundant Red complex organisms may be at higher risk for disease progression. Hence, it may not be surprising that our Bray-Curtis and Unifrac dissimilarity plots did not show a solid distinction in community composition as judged by the extensive overlap between samples. Further, although the color coded clustering of periodontal health associated organisms has been useful and informative, in light of the accumulating list of periodontitis indicator organisms from deep-sequencing studies it may be necessary to revise the constituent species in the colored complexes previously introduced by Socranksy and colleagues (112).

Organisms encompassing the Red cluster (112) are noteworthy: Traditionally, its constituents have been regarded as bacterial biomarkers of periodontitis, which have been used to monitor treatment outcomes. In addition to the classic periodontitis-associated pathogens in the Red complex, we and others (1,25,26,44,68,69) have repeatedly detected *F. alocis* in high prevalence in patients with increased periodontal destruction. Although this organism has been detected in tongue, SupG and SubG plaque, we obtained higher indicator values for its presence in SubG samples. It is noteworthy that *F. alocis* is a Gram positive facultative anaerobic rod that has gained notoriety as a periodontal pathogen and has repeatedly been identified by several other molecular and high-throughput studies (1,25,26,44,68,69). It is thought to have trypsin proteases, capacity to invade human cells,
resist oxidative stress and form biofilms—properties that are conducive to disease initiation and progression (9,90,107). In particular, in an epidemiological study done by Schlafer et al., prevalence of *F. alocis* was elevated in patients with generalized aggressive periodontitis and CP, whereas it was rarely detected in the control group of periodontal health (107). While it was shown to predominantly colonize apical parts of the pocket in close proximity to the soft tissues, using carrier-borne plaque it was shown to contribute to the structural integrity of these multi-species biofilms. Hence, *F. alocis* was suggested to be a powerful diagnostic marker organism for periodontal disease (107). Also, Colombo et al., 2012 has shown a significant and persistent presence of this organism in refractory periodontitis patients and mild reductions in numbers in stable periodontal patients. Further, while the levels of *F. alocis* were affected by smoking status, the overall prevalence of the organism was similar between smoker and non-smoker CP patients (29). In agreement with these authors, we propose that *F. alocis* could be included as a novel member in Socransky’s Red complex of periodontal pathogens. Further studies, perhaps repeating Socransky’s classic 1998 study and including DNA probes against *F. alocis* would be necessary for the addition of this species to the Red complex to occur. Since chronic periodontal destruction was predominantly attributed to proliferation of Gram-negative pathogens, inclusion of *F. alocis* in the Red cluster also changes this classic perspective. Association of *F. alocis* with CP also demands closer attention to study its strain variability, disease etiology and virulence mechanisms, etc.

In addition to our focus on *F. alocis* as a periodontal pathogen, a discussion of *R. dentocariosa* as an indicator organism of health is warranted. It is reasonable to expect that use of indicator organisms to monitor treatment success against CP does not necessarily
have to take into account the relative reduction in the abundance of pathogenic species. Such strategies should consider taking into account the ratios between disease-associated and health-associated organisms such as *R. dentocariosa*, to at least partly evaluate therapeutic successes. Although our study, as well as reports emerging from periodontal microbiome studies conducted by others have correlated this organism with good periodontal health (25,26,40,44), its inclusion as an indicator of health may prove to be controversial. While evidence is not clearly established, there are some groups who link this bacterium to inflammatory heart diseases (19,75) while others have documented increased prevalence in periodontal "stable" patients post-treatment (25). Although considered benign to a large extent, this organism was first isolated from carious dentine and there is accumulating evidence associating *R. dentocariosa* with other systemic illnesses, including bacterial endocarditis (14,18,106). *R. dentocariosa* is a Gram positive rod-shaped, aerobic bacterium isolated almost exclusively from oral and respiratory sites (19). Further, the introduction of a reducing environment has been shown to retard its growth in the laboratory (19) likely limiting the possibility of its persistence in oxygen-depleted periodontal pockets. Repeated detection of this bacterium, as a periodontal-health associated organism using recent high-throughput sequencing techniques suggests that it may be useful to examine strain variability of this species in health and in periodontal disease.

A notable difference between our study and other comparable reports was that, we did not obtain significant alpha-diversity changes in community structure between health and periodontal destruction. In addition to using the number of distinct OTUs, alpha diversity also takes into account the evenness in their distribution. Our finding that
statistically justified changes are not apparent between periodontal health and disease communities differs from Griffen et al., and Abusleme et al., who showed that substantially higher OTU-level diversity and taxonomic richness was observed under periodontal destruction (Griffen et al., 2012 and Abusleme et al., 2013). However, in agreement with other groups, we found Synergistes, T. forsythia and F. alocis as strong indicators of disease.

**Limitations**

There is potential for several limitations in the design, DNA extraction, sequencing and data analysis to affect the outcome of this work. These include, the small sample size, cross-sectional nature of the work, as well as the inclusion of smokers and alcohol consumers. Nevertheless, our study provides good ground-work for further studies with bigger sample sizes and of a longitudinal nature to ascertain the shift in bacterial ecology that occurs from health to disease as well as verify F. alocis as a good indicator of periodontal disease. Also, studies of a larger scale would allow for assessment of the validity of tongue samples in disease identification. Longitudinal studies could potentially verify the use of tongue samples in identifying treatment outcomes. Further, the method of DNA extraction from plaque samples can be one of the most significant steps that can alter the outcome of this work. Using a mock community, and by extracting DNA using three different methods by different operators, we only obtained consistent and reliable results with the GenScript BacReady DNA extraction kit (see Supplementary). Hence, we believe that this DNA extraction method offered the most comprehensive and accurate representation of community diversity in plaque samples from our patient cohorts.

High-throughput sequencing methods and application of diversity estimation
methods in computational biology are relatively new to the field of microbiome analysis. Here we used a 150bp segment of the 16S rRNA gene, which did not allow for phylotype resolution down to the species level. However, it is noteworthy that the use of this relatively short fragment is advantageous in significantly reducing the number of Chimeras and Singletons prior to data analysis. Also, the Illumina Solexa system used in this study allows for paired-end reads which reduces the formation of aforementioned Chimeras and generation of poor quality sequences (100).

Analysis of biofilm community structures is quite complex and involves super-computers and software packages such as QIIME. In this work, several databases were used to compare sequences, bin them into OTUs and then assign a taxonomic designation. Binning of OTUs at 97% similarity is commonly used in these studies but higher cut-offs of similarity can also be used. Data analysis can be done either by binning sequences into OTUs or by comparing sequences directly. Slightly different results can be obtained by either method as detailed by Morgan and others (92). Here, we used two different databases and an OTU based analysis of our sequences.

Clinical implications of this study:

Our evidence that tongue dorsum plaque may be used for disease indicator organisms has important clinical implications in epidemiologic studies as well as clinically evaluating or monitoring the success of treatment outcomes against CP. Also, the presence of key disease indicators in the tongue samples indicated that sampling the tongue may be useful in future chair-side diagnostic strategies for periodontal disease. However, further studies are necessary to validate the relevance of the tongue plaque in monitoring this
disease by examining the relative abundance of disease indicative bacterial biomarkers. The tongue provides an easily accessible, less invasive body site for plaque collection that can often be performed by persons without technical expertise on plaque extraction.

**Future Studies:**

In order to validate the use of tongue samples as possible sites for disease identification, large-scale cross-sectional or longitudinal studies are necessary. Also, further studies assessing the association of *F. alocis* with other periodontal pathogens are necessary if this species is to be included as a Red Complex pathogen. Studies looking at the interaction between *F. alocis* and other pathogens as well as inherent virulence features of this species are also necessary. Lastly, assessment of health-associated species is also relevant so as to have possible bacterial markers of transition to the healthy state.

**Conclusions:**

Here, we present a comprehensive snapshot of the microbial community structures associated with CP and periodontal health. We show that the diseased state is correlated with a unique shift in the microbial ecology present under health, which is reflected by the high abundance and distribution of selected taxa in these patients. The tongue as well as SupG and SubG sites harbored several common indicator species of disease that included *T. forsythia, F. alocis* and Synergistes OT 362 and 363. In our patient cohorts, the presence of these species in all three sites was indicative of periodontal disease. It is noteworthy that the tongue harbored the highest number of disease indicator species, which suggests the use of tongue plaque for the detection and enumeration of disease-associated bacterial biomarkers. So far, the clinical community has overlooked the use of tongue plaque to
assess treatment outcomes, develop novel therapeutics and determine the efficacy of treatments against periodontal disease. This issue is significant, since extracting tongue plaque is convenient and minimally-invasive. Such less invasive, personalized treatment methods is a hallmark of modern medicine and a direction that can be accomplished with the current advancements in science and technology.
References or Bibliography


