UNIVERSITY OF CALIFORNIA, SAN DIEGO

SAN DIEGO STATE UNIVERSITY

Oral Polymicrobial Communities and Impact on Human Health

A dissertation submitted in partial satisfaction of the

requirements for the degree Doctor of Philosophy

in

Biology

by

Karen Schwarzberg

Committee in charge:

University of California, San Diego

Professor Douglas Bartlett
Professor Joseph Pogliano

San Diego State University

Professor Scott T. Kelley, Chair
Professor Kelly Doran
Professor David Lipson

2013

The Dissertation of Karen Schwarzberg is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

_____

_____

_____

_____

_____

Chair

University of California, San Diego

San Diego State University

2013

DEDICATION

I dedicate this dissertation to my family who has supported me throughout this process: my sister Yael Rosen, my brother-in-law David Rosen, my two nephews Benjamin Rosen and Jacob Rosen, and my mother, Bat-Sheva Schwarzberg. My family may be small, but they more than make up for it in personality and love.

In addition, I dedicate this dissertation to Elan Lipson for his kindness, support, and love.

EPIGRAPH

A little light dispels much darkness.

Rabbi Israel Ben-Eliezer

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

AIAN	American Indian/Alaska Native
bp	base pairs
BOP	Bleeding on probing
CAL	Clinical attachment loss
ECF	Energy coupling factor
LPS	Lipopolysaccharide
NGS	Next-generation sequencing
OTU	Operational taxonomic unit
PCoA	Principal coordinates analysis
PCR	Polymerase chain reaction
PD	Pocket depth
QIIME	Quantitative Insights Into Microbial Ecology
rRNA	Ribosomal RNA
SM	Sodium magnesium

LIST OF FIGURES

ACKNOWLEDGEMENTS

I would like to express my gratitude to my mentor, Dr. Scott Kelley, who supported me and pushed me to think critically and seek meaning behind data. He is an incredible teacher who has helped me develop as a scientist and taught me how to effectively communicate scientific findings. He also taught me more about soccer and football than I thought I would ever know.

I would like to thank the rest of my committee members, Dr. Kelly Doran, Dr. David Lipson, Dr. Doug Bartlett and Dr. Joseph Pogliano for their insight and guidance throughout this process.

I would like to thank Dr. J. Gregory Caporaso from Northern Arizona University for lending us his expertise in assisting with our data analysis, and for his kindness and patience.

I would like to thank Drs. Mohamed Saber, Faisal Alonaizan and Jørgen Slots from the Herman Ostrow School of Dentistry at USC. Working with them was always a pleasure.

I would like to thank the members of the Kelley Laboratory, past and present, especially Dr. Beltran Rodriguez-Mueller, Kate Wall, Jenny Beth Cornell, Pedro Torres and Jennifer Fouquier. I would especially like to thank Rosalin Le for all of the hard work she put into the periodontal disease study. Without her diligence it could not have succeeded.

Chapter 1, in full, is a reprint of the material as it appears in the Journal of Endodontics 2012. Saber, Mohamed H; Schwarzberg, Karen; Alonaizan, Faisal; Kelley, Scott T; Sedghizadeh, Parish P; Furlan, Mike; Levy, Thomas A; Simon,

James H; Slots, Jørgen. Bacterial Flora of Dental Periradicular Lesions Analyzed by the 454-Pyrosequencing Technology. The dissertation author was the primary investigator and author of this paper.

Chapter 2, in full, has been submitted for publication of the material as it may appear in PLOS One, 2014. Schwarzberg, Karen; Le, Rosalin; Bharti, Balambal; Lindsay, Suzanne; Casaburi, Giorgio; Salvatore, Francesco; Saber, Mohamed H; Alonaizan, Faisal; Slots, Jørgen; Gottlieb, Roberta A; Caporaso, J. Gregory; Kelley, Scott T. The Personal Human Oral Microbiome Obscures the Effects of Treatment on Periodontal Disease. The dissertation author was the primary investigator and author of this paper.

VITA

Education:

- Hebrew University of Jerusalem (Jerusalem, Israel)
  B.Sc. in Biochemistry and Food Science, specializing in Biotechnology, June 2005

- University of California, Davis (Davis, California)
  M.Sc. in Food Science and Technology, June 2008

- University of California, San Diego/San Diego State University (San Diego, California)
  Ph.D. in Biology, December 2013

Association Memberships:

- Southern California Chapter of the American Society for Microbiology, since 2011

- American Society for Microbiology, since 2012

- Association for Women in Science, since 2013

Publications:

- Mohamed H. Saber*, Karen Schwarzberg*, Faisal A. Alonaizan, Scott T. Kelley, Parish P. Sedghizadeh, Mike Furlan, Thomas A. Levy, James H. Simon, Jørgen Slots. Bacterial Flora of Dental Periradicular Lesions Analyzed by the 454-pyrosequencing Technology,
  Journal of Endodontics - November 2012 (Vol. 38, Issue 11, Pages 1484-1488)

  * contributed equally to this work

- Karen Schwarzberg, Rosalin Le, Balambal Bharti, Suzanne Lindsay, Girogio Casaburi, Francesco Salvatore, Mohamed H. Saber, Faisal Alonaizan, Jørgen Slots, Roberta A. Gottlieb, J. Gregory Caporaso, Scott T. Kelley. The Personal Human Oral Microbiome Obscures the Effects of Treatment on Periodontal Disease. (Submitted for publication)

ABSTRACT OF THE DISSERTATION

Oral Polymicrobial Communities and Impact on Human Health

by

Karen Schwarzberg

Doctor of Philosophy in Biology

University of California, San Diego, 2013

San Diego State University, 2013

Professor Scott T. Kelley, Chair

The oral cavity is richly populated with polymicrobial communities. Bacteria associated with the oral cavity are associated with a number of diseases, such as periodontal disease, dental caries and endodontic infections. Moreover, bacteria from this environment have also been implicated in diseases elsewhere in the body, including cardiovascular disease, cancer and rheumatoid arthritis. The tipping point in the balance between health and disease in human microbiomics is still unclear.

Using next-generation sequencing, we investigated the bacterial content in endodontic lesions obtained by means of apical surgery. Our findings indicate that these lesions are populated with a complex mixture of bacteria that are dominated by periodontal pathogens, most notably *Fusobacterium*, *Prevotella* and *Porphyromonas*. We also investigated the bacterial environment in periodontal pockets, monitoring the changes that occur from standard treatment. This was done using the bacterial 16S ribosomal RNA marker gene as well as whole-genome shotgun sequencing for metagenomics analyses on a subset of these samples.

Our 16S study found changes in the abundance of some taxa in individuals whose condition improved with treatment. However, samples before and after treatment from the same individuals were most consistently similar to each other. Phylogenetic analyses of the periodontal pathogens *Prevotella* and *Fusobacterium* from these samples uncovered unexpected diversity and varied response to treatment amongst different species. Based on these findings, we hypothesize that differences in interpersonal microbiomes have an effect on the response to treatment.

Metagenomics analyses enabled us to better characterize the overall environment of a subset of the samples. Our taxonomic identification results are comparable with those from the 16S study with 19 identical genera in the top 25 genera identified in this subset. Two genera identified by metagenomics were not present in the 16S data at all: *Haemophilus* and *Eikenella*. In functional gene categories, we saw an increase in virulence factors after treatment. We

hypothesize that a longer period of time needs to pass after treatment before drawing more conclusions, allowing the bacteria time to reach a new steady state after perturbation.

INTRODUCTION OF THE DISSERTATION

Bacteria and in the Oral Cavity and Human Diseases

The mouth is a unique environment in the body. It is comprised of soft tissue and hard tissue that can contain both aerobic and anaerobic environments for microbial growth in different sites such as the tongue, periodontal pockets, dental caries, mucous membranes, and endodontic lesions. The mouth is host to bacteria (Paster, Olsen et al. 2006), archaea (Lepp, Brinig et al. 2004), viruses (Sabeti and Slots 2004; Slots 2010) and fungi (Naglik, Tang et al. 2013). There are over 700 bacterial species that have been identified in the human oral cavity, over 400 of these in periodontal pockets and the remaining 300 from other sites (Paster, Olsen et al. 2006).

Oral bacteria have been implicated in systemic diseases as well. Oral bacteria have been found to be associated with cardiovascular disease (Gaetti-Jardim, Marcelino et al. 2009; Koren, Spor et al. 2011; das Merces Batista, Rosetti et al. 2012; Lockhart, Bolger et al. 2012; Yakob, Meurman et al. 2012), ulcerative colitis (Kojima, Nakano et al. 2012), oral and gastrointestinal cancer (Ahn, Chen et al. 2012), pancreatic diseases and cancer (Farrell, Zhang et al. 2012), breast cancer (Soder, Yakob et al. 2011), as well as rheumatoid arthritis (Detert, Pischon et al. 2010). The link between these diseases and oral bacteria is still uncertain. However, it is known that periodontal disease causes inflammation in the body (Darveau 2010). Inflammation plays a critical role in the aforementioned diseases, such as in cancer (Coussens and Werb 2002) and in cardiovascular disease (Yakob, Meurman et al. 2012). In some cases there is

evidence of inflammation in the body before there are cancerous changes in cells (Mantovani, Allavena et al. 2008). It has been stipulated that oral bacteria up-regulate inflammatory compounds that may be involved in carcinogenesis (Meurman 2010).

Bacteria and Endodontic Infections

Endodontic infections (also known as periradicular or apical root tip lesions) are oral infections in humans (Eriksen 1991) that originate in infected pulp tissue in the root canal of the tooth and progress into an infected lesion in the upper or lower jawbone (Kakehashi, Stanley et al. 1965; Seltzer and Farber 1994). The inflammation that is caused by the host defense response to the bacteria in the apical tip of the root canal causes bone resorption, destruction of apical root tissues, and formation of the periapical lesion. The lesion itself is a granulation tissue that contains macrophages, neutrophils, lymphocytes, and plasma cells (Nair 2004).

Until recently, it was not a commonly held belief that endodontic infections are caused by microorganisms, rather that the lesions are sparsely populated by microorganisms or that they contain none at all (Andreasen and Rud 1972; Langeland, Conn et al. 1977). A study by Andreasen and Rud in 1972 examined 66 lesions surgically removed from patients in an attempt to better understand where bacteria were located in the lesions and how they were connected to inflammation. They detected bacteria using histobacteriologic examination. They did not locate bacteria in most samples and concluded that the amount and

location of bacteria had little if any effect on the amount of inflammation in the host (Andreasen and Rud 1972). A study by Langeland *et al.* in 1977 examined lesions from 35 patients using similar methods. They found bacteria in only five of their samples (Langeland, Conn et al. 1977).

With advances in technology, recent studies found bacteria as well as viruses in endodontic lesions (Slots 2010), changing the traditional perspective that bacteria were not an important factor in endodontic infections. DNA sequencing techniques advanced the current understanding of bacterial diversity in other parts of the oral cavity, such as in root canals. In 2010, Li *et al*. saw a 600-fold increase in the depth of sequencing coverage attainable by pyrosequencing when compared to traditional Sanger capillary sequencing (Li, Hsiao et al. 2010). A pyrosequencing study by Siqueira *et al*. in 2011 found a high degree of bacterial diversity in root tips from necrotic teeth (Siqueira Jr, Alves et al. 2011). Another study by Özok *et al*. in 2012 examined infected root canals and identified 606 taxa. They found that the bacterial component of endodontic infections is more complex than originally understood, with the results showing that there were different bacteria in different niches within the root canal (Ozok, Persoon et al. 2012).

While these advances were made with pyrosequencing, there was a lack of information regarding the bacterial diversity of endodontic lesions that could be uncovered using next-generation sequencing. Such studies would confirm the presence of bacteria in samples and refute the original approach that suggested that bacteria had a minimal impact on these infections while advancing the

understanding of which bacteria are capable of causing these inflammations and lesions in that most interesting of locations: human bone.

Bacteria in Periodontal Disease

Periodontal disease is a polymicrobial inflammation of the gums in the mouth (Pihlstrom, Michalowicz et al. 2005). The term “periodontal disease” usually refers to gingivitis and periodontitis, which are both caused by bacteria in the plaque that accumulates on teeth. Gingivitis is milder than periodontitis and is reversible with proper oral hygiene. Periodontitis creates pockets that occur between the gums and the root of the tooth, and loss of connective tissue and bone support of teeth, which can lead to tooth loss. Oral microorganisms are the main cause of periodontal diseases, while other factors exacerbate the disease, such as genetics, tobacco and alcohol use, osteoporosis, diabetes and more (Pihlstrom, Michalowicz et al. 2005).

Over 700 species of bacteria have been identified in the human mouth, most of which colonize specific sites within the oral cavity (Paster, Olsen et al. 2006). Most of the bacteria in the mouth accumulate in surface-attached biofilms. According to the “successional integration” theory on biofilm formation in the mouth, the first organisms to attach, the so-called “early colonizers”, include streptococci and actinomyces. Other species that attach to the early colonizers are called secondary colonizers, which can be a variety of species. *Fusobacterium nucleatum* acts as a bridging organism to bacteria that would not normally integrate into the biofilm. These bacteria are pathogens, which can

interact with *F. nucleatum* and invade the biofilm. Examples of such pathogens are *Porphyromonas gingivalis* and *Treponema denticola,* which are well-known periodontal pathogens. The pathogens are considered late colonizers, and their integration into the biofilm decreases the species diversity (Kolenbrander, Palmer et al. 2006).

Many studies have investigated the bacterial diversity in the periodontal pocket in health and in disease. In 2000, Ximenez-Fyvie *et al*. compared the supra- and subgingival plaque in adults with periodontitis in two separate studies using whole genomic probes and checkerboard DNA-DNA hybridization. They found that subgingival samples harbored higher proportions of bacteria from what is known as the "red" species, which are bacteria highly associated with periodontal disease (*Porphyromonas gingivalis, Treponema denticola* and *Tannerella forsythia*) and that healthy patients have different proportions of these bacteria (Ximenez-Fyvie, Haffajee et al. 2000; Ximenez-Fyvie, Haffajee et al. 2000). Kumar *et al*. conducted a study in 2005 using 16S rRNA cloning and sequencing to investigate bacterial diversity in periodontitis and health. They identified periodontal pathogens such as *Campylobacter, Tannerella,* and *Treponema* in periodontal patients and found higher levels of *Streptococcus* and *Veillonella* in healthy controls (Kumar, Griffen et al. 2005).

More recent studies have used next-generation sequencing of 16S rRNA genes and whole-genome shotgun metagenomic sequencing to examine the microbial community composition in the periodontium. One such study combined both techniques to compare periodontal disease and health. Liu *et al*. used 454-

pyrosequencing to identify the microbiota in samples from 2 periodontal patients and 3 healthy controls as well as whole metagenome shotgun sequencing on the Illumina platform. This study was groundbreaking in identifying metabolic differences in the oral microbiome in health and disease as well as the taxonomic composition of these two states. The pyrosequencing results showed that the most dominant genera in the diseased samples were *Prevotella* and *Fusobacterium*, and in health the most dominant genera were *Streptococcus, Peptostreptococcus, Leptotrichia, Actinomyces, Neisseria and Kingella*. One healthy control sample contained *Fusobacterium* as a dominant genus, but the patient had early signs of gingivitis. The metagenome results showed that the most dominant genus in the diseased samples was *Prevotella*, and in health the most dominant genus was *Streptococcus* in one sample and *Actinomyces* in the other (Liu, Faller et al. 2012).

Another study that rigorously investigated the taxonomic differences found in periodontal health and disease was the study by Griffen *et al*. in 2012. Using 454-pyrosequencing, they compared the oral microbial taxa from 29 periodontal patients and 29 healthy controls. Similar to other studies, they found higher microbial diversity in diseased samples, where the most abundant phyla were Spirochaetes, Synergistetes, and Bacteroidetes. In healthy control samples, Proteobacteria were found to be more abundant (Griffen, Beall et al. 2012).

A similar study in 2013 by Abusleme *et al*. examined the microbial diversity of 22 periodontal patient samples and 10 healthy controls using 454-pyrosequencing of the 16S rRNA gene. Their findings indicate that there were

higher levels of Spirochaetes, Synergistetes, Firmicutes and Chloroflexi in diseased samples, whereas the healthy controls samples had higher levels of Actinobacteria (Abusleme, Dupuy et al. 2013).

Wang *et al*., who examined 2 patients with periodontal disease and 3 healthy controls, also combined 454-pyrosequencing and whole-genome shotgun sequencing in their study. On the phylum level, they found differences between health and disease: Bacteroidetes was more abundant in the periodontal disease samples and Actinobacteria and Proteobacteria were most abundant in the healthy control samples. At the genus level, they did not find that any groups were consistently more abundant in the healthy controls, but in the diseased samples they found that *Prevotella* was consistently higher. Metabolic pathways overrepresented in disease involved chemotaxis, flagellar assembly and toxin biosynthesis (Wang, Qi et al. 2013).

The technological advances in both sequencing and the bioinformatics tools that can accommodate the mass of data the sequencing generates have increased our understanding of periodontal disease pathogens and the changes that occur in the microbial community as it shifts from health to disease. The studies that have recently been published shed light on the community composition and the metabolic functions that it contains. However, these studies have been based on a small number of samples. Repeating these studies to verify if the findings are consistent in additional samples is important. Moreover, none of these studies have examined the effects of treating periodontal disease on the bacterial community composition.

REFERENCES

Abusleme, L., A. K. Dupuy, et al. (2013). "The subgingival microbiome in health and periodontitis and its relationship with community biomass and inflammation." ISME J. 2013 Jan 10. doi: 10.1038/ismej.2012.174.(1751-7370 (Electronic)): T - aheadofprint.

Ahn, J., C. Y. Chen, et al. (2012). "Oral microbiome and oral and gastrointestinal cancer risk." Cancer Causes & Control **23**(3): 399-404.

Andreasen, J. and J. Rud (1972). "A histobacteriologic study of dental and periapical structures after endodontic surgery." International journal of oral surgery **1**(5): 272-281.

Coussens, L. M. and Z. Werb (2002). "Inflammation and cancer." Nature **420**(6917): 860-867.

Darveau, R. P. (2010). "Periodontitis: a polymicrobial disruption of host homeostasis." Nature Reviews Microbiology **8**(7): 481-490.

das Merces Batista, R., E. P. Rosetti, et al. (2012). "Association between periodontal disease and subclinical atherosclerosis: the ELSA-Brasil study " Cad. Saude Publica **28**(5): 965-976.

Detert, J., N. Pischon, et al. (2010). "The association between rheumatoid arthritis and periodontal disease." Arthritis Research and Therapy **12**(5): 218.

Eriksen, H. M. (1991). "Endodontology: epidemiologic considerations." Dental Traumatology **7**(5): 189-195.

Farrell, J. J., L. Zhang, et al. (2012). "Variations of oral microbiota are associated with pancreatic diseases including pancreatic cancer." Gut **61**(4): 582-588.

Gaetti-Jardim, E., S. L. Marcelino, et al. (2009). "Quantitative detection of periodontopathic bacteria in atherosclerotic plaques from coronary arteries." Journal of medical microbiology **58**(12): 1568-1575.

Griffen, A. L., C. J. Beall, et al. (2012). "Distinct and complex bacterial profiles in human periodontitis and health revealed by 16S pyrosequencing." Isme Journal **6**(6): 1176-1185.

Kakehashi, S., H. Stanley, et al. (1965). "The effects of surgical exposures of dental pulps in germ-free and conventional laboratory rats." Oral Surgery, Oral Medicine, Oral Pathology **20**(3): 340-349.

Kojima, A., K. Nakano, et al. (2012). "Infection of specific strains of Streptococcus mutans, oral bacteria, confers a risk of ulcerative colitis." Scientific reports **2**.

Kolenbrander, P. E., R. J. Palmer, Jr., et al. (2006). "Bacterial interactions and successions during plaque development." Periodontology 2000 **42**: 47-79.

Koren, O., A. Spor, et al. (2011). "Human oral, gut, and plaque microbiota in patients with atherosclerosis." Proceedings of the National Academy of Sciences **108**(Supplement 1): 4592-4598.

Kumar, P. S., A. L. Griffen, et al. (2005). "Identification of candidate periodontal pathogens and beneficial species by quantitative 16S clonal analysis." Journal of clinical microbiology **43**(8): 3944-3955.

Langeland, K., F. Conn, et al. (1977). "A histopathologic and histobacteriologic study of 35 periapical endodontic surgical specimens." Journal of endodontics **3**(1): 8-23.

Lepp, P. W., M. M. Brinig, et al. (2004). "Methanogenic Archaea and human periodontal disease." Proceedings of the National Academy of Sciences of the United States of America **101**(16): 6176-6181.

Li, L., W. Hsiao, et al. (2010). "Analyzing endodontic infections by deep coverage pyrosequencing." Journal of dental research **89**(9): 980-984.

Liu, B., L. L. Faller, et al. (2012). "Deep sequencing of the oral microbiome reveals signatures of periodontal disease." Plos One **7**(6).

Lockhart, P. B., A. F. Bolger, et al. (2012). "Periodontal disease and atherosclerotic vascular disease: does the evidence support an independent association? A scientific statement from the American Heart Association." Circulation **125**(20): 2520-2544.

Mantovani, A., P. Allavena, et al. (2008). "Cancer-related inflammation." Nature **454**(7203): 436-444.

Meurman, J. H. (2010). "Oral microbiota and cancer." Journal of oral microbiology **2**.

Naglik, J. R., S. X. Tang, et al. (2013). "Oral Colonization of Fungi." Current Fungal Infection Reports: 1-8.

Nair, P. (2004). "Pathogenesis of apical periodontitis and the causes of endodontic failures." Critical Reviews in Oral Biology & Medicine **15**(6): 348-381.

Ozok, A., I. Persoon, et al. (2012). "Ecology of the microbiome of the infected root canal system: a comparison between apical and coronal root segments." International endodontic journal **45**(6): 530-541.

Paster, B. J., I. Olsen, et al. (2006). "The breadth of bacterial diversity in the human periodontal pocket and other oral sites." Periodontology 2000 **42**: 80-87.

Pihlstrom, B. L., B. S. Michalowicz, et al. (2005). "Periodontal diseases." Lancet **366**(9499): 1809-1820.

Sabeti, M. and J. Slots (2004). "Herpesviral-bacterial coinfection in periapical pathosis." Journal of endodontics **30**(2): 69-72.

Seltzer, S. and P. A. Farber (1994). "Microbiologic factors in endodontology." Oral Surgery, Oral Medicine, Oral Pathology **78**(5): 634-645.

Siqueira Jr, J. F., F. R. Alves, et al. (2011). "Pyrosequencing analysis of the apical root canal microbiota." Journal of endodontics **37**(11): 1499-1503.

Slots, J. (2010). "Herpesviral-bacterial interactions in periodontal diseases." Periodontology 2000 **52**(1): 117-140.

Soder, B., M. Yakob, et al. (2011). "Periodontal disease may associate with breast cancer." Breast cancer research and treatment **127**(2): 497-502.

Wang, J., J. Qi, et al. (2013). "Metagenomic sequencing reveals microbiota and its functional potential associated with periodontal disease." Scientific reports **3**.

Ximenez-Fyvie, L. A., A. D. Haffajee, et al. (2000). "Comparison of the microbiota of supra- and subgingival plaque in health and periodontitis." Journal of Clinical Periodontology **27**(9): 648-657.

Ximenez-Fyvie, L. A., A. D. Haffajee, et al. (2000). "Microbial composition of supra- and subgingival plaque in subjects with adult periodontitis." Journal of Clinical Periodontology **27**(10): 722-732.

Yakob, M., J. H. Meurman, et al. (2012). "C-reactive protein in relation to early atherosclerosis and periodontitis." Clinical oral investigations **16**(1): 259-265.

CHAPTER I:

Bacterial Flora of Dental Periradicular Lesions Analyzed by the 454-Pyrosequencing Technology

INTRODUCTION

Periradicular inflammatory disease of endodontic origin is a prevalent human infection (Eriksen 1991), which is initiated by bacteria from an infected pulp (Kakehashi, Stanley et al. 1965; Seltzer and Farber 1994). The endodontic bacteria and their products induce innate and adaptive host responses which result in the formation of granulation tissue containing macrophages, neutrophils, lymphocytes and plasma cells (Nair 2004).The traditional viewpoint is that the periradicular lesion itself is sparsely populated by low-grade pathogenic bacteria, or is entirely devoid of microorganisms (Andreasen and Rud 1972; Langeland, Conn et al. 1977). However, recent studies have found bacteria and herpesviruses in a high proportion of symptomatic and large-sized periradicular lesions (Sabeti and Slots 2004).

Metagenomics is the functional and sequence-based analysis of the collective microbial genomes present in an ecosystem (Riesenfeld, Schloss et al. 2004). The current standard for microbial identification involves polymerase chain reaction (PCR) for DNA amplification of targeted 16S ribosomal RNA (rRNA) genes and Sanger (dideoxy) sequencing (Weisburg, Barns et al. 1991; Siqueira Jr and Rocas 2005; Pozhitkov, Beikler et al. 2011). PCR-based microbial identification has revealed several previously undetected taxa in endodontic disease (Siqueira Jr and Rocas 2005; Siqueira Jr and Rocas 2006; Handal, Caugant et al. 2009). However, labor-intensiveness and high cost of molecular cloning of community 16S rRNA PCR products are limiting factors for in-depth

microbial identification (Petrosino, Highlander et al. 2009; Pozhitkov, Beikler et al. 2011).

New molecular technologies have the potential to further elucidate the microbial diversity of environmental and human ecosystems (Pozhitkov, Beikler et al. 2011). The release, in 2005, of the first high-throughput parallel DNA sequencer based on the pyrosequencing concept gave rise to what is now referred to as Next-Generation sequencing. The 454-pyrosequencing technology [Genome Sequencer (GS) FLX sequence system from Roche/454 Life Sciences (Indianapolis, IN)] can sequence 400-600 million base pairs per run with 400-600 base pair read lengths (Pozhitkov, Beikler et al. 2011).

High-throughput DNA sequencing methods have provided a deeper understanding of the oral microbiota (Keijser, Zaura et al. 2008; Siqueira Jr, Fouad et al. 2012). Keijser *et al*. used the 454 pyrosequencing technology to sequence 16S rRNA amplicons derived from saliva and subgingival plaque, and found a bacterial diversity far greater than previously recognized (Keijser, Zaura et al. 2008). Zaura *et al*., using the pyrosequencing technology, found identical sequences in a major portion of the oral microflora of healthy unrelated individuals (Zaura, Keijser et al. 2009). Lazarevic *et al*. used pyrosequencing to determine inter- and intra-individual variations in the salivary microbiota (Lazarevic, Whiteson et al. 2010), and other studies have also employed pyrosequencing for characterization of the oral microbiome (Nasidze, Quinque et al. 2009; Ling, Kong et al. 2010). In endodontics, pyrosequencing has been used to elucidate the bacterial diversity in necrotic root canals. Li *et al*. demonstrated

that a 600-fold increase in the “depth of coverage” can be obtained with pyrosequencing compared to traditional Sanger capillary sequencing (Li, Hsiao et al. 2010). Siqueira *et al*. revealed a high degree of bacterial diversity in resected root tips of necrotic teeth using pyrosequencing (Siqueira Jr, Alves et al. 2011). Özok *et al*. identified 606 taxa (species or higher taxon) in infected root canals, representing 25 microbial phyla or divisions (Ozok, Persoon et al. 2012).

To the best of our knowledge, the presence and diversity of bacteria in persistent periradicular lesions following orthograde endodontic treatment have not been studied using Next-Generation sequencing. Therefore, to further elucidate the bacterial content of periradicular lesions, this study employed the pyrosequencing technology of bacterial identification to analyze periradicular samples obtained by means of apical surgery.

METHODS

Patient Selection

Thirteen patients, 7 males and 6 females, with periradicular pathosis were included in the study. Study participants were selected among patients scheduled for apical surgery at the Postgraduate Endodontic Clinic, Herman Ostrow School of Dentistry at the University of Southern California. The median age of the patients was 49 years (range 32-65 years). Eleven of the thirteen study teeth were symptomatic. The largest diameter of the radiographic lesions averaged 5.7±1.9 mm.

The study was approved by the IRB ethics committee at the University of Southern California (IRB # FWA 00007099). The aim of the study and the procedures were explained to each patient and informed consent was obtained. The inclusion criteria were: 1) English-speaking patients who could give informed consent; 2) Patients were at least 18 years old at the time of surgery; 3) Teeth had conventional root canal treatment performed at the University of Southern California but showed either persistent symptoms of tenderness to percussion or palpation, or revealed no radiographic evidence of healing for at least 6 months; and 4) Teeth had a satisfactory permanent coronal seal. The exclusion criteria were: 1) Active systemic disease or infection; 2) Antibiotic use in the past eight weeks prior to the endodontic surgery; 3) Clinical communication between the periradicular lesion and the oral cavity; 4) Teeth with suspected fracture or advanced periodontal disease.

Microbial Sampling

To avoid contamination by intra-radicular bacteria, periradicular lesions were sampled in conjunction with apical surgery, and not through the root canal. Surgical samples that fit the ascertainment criteria were obtained as follows. Scaling and root planing was performed one week prior to surgery and patients were instructed to rinse with 0.12% chlorhexidine twice daily. Patients were also asked to rinse with 0.12% chlorhexidine mouth rinse for 30 seconds immediately before surgery, and the surgical site was disinfected with 0.12% chlorhexidine prior to the administration of local anesthesia. A full-thickness mucoperiosteal flap with intrasulcular horizontal incision was reflected using a sterile #15 blade and periosteal elevators. Osteotomy was performed with a sterile #4 round bur in conjunction with sterile saline coolant. Sterile curettes and/or spoon excavators were used for specimen removal. Specimens were placed in sterile Eppendorf tubes containing 1 ml of sterile sodium-magnesium buffer (pH 7.4) and immediately placed in a freezer at -20°C. The sodium-magnesium buffer contained (per liter): 5.8 gr NaCl, 2 gr $MgSO_4$, 50 mL 1M Tris-Cl and 950 mL water.

DNA Extraction, PCR Amplification and 454-Pyrosequencing

DNA was extracted using the NucleoSpin Tissue Nucleic Acid and Protein Purification Kit from Macherey-Nagel GmbH & Co. (Düren, Germany). Extracted DNA was stored at -20 ^{o}C. PCR reactions utilized the 27F and 338R universal primers, which target the V1–V2 hypervariable regions of 16S rRNA genes (Integrated DNA Technologies, San Diego, CA). The 338R primer included a

unique 128-bp sequence tag to barcode each sample. Each pyrosequenced sample would then acquire a unique barcode, which allowed sequences to be traced back to specific clinical samples. The PCR reactions contained 5 µL of DNA, 25 µL Taq98 Hot Start 2X Master Mix (Lucigen Corporation, Middleton, WI), 0.5 µL of 27F primer, 0.5 µL of barcoded 338R primer (final primer concentration for both primers was 0.5 µM), and 19 µL sterile water (Sigma-Aldrich Corporation, St. Louis, MO). The DNA quantity varied between 20 and 50 ng per reaction. Thermocycling was carried out for 35 cycles using conditions recommended by the Lucigen Corporation for the Taq98 Hot Start 2X Master Mix. All PCR reactions included a negative control reaction in which sterile water was used instead of DNA to control for contamination. All PCR products were run on a 1% agarose gel with a digested size ladder to verify correct product size and absence of contamination. PCR products were submitted to the core sequencing facility at the University of Pennsylvania for pyrosequencing on the Roche 454 GS FLX instrument.

The bacterial identification, usually at the genus level or higher, was performed at the finest level of taxonomic resolution possible given the sequences and current state of databases. Sequencing data were analyzed using the Quantitative Insights into Microbial Ecology (QIIME) software (Caporaso, Kuczynski et al. 2010). The results were filtered for quality by removing sequences that were shorter than 200 bp, with a quality score lower than 25, without the primer, without the exact barcodes, or with ambiguous characters. The remaining sequences had the sample-identifying barcode

nucleotides incorporated into them. Representative Operational Taxonomic Units (OTUs) were selected and taxonomic identity determined. The data set was subsequently subjected to noise-reduction, chimera checking, length trimming and verification that sequences were found in at least 2 samples in order to be included. Contaminants of non-human bacteria were identified by the SourceTracker software program, which is designed to address the problem of laboratory contamination in high-throughput sequencing studies (Knights, Kuczynski et al. 2011).

RESULTS

PCR amplification yielded amplicons in 7 of 13 periradicular samples (53.8%), which all originated from symptomatic teeth. The presence of bacterial DNA in periapical samples was illustrated by direct staining with SYBR® Gold (performed as described by the manufacturer, Invitrogen, Grand Island, NY) (Figure 1.1). Sequencing of PCR amplicons resulted in 35,731 high-quality reads after discarding poor quality sequences and suspected contaminants (e.g., water- and soil-associated bacteria). The contaminants were present in low numbers (less than 0.1%) and tended to be common laboratory contaminants, such as the hardy spore-forming *Geobacillus* bacterium, which can appear in PCR-based studies including oral cavity studies.

The QIIME software mapped the sequenced amplicons from the 7 study lesions into 10 phyla and 73 genera, and the bacterial taxa in individual samples ranged from 23 to 54 (mean, 40). The phyla containing most bacterial taxa were: *Proteobacteria* (proportion of total bacterial taxa, 33.3%), *Firmicutes* (30.9%), *Actinobacteria* (12.2%), *Bacteroidetes* (11.4%) and *Fusobacteria* (4.1%). The most abundant genera were: *Fusobacterium* (average of total sequences, 21.0%), *Streptococcus* (8.0%), *Prevotella* (7.5%), *Corynebacterium* (7.2%), *Porphyromonas* (6.0%) and *Actinomyces* (5.8%). Bacteria averaging between 4.0% and 1.0% of the periradicular microbiota were (listed in declining order of occurrence): *Treponema*, *Neisseriaceae*, *Capnocytophaga*, *Parvimonas*, *Kingella*, *Tannerella*, *Clostridiales* and *Veillonella*. Bacteria that comprised less

than 1.0% included: *Ralstonia, Leptotrichia,* TM7, *Dialister, Rothia, Campylobacter, Eubacterium, Propionibacterium, Granulicatella, Selenomonas, Gemella, Staphylococcus, Anaeroglobus, Solobacterium, Rhodococcus, Chryseobacterium, Lactobacillales, Lactococcus, Burkholderiaceae*, *Cardiobacterium, Mycoplasma, Microbacterium, Olsenella, Xylanibacter, Abiotrophia, Fructobacillus, Peptostreptococcus, Centipeda, Escherichia/Shigella, Enterobacteriaceae, Aggregatibacter, Heamophilus, Pasteurellaceae, Pseudomonas*, *Spirochaetales*, plus a number of unclassifiable reads.

Figure 1.2 provides a list of the bacterial genera that were found in at least two different samples after eliminating putative contaminants, and demonstrates a marked sample-to-sample variation in the occurrence of individual genera. For example, Endo05 yielded 37.8% *Porphyromonas* and 23.8% *Fusobacterium*, Endo07 yielded 65.4% *Fusobacterium* and 0% *Porphyromonas*, and Endo02 yielded 25.1% *Actinomyces* and 23.6% *Streptococcus*. Endo01 revealed *Streptococcus* (13.5%), *Actinomyces* (13.3%) and a remarkably high level of *Corynebacterium* (33.5%).

DISCUSSION

The 325-year-old history of endodontic microbiology began with Antonie van Leeuwenhoek's microscopic description of bacteria in the dental pulp in 1683, was continued with the refinement of bacterial culture methodology by Åke Möller in the 1960s, and became metagenomic-based about 15 years ago. Studies using Sanger sequencing platforms or pyrosequencing of 16S rRNA amplicons have greatly expanded our knowledge of the human oral microbiome, but are not without challenges and limitations (Pozhitkov, Beikler et al. 2011). In Figure 1.2, we chose to report only bacterial genera that were detected in at least two different samples primarily because many of the genera occurred in small quantities and should be considered somewhat suspect with low microbial biomass samples. Sequencing errors due to insertion and deletion of nucleotides in highly variable 16S rRNA regions can cause misalignment to the master sequence in homopolymeric pyrosequencing runs and give rise to misdiagnosis. Also, some studies provide "species-level" descriptions based on 16S rRNA sequence analysis despite that bacterial species within many genera are not easily distinguished with this gene marker (i.e., very few nucleotide positions vary amongst species within a particular genus). Guided by a conservative approach to microbial identification, our study focused on genus-level taxon assignments.

Metagenomic identification of bacteria determines the mere presence of specific DNA and not viability (Siqueira Jr and Rocas 2005). As bacterial DNA can remain PCR-detectable for up to 1 year after cell death *in vitro*,

metagenomics may overestimate current bacterial load (Young, Turner et al. 2007). However, an assessment of both alive and dead micro-organisms in endodontic and other complex diseases can help to identify bacteria that may predominate the early phases of disease, but become underrepresented or even absent in later disease states. Furthermore, a single microbiological examination cannot determine if specific bacteria are permanent or transient residents, or primary pathogens or merely bystanders to disease. Endodontic yeasts and viruses do not contain 16S rRNA genes and were not diagnosed in this study.

The present study identified a complex mixture of periradicular bacteria. The 7 bacteria-positive samples yielded 10 phyla and 73 genera, with an average of 40 genera per sample. Metagenomic studies have described even larger arrays of intraradicular bacteria in teeth with periapical pathosis (Li, Hsiao et al. 2010; Siqueira Jr, Alves et al. 2011; Ozok, Persoon et al. 2012). Differences in microbial content among studies may be due to selection of study teeth, sampling methodology, geographic location, PCR and pyrosequencing techniques, and database used for bacterial identification (Baumgartner, Siqueira Jr et al. 2004; Siqueira and Rocas 2009). In teeth with periradicular lesion, Özok *et al*. identified 24 phyla, 317 genera and 606 species or higher taxon (Ozok, Persoon et al. 2012), Siqueira *et al*. identified 10 phyla, 84 genera and 187 species (Siqueira Jr, Alves et al. 2011), and Li *et al*. identified 13 phyla and 179 genera (Li, Hsiao et al. 2010). Ribeiro *et al*. detected between 3 and 21 bacterial taxa per canal with asymptomatic periapical lesions, and 65.7% of the cloned sequences represented previously uncultivated phylotypes (Ribeiro, Matarazzo

et al. 2011). Yang *et al*. studied acute periradicular abscesses in children aged 5-7 years and found between 1 and 8 bacterial genera per abscess, with the predominant genera being *Prevotella* (24%), *Fusobacterium* (18%), *Porphyromonas* (14%), *Lactobacillus* (11%), *Peptostreptococcus* (8%) and *Streptococcus* (6%) (Yang, Fan et al. 2010). The present study and that of Yang *et al*. (Yang, Fan et al. 2010) included exclusively symptomatic periradicular lesions and showed many microbial similarities. Apparently, despite a great bacterial diversity, symptomatic periradicular lesions tend to harbor a core microbial community consisting of anaerobic bacteria, especially *Fusobacterium*, *Prevotella* and *Porphyromonas*, and of facultative *Streptococcus* and *Actinomyces*.

Non-dental bacteria may play a role in individual periradicular lesions, such as *Corynebacterium,* which comprised one-third of the microbiota in one of the study samples. Özok *et al*. detected *Corynebacterium* in 22 of 23 teeth with endodontic pathosis (Ozok, Persoon et al. 2012). *Corynebacterium* and *Propionibacterium*, two essentially skin and non-oral mucosal bacteria, are rare isolates from dental caries and marginal periodontitis and may have entered necrotic dental pulps and periradicular sites via the systemic circulation (Cogen, Nizet et al. 2008). This may also have been the case for some of the bacterial taxa, which previously have not been described in the oral microbiome, but were identified in small amounts in this study. The colonization of endodontic bacteria in teeth with an intact crown and a normal periodontium, but with a necrotic pulp due to trauma, is most likely also the consequence of transient bacteremia

(Sundqvist 1976). Herpesviruses reside in inflammatory cells and positively gain access to periradicular sites via in the blood stream (Sabeti and Slots 2004). In view of the available information, it seems appropriate to reevaluate the concept of anachoresis as a mechanism of microbial colonization of damaged or necrotic pulps or periapical sites.

The present study may help to clarify the long-standing debate over a possible bacterial presence in periradicular lesions. To obtain bacteria exclusively from periradicular lesions, and as sampling through the root canal will invariably include pulpal bacteria, we opted for microbial sampling in conjunction with periapical surgery. We also instituted pre-surgical antiseptic treatments to minimize bacterial contamination from saliva. Our finding of a high occurrence of anaerobic bacteria, which are not prominent in saliva or oral mucosa, is consistent with an effective sampling methodology of the periradicular area.

We recovered bacterial DNA from 7 of 11 symptomatic lesions, but not from 4 symptomatic and 2 asymptomatic lesions (54% recovery rate). The 6 periradicular lesions that yielded no bacterial DNA may indeed have been bacteria-negative or, less likely, were the result of a failure to sample bacteria-positive areas within the periradicular lesions or technical problems with the PCR/pyrosequencing procedures. Our findings are consistent with those of Desai *et al*. (Desai, Love et al. 2011), who showed a high level of Toll-like receptor-2 expression in symptomatic refractory periradicular lesions, an indication of the presence of bacterial or herpesvirus antigens (36). Sunde *et al*. (Sunde, Olsen et al. 2002) detected bacteria in 35 of 36 periradicular lesions by using culture and

scanning electron microscopy, and in 20 of 39 surgically excised periradicular lesions by using fluorescence in situ hybridization in combination with epifluorescence and confocal laser scanning microscopy (Sunde, Olsen et al. 2003). In a 16S rRNA clone library study, Handal *et al*. (Handal, Caugant et al. 2009) detected bacterial DNA in 17 of 20 periradicular lesions (85%). Several studies thus establish presence of a great variety of bacteria in periradicular lesions.

The presence or absence of periradicular bacteria is determined by the virulence of the resident bacteria and the efficiency of local host defenses (Nair 2004). As demonstrated in monkeys, pulpal bacteria play a critical role in the development of periradicular lesions (Moller, Fabricius et al. 2004). After establishing the initial periradicular lesion, bacteria from the necrotic pulp may periodically egress into the lesion site, where their survivability depends on the effectiveness of local immune responses (Nair 2004). As hypothesized recently, an active periodontal herpesvirus infection capable of triggering a strong pro-inflammatory cytokine response and impeding neutrophil functions can suppress anti-bacterial host defenses and cause an overgrowth of pathogenic bacteria (Slots 2010). The majority of large symptomatic periradicular lesions harbor one or more herpesviruses, and herpesvirus-associated lesions tend to show elevated levels of suspected endodontopathic bacteria (Sabeti and Slots 2004). Because herpesviruses occur in an active and a latent state, it may be that periradicular lesions experiencing frequent viral reactivation are prone to bacterial colonization, whereas lesions free of herpesviruses or with prolonged

periods of viral latency can remain essentially bacteria-negative. Further research is necessary to determine the possible impact of herpesviruses on the colonization and persistence of periradicular bacteria.

The finding of a great bacterial diversity in some periradicular lesions may have clinical implications. This study showed that even well performed root canal procedures cannot ensure the absence of periradicular microorganisms. A study in monkeys revealed that bacteria in root canals with no communication to the oral cavity were able to survive for 3 years (Fabricius, Dahlen et al. 1982). Persistent bacteria may compromise healing (Nair 2004), and methods for their elimination need to be identified.

In conclusion, this study demonstrated that a great variety of extraradicular bacteria or their DNA can persist in symptomatic periapical lesions following orthograde root canal therapy. Whether a similar bacterial diversity exists in periradicular lesions of teeth with no prior endodontic treatment is unknown. Most bacteria belonged to the genera *Fusobacterium*, *Prevotella, Porphyromonas*, *Streptococcus* and *Actinomyces*. However, 46% of the study lesions revealed no detectable bacterial DNA. The determinants of bacterial colonization of periradicular lesions and the clinical impact of such colonization have still to be established. Whether the root canal and the periradicular area harbor different microbiotas also warrants further study. Increased insights into the microbiota of endodontic pathosis will enhance our understanding of endodontic diseases and hopefully improve endodontic treatment.

Chapter 1, in full, is a reprint of the material as it appears in the Journal of Endodontics 2012. Saber, Mohamed H; Schwarzberg, Karen; Alonaizan, Faisal; Kelley, Scott T; Sedghizadeh, Parish P; Furlan, Mike; Levy, Thomas A; Simon, James H; Slots, Jørgen. Bacterial Flora of Dental Periradicular Lesions Analyzed by the 454-Pyrosequencing Technology. The dissertation author was the primary investigator and author of this paper.

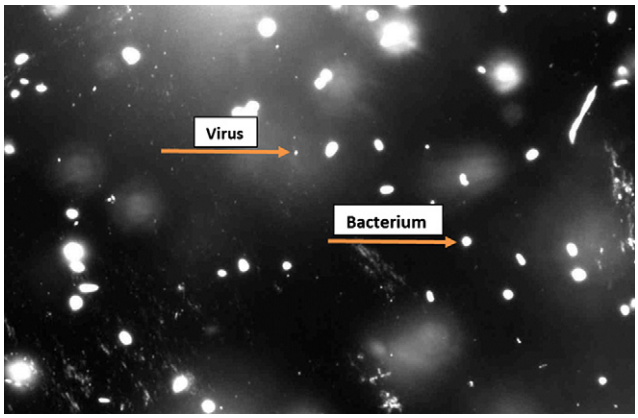

Figure 1.1: Periradicular sample (10 *µ*l) stained with SYBR Gold for detection of nucleic acids, 100X magnification

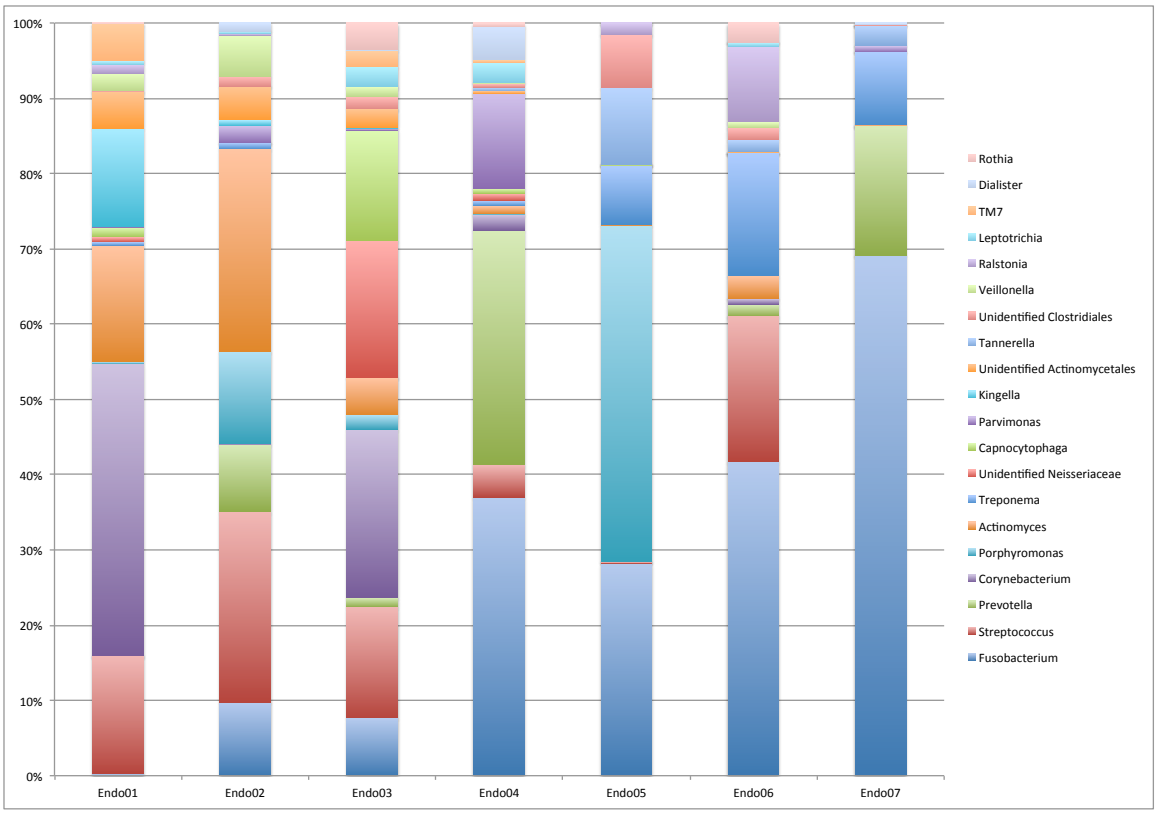

Figure 1.2: Distribution in descending order of frequency of the top 20 bacterial taxa in the 7 PCR-amplicon–positive periradicular lesions.

REFERENCES

Andreasen, J. and J. Rud (1972). "A histobacteriologic study of dental and periapical structures after endodontic surgery." International journal of oral surgery **1**(5): 272-281.

Baumgartner, J., J. Siqueira Jr, et al. (2004). "Geographical differences in bacteria detected in endodontic infections using polymerase chain reaction." Journal of endodontics **30**(3): 141-144.

Caporaso, J. G., J. Kuczynski, et al. (2010). "QIIME allows analysis of high-throughput community sequencing data." Nat Methods **7**(5): 335-336.

Cogen, A., V. Nizet, et al. (2008). "Skin microbiota: a source of disease or defence?" British Journal of Dermatology **158**(3): 442-455.

Desai, S., R. Love, et al. (2011). "Toll-like receptor 2 expression in refractory periapical lesions." International endodontic journal **44**(10): 907-916.

Eriksen, H. M. (1991). "Endodontology: epidemiologic considerations." Dental Traumatology **7**(5): 189-195.

Fabricius, L., G. Dahlen, et al. (1982). "Predominant indigenous oral bacteria isolated from infected root canals after varied times of closure." European journal of oral sciences **90**(2): 134-144.

Handal, T., D. A. Caugant, et al. (2009). "Bacterial diversity in persistent periapical lesions on root-filled teeth." Journal of oral microbiology **1**.

Kakehashi, S., H. Stanley, et al. (1965). "The effects of surgical exposures of dental pulps in germ-free and conventional laboratory rats." Oral Surgery, Oral Medicine, Oral Pathology **20**(3): 340-349.

Keijser, B., E. Zaura, et al. (2008). "Pyrosequencing analysis of the oral microflora of healthy adults." Journal of dental research **87**(11): 1016-1020.

Knights, D., J. Kuczynski, et al. (2011). "Bayesian community-wide culture-independent microbial source tracking." Nature methods **8**(9): 761-763.

Langeland, K., F. Conn, et al. (1977). "A histopathologic and histobacteriologic study of 35 periapical endodontic surgical specimens." Journal of endodontics **3**(1): 8-23.

Lazarevic, V., K. Whiteson, et al. (2010). "Study of inter-and intra-individual variations in the salivary microbiota." BMC genomics **11**(1): 523.

Li, L., W. Hsiao, et al. (2010). "Analyzing endodontic infections by deep coverage pyrosequencing." Journal of dental research **89**(9): 980-984.

Ling, Z., J. Kong, et al. (2010). "Analysis of oral microbiota in children with dental caries by PCR-DGGE and barcoded pyrosequencing." Microbial ecology **60**(3): 677-690.

Moller, A. J., L. Fabricius, et al. (2004). "Apical periodontitis development and bacterial response to endodontic treatment. Experimental root canal infections in monkeys with selected bacterial strains." European journal of oral sciences **112**(3): 207-215.

Nair, P. (2004). "Pathogenesis of apical periodontitis and the causes of endodontic failures." Critical Reviews in Oral Biology & Medicine **15**(6): 348-381.

Nasidze, I., D. Quinque, et al. (2009). "Comparative analysis of human saliva microbiome diversity by barcoded pyrosequencing and cloning approaches." Analytical biochemistry **391**(1): 64-68.

Ozok, A., I. Persoon, et al. (2012). "Ecology of the microbiome of the infected root canal system: a comparison between apical and coronal root segments." International endodontic journal **45**(6): 530-541.

Petrosino, J. F., S. Highlander, et al. (2009). "Metagenomic pyrosequencing and microbial identification." Clinical chemistry **55**(5): 856-866.

Pozhitkov, A. E., T. Beikler, et al. (2011). "High-throughput methods for analysis of the human oral microbiome." Periodontology 2000 **55**(1): 70-86.

Ribeiro, A. C., F. Matarazzo, et al. (2011). "Exploring bacterial diversity of endodontic microbiota by cloning and sequencing 16S rRNA." Journal of endodontics **37**(7): 922-926.

Riesenfeld, C. S., P. D. Schloss, et al. (2004). "Metagenomics: genomic analysis of microbial communities." Annu. Rev. Genet. **38**: 525-552.

Sabeti, M. and J. Slots (2004). "Herpesviral-bacterial coinfection in periapical pathosis." Journal of endodontics **30**(2): 69-72.

Seltzer, S. and P. A. Farber (1994). "Microbiologic factors in endodontology." Oral Surgery, Oral Medicine, Oral Pathology **78**(5): 634-645.

Siqueira, J. and I. Rocas (2009). "Diversity of endodontic microbiota revisited." Journal of dental research **88**(11): 969-981.

Siqueira Jr, J. and I. Rocas (2005). "Exploiting molecular methods to explore endodontic infections: part 1 - current molecular technologies for microbiological diagnosis." Journal of endodontics **31**(6): 411-423.

Siqueira Jr, J. and I. Rocas (2005). "Exploiting molecular methods to explore endodontic infections: part 2 - redefining the endodontic microbiota." Journal of endodontics **31**(7): 488-498.

Siqueira Jr, J. F., F. R. Alves, et al. (2011). "Pyrosequencing analysis of the apical root canal microbiota." Journal of endodontics **37**(11): 1499-1503.

Siqueira Jr, J. F., A. F. Fouad, et al. (2012). "Pyrosequencing as a tool for better understanding of human microbiomes." Journal of oral microbiology **4**.

Siqueira Jr, J. F. and I. N. Rocas (2006). "Catonella morbi and Granulicatella adiacens: new species in endodontic infections." Oral Surgery, Oral Medicine, Oral Pathology, Oral Radiology, and Endodontology **102**(2): 259-264.

Slots, J. (2010). "Herpesviral-bacterial interactions in periodontal diseases." Periodontology 2000 **52**(1): 117-140.

Sunde, P. T., I. Olsen, et al. (2002). "Microbiota of periapical lesions refractory to endodontic therapy." Journal of endodontics **28**(4): 304-310.

Sunde, P. T., I. Olsen, et al. (2003). "Fluorescence in situ hybridization (FISH) for direct visualization of bacteria in periapical lesions of asymptomatic root-filled teeth." Microbiology **149**(5): 1095-1102.

Sundqvist, G. (1976). Bacteriologic studies of necrotic dental pulps. Umea, Sweden, Umea University Odontological Disseration no. 7.

Weisburg, W. G., S. M. Barns, et al. (1991). "16S ribosomal DNA amplification for phylogenetic study." Journal of bacteriology **173**(2): 697-703.

Yang, Q.-B., L.-N. Fan, et al. (2010). "Polymerase Chain Reaction - Denaturing Gradient Gel Electrophoresis, Cloning, and Sequence Analysis of Bacteria Associated with Acute Periapical Abscesses in Children." Journal of endodontics **36**(2): 218-223.

Young, G., S. Turner, et al. (2007). "Bacterial DNA persists for extended periods after cell death." Journal of endodontics **33**(12): 1417-1420.

Zaura, E., B. J. Keijser, et al. (2009). "Defining the healthy "core microbiome" of oral microbial communities." BMC microbiology **9**: 259.

CHAPTER II:

The Personal Human Oral Microbiome Obscures the Effects of Treatment on Periodontal Disease

INTRODUCTION

Periodontitis is a complex, polymicrobial infection of the periodontium. The disease is caused by dental plaque microorganisms that migrate into the periodontal pocket and give rise to inflammation of the gingiva (Dentino, Lee et al. 2013). Left untreated, the inflammatory process may lead to loss of tooth-supporting connective tissue and bone, and eventually to edentulism (Teles, Teles et al. 2013). While oral microbes are the principal cause of periodontitis, factors such as tobacco use, osteoporosis, obesity, and diabetes exacerbate the disease (Genco and Borgnakke 2013). Periodontitis has also been associated with systemic diseases, including atherosclerosis, preterm birth, and diabetes (Cullinan and Seymour 2013).

Conventional diagnostic techniques in periodontics are based on clinical examination and occasionally on laboratory tests. Clinical examination assesses gingival health status, periodontal pocket depth, clinical attachment loss, radiographic alveolar bone level, oral hygiene performance, and other clinical variables (Slots 2013). Laboratory testing may include microbiological analysis for periodontal pathogens, blood tests for systemic health status, and histological evaluation of tissue changes. The obtained information allows a classification of periodontal disease into gingivitis and mild, moderate and severe periodontitis. However, the current diagnostic tests are not particularly sensitive and specific for periodontal disease activity and have limited prognosticative value. Rapid molecular techniques capable of identifying

periodontal bacteria and viruses with great accuracy may eventually provide a better classification and diagnosis of various types of periodontal disease and aid significantly in clinical decision-making (Slots 2013).

Thus far, most of what we know about bacteria in periodontal disease has been learned through anaerobic culturing, but the immense bacterial diversity in periodontal pockets will require molecular methods able to simultaneously investigate all members of periodontal pocket communities, including those that we cannot currently grow in culture (Paster, Olsen et al. 2006; Keijser, Zaura et al. 2008; Liu, Faller et al. 2012). Recent studies by Griffen et al. (2012) and Abusleme et al. (2013) using Next-Generation Sequencing (NGS) of bacterial small-subunit ribosomal RNA (16S rRNA) genes showed the promise of these methods for investigating periodontal disease (Griffen, Beall et al. 2012; Abusleme, Dupuy et al. 2013). These studies analyzed patterns of microbial diversity in healthy and diseased periodontal pockets and showed clear community level differences among, and even within, individuals.

Here, we used NGS methods to determine how standard periodontal disease treatment, namely scaling and root planing and oral hygiene instruction, altered polymicrobial diversity in periodontal pockets. The study design and analytical methods allowed us to investigate differences in microbial community diversity among periodontal health and disease states, and whether there were consistent associations of particular bacteria with health or disease.

METHODS

Study Population, Clinical Assessment and Treatment

Thirty-six subjects aged 21-40 with gingivitis, mild-to-moderate periodontitis, or severe periodontitis, along with 4 healthy controls were recruited from an American Indian/Alaska Native (AIAN) population in Southern California. The AIAN population is known to have a higher incidence of periodontal disease than the general population, making it an important subject of study for this community (Skrepcinski and Niendorff 2000). The study was reviewed and approved by the human subjects committee and all subjects gave informed consent. Degree of periodontal disease was assessed by measuring probing pocket depths (PD), clinical attachment loss (CAL), plaque scores, and bleeding on probing (BOP). Twenty-three patients aged 21-40 with gingivitis (CAL ≤ 3 mm, PD ≤ 4 mm, BOP > 10%), twelve patients with mild-moderate periodontitis (CAL ≥ 4 mm, PD ≥ 5 mm, BOP ≥ 30%), one patient with severe periodontitis (CAL ≥ 6 mm, pocket depths ≥ 7 mm, BOP ≥ 30%), along with 4 healthy controls (CAL ≤ 3 mm, PD ≤ 3 mm, BOP ≤ 10%) all aged 21-40 were enrolled in the study. Following completion of periodontal treatment (at least 6 weeks later), patients returned for a follow-up visit.

Patients received a baseline dental examination which included a full dental screening and measurement of periodontal pocket depths of all teeth. Following the clinical examination, microbial samples were collected from the two deepest periodontal pockets of the dentition using a periodontal scaler. The

sample material was wiped onto sterile Whatman filters and submerged into 10 mL of sterile Sodium-Magnesium buffer (SM buffer) and kept at 4^{o}C. DNA was extracted with the NucleoSpin Tissue Nucleic Acid and Protein Purification Kit (Macherey-Nagel GmbH & Co, Germany) from the supernatant after vigorous vortexing. The same procedure was repeated at least six weeks following completion of standard periodontal disease treatment. Patients were classified as improved if their average pocket depth decreased (twelve patients), worsened if their average pocket depth increased (eighteen patients), and no change if their average pocket depth remained the same (6 patients) (Badersten, Nilveus et al. 1981; Cobb 2002).

Next-Generation Sequencing and Bioinformatics

The 27F and 338R primers targeting the V1–V2 hypervariable regions of 16S rRNA genes were used in the PCR reactions (Fierer, Hamady et al. 2008). The primers were barcoded following Fierer *et al.* (2008), using the same PCR thermocycling parameters. PCR products were submitted to the core sequencing facility at the University of Pennsylvania for purification, equimolar dilution and pyrosequencing on a Roche 454 GS FLX instrument. The dataset sequences were deposited into the publically accessible QIIME Database at http://www.microbio.me/qiime.

Sequencing data were analyzed using QIIME 1.6.0-dev (Caporaso, Kuczynski et al. 2010). Briefly, sequences were clustered into 97% using a uclust-based (Edgar 2010) open-reference OTU picking protocol using the Greengenes 12_10 reference sequences (McDonald, Price et al. 2012).

Taxonomy was assigned to sequences using the RDP Classifier (Wang, Garrity et al. 2007), retrained on Greengenes 12_10, via QIIME. Representative sequences, which were selected as the centroid sequence of each OTU, were aligned with PyNAST (Caporaso, Bittinger et al. 2010), and trees were constructed using FastTree (Price, Dehal et al. 2010) for phylogenetic diversity calculations. Procrustes analysis (Gower 1975) was performed using QIIME with 1000 Monte Carlo iterations. OTU counts for specific taxonomic groups (e.g., *Streptococcus*) were exported from QIIME for statistical analyses in R version 2.15.1 (Team 2008). Representative *Fusobacterium* and *Prevotella* sequences were exported for multiple sequence alignment and phylogenetic analyses (see Figures 2.6 and 2.7).

RESULTS

A total of 76 periodontal pocket microbial community samples were analyzed via 454 pyrosequencing of bacterial 16S rRNA amplicons. Pyrosequencing yielded a combined total of 759,717 sequences across all samples with a median sequence count of 9,676. From these data, we identified 87 bacterial genera belonging to 12 different divisions, the majority of which were common members of periodontal pocket microbiota. Community-level analyses (Unifrac-based PCoA) did not uncover clear differences between samples collected prior to treatment with those collected post-treatment, even after accounting for the treatment effectiveness. On the contrary, post-treatment samples remained most similar to pre-treatment samples from the same individual (Figure 2.1).

Deeper analyses of the distributions of specific bacterial taxa associated with either health (*Streptococcus*, *Veillonella*) or disease (*Fusobacterium*, *Prevotella* and *Leptotrichia*) (Griffen, Beall et al. 2012; Liu, Faller et al. 2012; Abusleme, Dupuy et al. 2013) found only *Fusobacterium* to be significantly correlated with pocket depth over all samples (Figure 2.2a). As expected, we found an inverse correlation between the abundance of *Fusobacterium* and *Streptococcus* (data not shown) and between *Streptococcus* and *Prevotella* (Figure 2.2b), with the association primarily driven by the negative correlation between *Streptococcus* and *P. loescheii* (Figure 2.2c). *Fusobacterium*, especially *F. nucleatum*, plays a key role in periodontal biofilm development by

bridging early and late colonizers, according to the successional integration theory (Kolenbrander, Palmer et al. 2006). *Streptococcus* species establish the biofilm and *P. loescheii* attaches directly to *Streptococcus*, unlike the other *Prevotella* species. The roles played by these bacterial genera may make them particularly responsive to biofilm disturbance, and perhaps make them useful indicators of periodontal treatment efficacy.

DISCUSSION

In interpreting patient response to treatment, accounting for the personal microbiome of individual patients proved critical. This interpersonal variability also explains why we do not observe pre- and post-treatment clustering in PCoA space (Figure 2.1). While there are consistent changes associated with recovery from periodontal disease (e.g., a decrease in *Prevotella* abundance), the "healthy" amount of *Prevotella* differs on an individual basis. Moreover, the flora of some individuals changed contrary to the prevailing trends, notably in the *Fusobacterium* and *Prevotella*. *Streptococcus* remained steady or slightly increased in patients that improved, except two individuals who experienced dramatic declines post-treatment (Figure 2.3c). We also did not observe an expected increase in *Veillonella* in improving individuals post-treatment (Figure 2.3d).

Understanding the behavior of the biofilm response also appeared, at least in the case of *Prevotella*, to require more species-specific knowledge. Having successfully differentiated a number of oral *Prevotella* species (Figure 2.6), we found the abundance of *P. melaninogenica* and *P. loescheii* changed in opposite directions, while other *Prevotella* showed highly variable response post-treatment (Figure 2.4). A closer examination of *Fusobacterium* diversity also provided intriguing insight into periodontal biofilms. OTU clustering and phylogenetic analysis determined as many as 73 different species (Figure 2.5; Figure 2.7). Only four of these were abundant across all samples, and only two

were found in every sample (Figure 2.5), supporting recent findings that the core human microbiome in unrelated individuals tends to be minimal at lower taxonomic levels (Faith, Guruge et al. 2013). These rarer species may increase the overall immune response and metabolic activity, but our data also suggest the presence of biofilm “cheaters” who contribute little to actual biofilm stability.

In the past, it was common to focus on the presence or absence of the bacteria that comprise the “red complex” (*Porphyromonas gingivalis, Tannerella forsythia* and *Treponema denticola*), which were implicated in disease (Socransky, Haffajee et al. 1998). However, it is clear from recent studies that culturing and emphasis on specific bacteria will not capture all the variability in the diseased periodontium (Griffen, Beall et al. 2012; Liu, Faller et al. 2012; Abusleme, Dupuy et al. 2013). This leads us to question the use of antibiotics in treatment of periodontal disease due to the variability of bacteria found in different diseased patients and the varied susceptibility of bacteria to different kinds of antibiotics.

Systemic antibiotic therapy is often used in periodontics to reduce or eradicate periodontopathic bacteria that are invading gingiva or are otherwise not reachable by topical antimicrobial treatment (Slots 2012). The selection of antibiotics is challenging because deep periodontal pockets can harbor several pathogens which exhibit diverse susceptibility to common antibiotics. Reference laboratories are available to identify periodontal pathogens and their antibiotic susceptibility, but most dentists institute antibiotic therapy empirically based on the best estimate of the most probable pathogen(s) and their usual antibiotic

susceptibility pattern. Combination antibiotic therapy is frequently employed to cover a broader spectrum of pathogens. However, even though properly prescribed antibiotics can help provide resolution of severe periodontitis, the widespread use of antibiotics carries risks of inducing antibiotic resistance in important medical pathogens. It is expected that increased insights into the composition of the periodontal microbiome will lead to a better definition of patients who may, or may not, benefit from adjunctive antibiotic therapy.

Altogether, our results highlight the importance of understanding each patient's personal oral microbiome, a goal achievable by collecting and analyzing pre- and post-treatment samples. Furthermore, they lead us to believe that there is not a single composition that represents a healthy periodontal state and that recovery from periodontal disease appears to reflect a shift from a personalized disease state to a personalized healthy state. While there is consensus that particular communities should shift with response to disease, there may not be a "healthy amount" of these bacteria that is consistent across individuals. Further research with a larger patient sample size and more sampling over a longer time period will be necessary to confirm this hypothesis.

Chapter 2, in full, has been submitted for publication of the material as it may appear in PLOS One, 2014. Schwarzberg, Karen; Le, Rosalin; Bharti, Balambal; Lindsay, Suzanne; Casaburi, Giorgio; Salvatore, Francesco; Saber, Mohamed H; Alonaizan, Faisal; Slots, Jørgen; Gottlieb, Roberta A; Caporaso, J. Gregory; Kelley, Scott T. The Personal Human Oral Microbiome Obscures the

Effects of Treatment on Periodontal Disease. The dissertation author was the primary investigator and author of this paper.

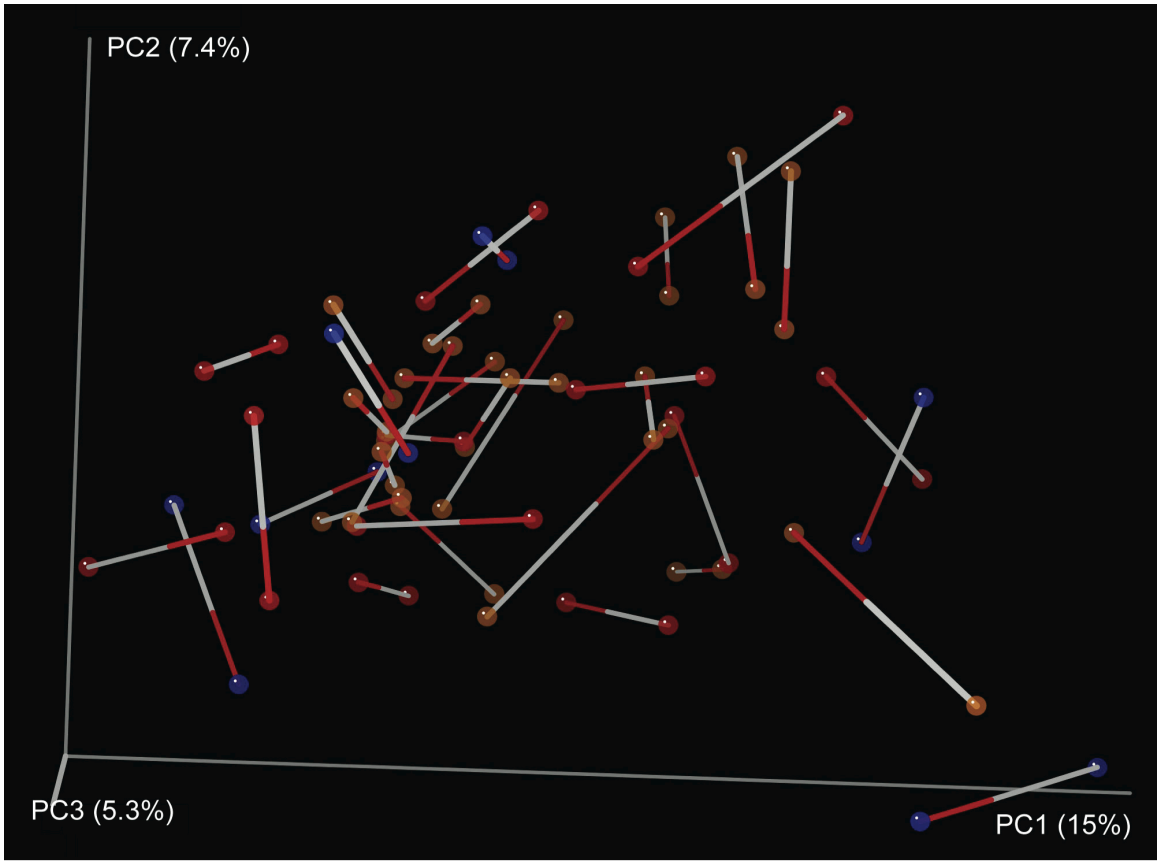

Figure 2.1: Procrustes analysis of samples before and after periodontal treatment. Procrustes M^2 value = 0.420 (dissimilarity of the two datasets), *P*-value = 0.00 based on 1000 Monte Carlo iterations. This analysis is a visualization of a principal coordinates analysis (PCoA) of the Unifrac distances between samples, showing the best superimposition of one Unifrac plot on the other. Samples collected from the same patient before and after treatment are connected by a line, the white end indicating the before-treatment sample red end indicating the after-treatment sample. Patients were classified as improved (red circles), worsened (brown circles) or no change (blue circles). Determination of patient improvement or decline was based on changes in observed pocket depth, a standard approach used in periodontal research (Badersten, Nilveus et al. 1981; Cobb 2002).

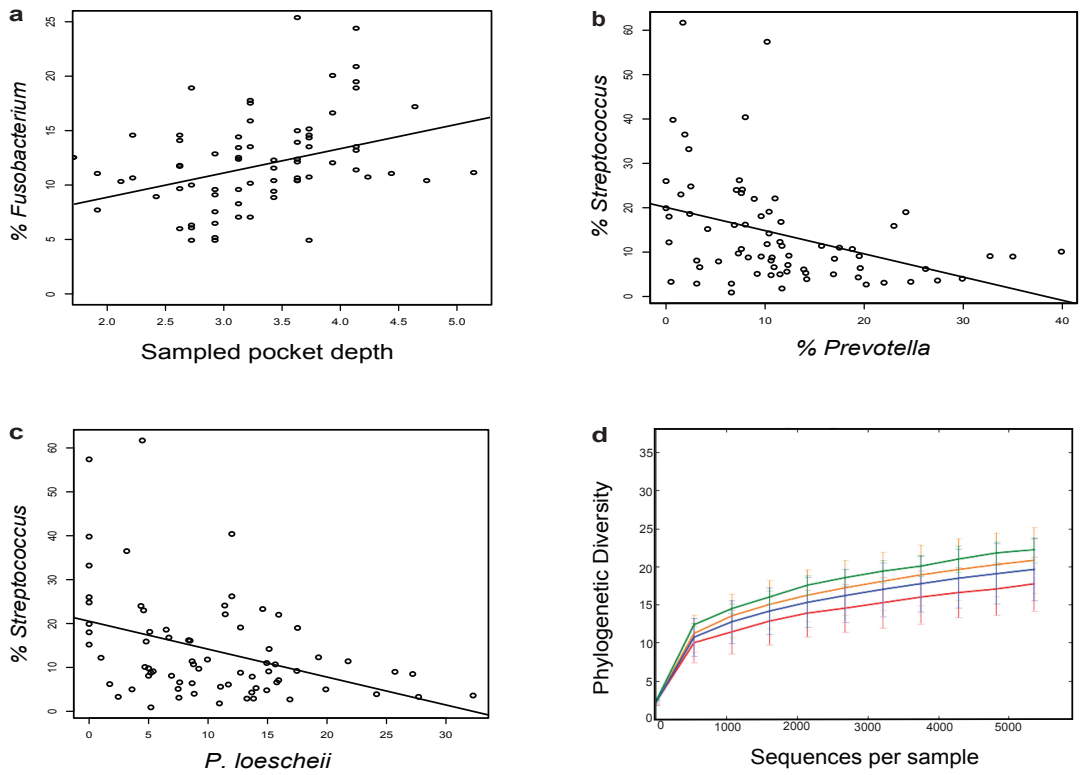

Figure 2.2: Statistical trends in samples. a Percent of *Fusobacterium* relative to pocket depth of sampled teeth (r=0.2413, *P*=0.0411).
b Percent of *Streptococcus* relative to *Prevotella* (r=-0.3846, *P*=0.0008).
c Percent of *Streptococcus* relative to single *Prevotella* species, *P. loescheii* (r=-0.3055, *P*=0.0090).
d Rarefaction trends: distribution of number of sequences per sample. Samples were classified as Healthy Controls (red line), gingivitis (blue line), mild/moderate periodontitis (orange line) and severe periodontitis (green line).

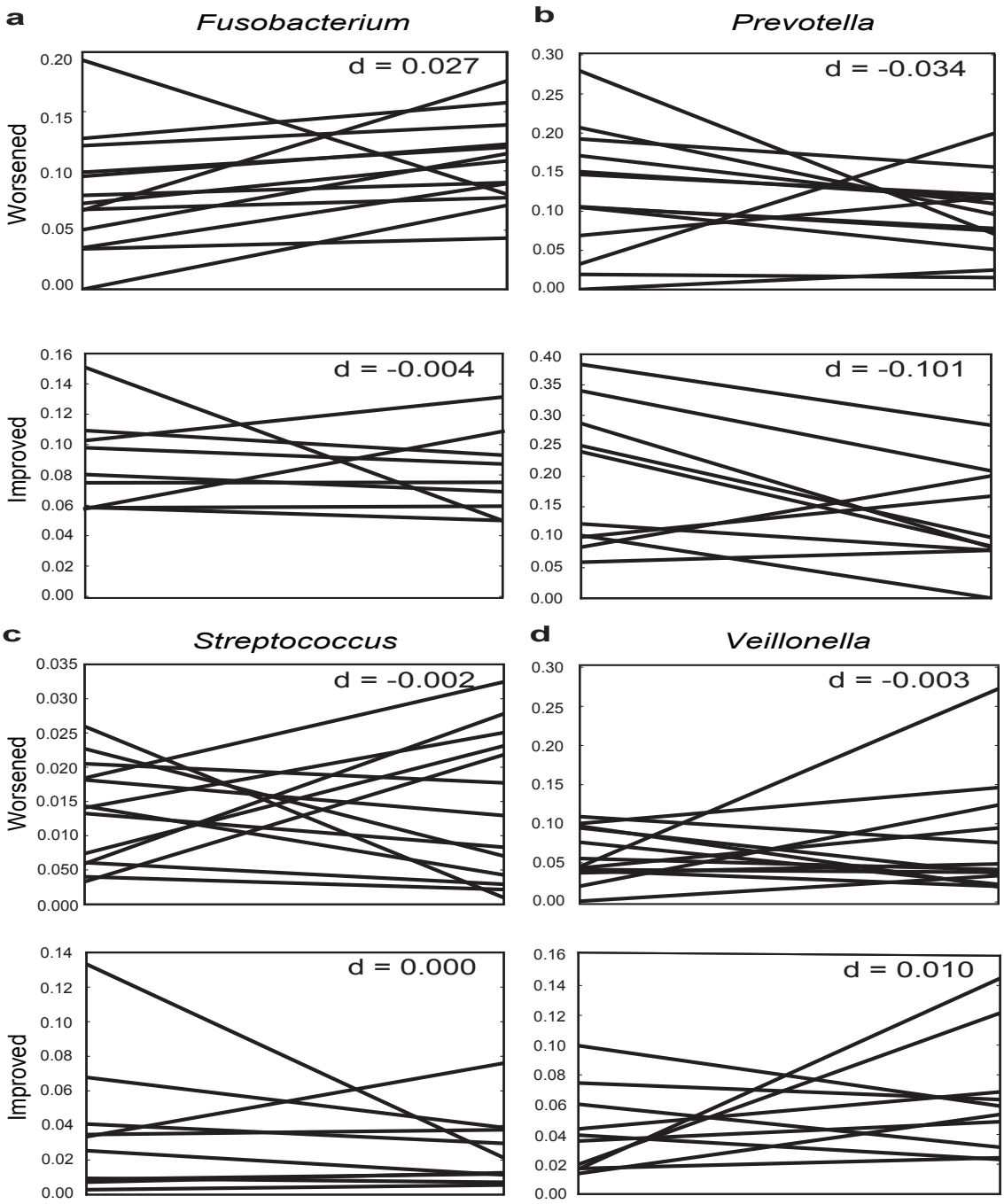

Figure 2.3: Trends of bacterial genera associated with health or disease, separated by whether individuals improved or worsened after treatment. An analysis of average periodontal pocket depth before and after treatment showed that less than half (N=12) the treated individuals improved post-treatment, while the rest stayed the same (N=6) or worsened (N=18). Lines indicate the proportion for a particular individual. The d-scores indicate the median line slope. **a** *Fusobacterium,* **b** *Prevotella*, **c** *Streptococcus*, **d** *Veillonella*. Note that the scale of the y-axis differs to highlight difference in individual responses to treatment.

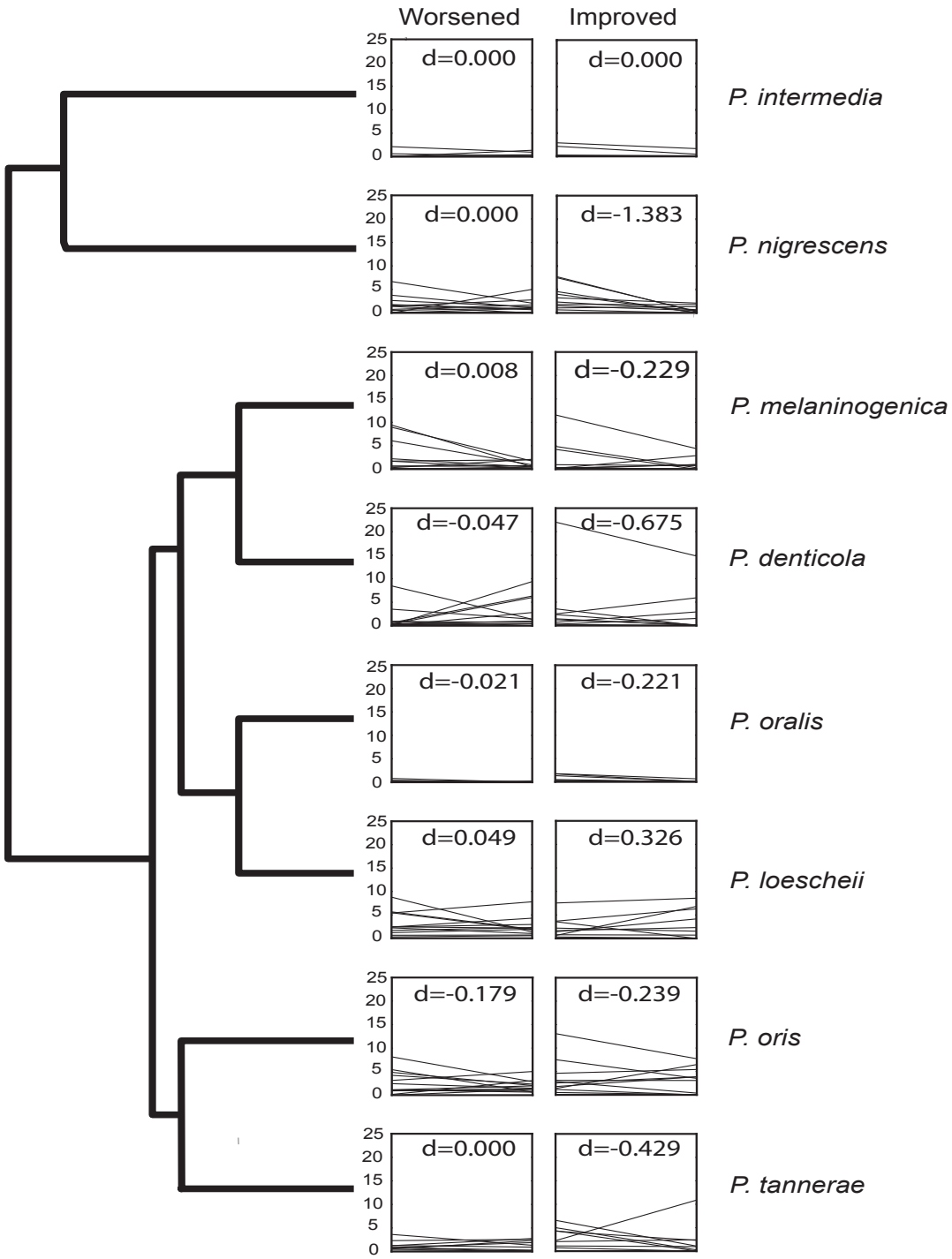

Figure 2.4: Representative cladogram of Prevotella species determined in this study. This is based on phylogenetic analysis shown in Figure 6, with plots of relative abundance of specific species divided into patients that improved and patients that worsened. The d-scores indicate the median line slope. In many cases, changes in relative proportions before and after treatment appeared to be species dependent.

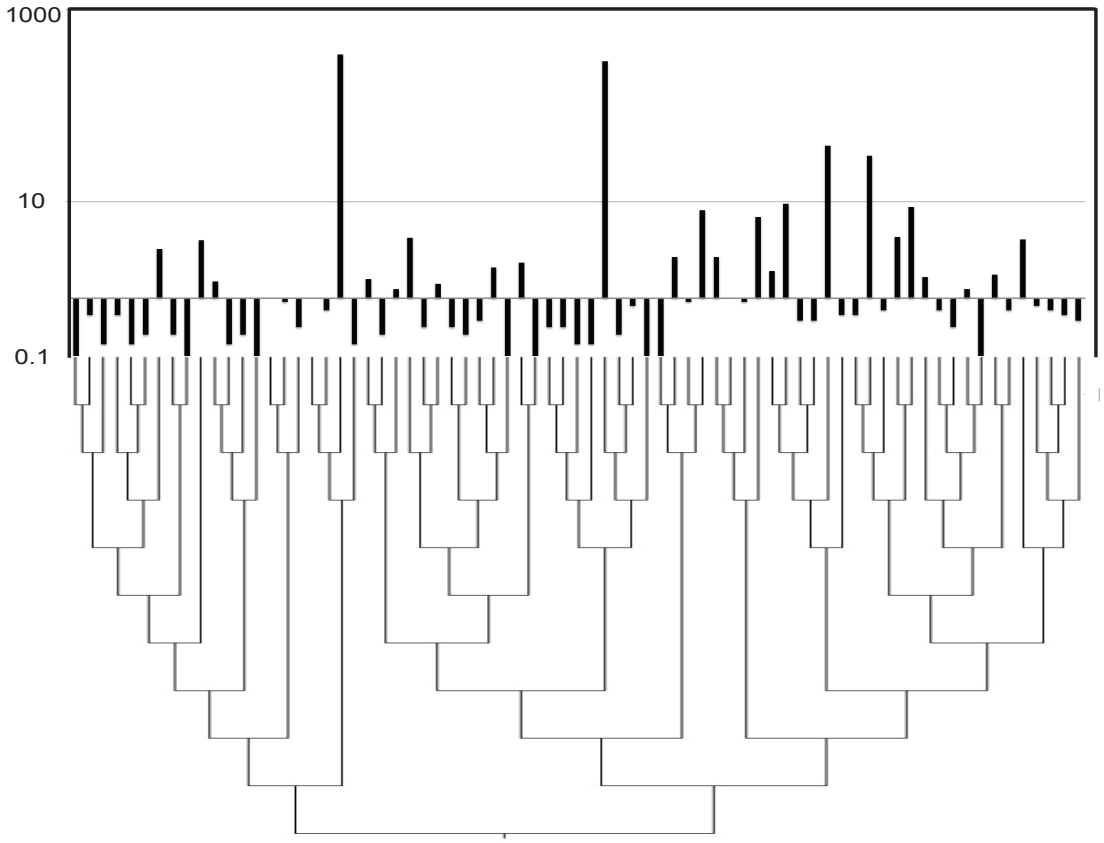

Figure 2.5: Cladogram of 73 different Fusobacterium-species sequences. OTUs were clustered at 97%. The histogram shows the log OTU-count abundance of these same species. Most OTUs were sparse and the overall diversity within and among pockets was considerable.

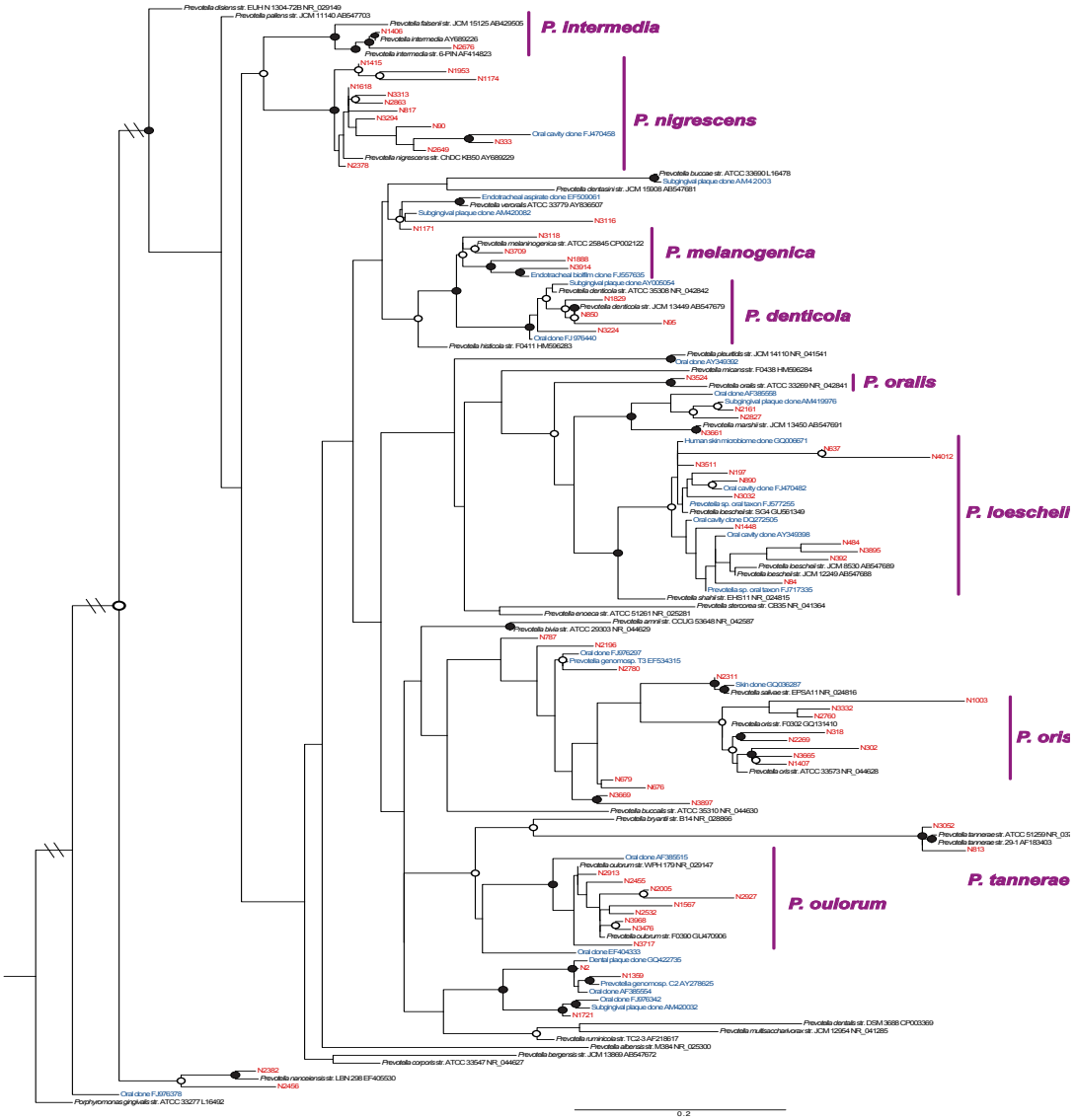

Figure 2.6: Maximum likelihood tree of Prevotella-related small-subunit ribosomal RNA gene sequences. The sequences highlighted in red were obtained in this study, while the rest include both cultured and uncultured sequences obtained from GenBank.

To be included in the phylogenetic analysis, sequences identical to the representative OTU had to be found in at least three independent periodontal pocket samples. Sequences from cultured and uncultured organisms were also included in the alignments. Alignments were trimmed to ~300 nucleotides and checked for accuracy and edited manually. Maximum-likelihood trees were created using RAxML HPC-BlackBox on CIPRES ((Miller 2010); http://www.phylo.org/). Black circles indicate bootstrap values of > 70% while white circles indicate bootstrap values between 50 and 70%.

Figure 2.7: Maximum likelihood tree of Fusobacterium-related small-subunit ribosomal RNA gene sequences. The sequences highlighted in red were obtained in this study. The orange highlighted sequences were obtained from a study of bacteria in periradicular lesions by Saber *et al.* (2012) (Saber, Schwarzberg et al. 2012).
See Figure 2.6 for details on the phylogenetic methods.

REFERENCES

Abusleme, L., A. K. Dupuy, et al. (2013). "The subgingival microbiome in health and periodontitis and its relationship with community biomass and inflammation." ISME J. 2013 Jan 10. doi: 10.1038/ismej.2012.174.(1751-7370 (Electronic)): T - aheadofprint.

Badersten, A., R. Nilveus, et al. (1981). "Effect of nonsurgical periodontal therapy. I. Moderately advanced periodontitis." Journal of Clinical Periodontology **8**(1): 57-72.

Caporaso, J. G., K. Bittinger, et al. (2010). "PyNAST: a flexible tool for aligning sequences to a template alignment." Bioinformatics **26**(2): 266-267.

Caporaso, J. G., J. Kuczynski, et al. (2010). "QIIME allows analysis of high-throughput community sequencing data." Nature Methods **7**(5): 335-336.

Cobb, C. M. (2002). "Clinical significance of non-surgical periodontal therapy: an evidence-based perspective of scaling and root planing." Journal of Clinical Periodontology **29**: 22-32.

Cullinan, M. P. and G. J. Seymour (2013). "Periodontal disease and systemic illness: will the evidence ever be enough?" Periodontology 2000 **62**(1): 271-286.

Dentino, A., S. Lee, et al. (2013). "Principles of periodontology." Periodontology 2000 **61**(1): 16-53.

Edgar, R. C. (2010). "Search and clustering orders of magnitude faster than BLAST." Bioinformatics **26**(19): 2460-2461.

Faith, J. J., J. L. Guruge, et al. (2013). "The Long-Term Stability of the Human Gut Microbiota." Science **341**(6141).

Fierer, N., M. Hamady, et al. (2008). "The influence of sex, handedness, and washing on the diversity of hand surface bacteria." Proceedings of the National Academy of Sciences **105**(46): 17994-17999.

Genco, R. J. and W. S. Borgnakke (2013). "Risk factors for periodontal disease." Periodontology 2000 **62**(1): 59-94.

Gower, J. C. (1975). "Generalized procrustes analysis." Psychometrika **40**(1): 33-51.

Griffen, A. L., C. J. Beall, et al. (2012). "Distinct and complex bacterial profiles in human periodontitis and health revealed by 16S pyrosequencing." Isme Journal **6**(6): 1176-1185.

Keijser, B. J. F., E. Zaura, et al. (2008). "Pyrosequencing analysis of the oral microflora of healthy adults." Journal of Dental Research **87**(11): 1016-1020.

Kolenbrander, P. E., R. J. Palmer, Jr., et al. (2006). "Bacterial interactions and successions during plaque development." Periodontology 2000 **42**: 47-79.

Liu, B., L. L. Faller, et al. (2012). "Deep sequencing of the oral microbiome reveals signatures of periodontal disease." Plos One **7**(6).

McDonald, D., M. N. Price, et al. (2012). "An improved Greengenes taxonomy with explicit ranks for ecological and evolutionary analyses of bacteria and archaea." ISME J **6**(3): 610-618.

Paster, B. J., I. Olsen, et al. (2006). "The breadth of bacterial diversity in the human periodontal pocket and other oral sites." Periodontology 2000 **42**: 80-87.

Price, M. N., P. S. Dehal, et al. (2010). "FastTree 2--approximately maximum-likelihood trees for large alignments." Plos One **5**(3): e9490.

Skrepcinski, F. B. and W. J. Niendorff (2000). "Periodontal Disease in American Indians and Alaska Natives." Journal of Public Health Dentistry **60**: 261-266.

Slots, J. (2012). "Low-cost periodontal therapy." Periodontology 2000 **60**(1): 110-137.

Slots, J. (2013). "Periodontology: past, present, perspectives." Periodontology 2000 **62**(1): 7-19.

Socransky, S., A. Haffajee, et al. (1998). "Microbial complexes in subgingival plaque." Journal of Clinical Periodontology **25**(2): 134-144.

Team, R. C. (2008). "R: A language and environment for statistical computing." R Foundation Statistical Computing, Vienna, Austria.

Teles, R., F. Teles, et al. (2013). "Lessons learned and unlearned in periodontal microbiology." Periodontology 2000 **62**(1): 95-162.

Wang, Q., G. M. Garrity, et al. (2007). "Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy." Appl Environ Microbiol **73**(16): 5261-5267.

CHAPTER III:

Effects of Periodontal Treatment on Bacteria in Periodontal Pockets:

a Metagenomics Approach

INTRODUCTION

Advances in technology have made characterizing the microbial composition in an environmental sample more effective than culture-based methods. Whole-genome shotgun sequencing applied to all microbial populations in a sample is a step beyond examining single genes such as 16S ribosomal RNA (rRNA). This genomic approach allows for better overall environment characterization (Venter, Remington et al. 2004). These recent advances have uncovered essential information for understanding complex microbial interactions in environmental samples. For example, The Human Microbiome Project found that there are over 100 times more genes in the microbiome than in our own genome (Methe, Nelson et al. 2012).

A deeper understanding of the human microbiome is a complicated undertaking that has promise to uncover important key functions that are not yet understood (Ley, Peterson et al. 2006; Kuramitsu, He et al. 2007; Turnbaugh, Ley et al. 2007; Belda-Ferre, Alcaraz et al. 2011; Methe, Nelson et al. 2012). Using metagenomics approaches instead of polymerase chain reaction (PCR) studies bypasses biases introduced into the estimates of microbial diversity (de Lillo, Ashley et al. 2006). Few studies have used metagenomics approaches to investigate dental caries and periodontal pocket microbial communities (Belda-Ferre, Alcaraz et al. 2011; Liu, Faller et al. 2012). The study focusing on periodontal pockets by Liu *et al.* investigated the microbial communities in health and disease. Using a metagenomics approach, they were able to see that the

diseased microbiome is enriched in metabolic functions that are consistent with a parasitic lifestyle. The bacteria in their samples appeared to be able to utilize nutrients derived from the degradation of host tissue and other bacteria that were killed by the host immune response. They found an increase of genes for fatty acid metabolism, acetyl-CoA degradation, aromatic amino acid degradation, ferrodoxin oxidation, and energy-coupling factor (ECF) class transporters. In addition, they found drug and metal resistance genes, conjugative transposons, type IV secretion systems, biosynthesis of lipid-A of the lipopolysaccharide and biosynthesis of toxic factors (e.g., acetone, butanol). Their study was a great contribution to the understanding of metabolic pathways in diseased periodontal pockets and in healthy patients, however, they had a limited amount of samples for each disease class (3 healthy controls and 2 periodontal patients).

To the best of our knowledge, the effects of periodontal treatment have not been studied using whole-genome shotgun sequencing. Our study investigated the metagenomes from 12 periodontal patients to determine how standard periodontal treatment, scaling and root planing and oral hygiene instruction altered the polymicrobial diversity and metabolic profiles in periodontal pockets. We hypothesized that after treating periodontal disease we would see a reduction in the number of hits to the factors identified by Liu *et al.* in periodontitis samples.

METHODS

Study Population, Clinical Assessment and Treatment

Twelve subjects aged 21-40 with gingivitis or mild-to-moderate periodontitis were recruited from an American Indian/Alaska Native (AIAN) population in Southern California. The AIAN population is known to have a higher incidence of periodontal disease than the general population, making it an important subject of study for this community (Skrepcinski and Niendorff 2000). The study was reviewed and approved by the human subjects committee and all subjects gave informed consent. Degree of periodontal disease was assessed by measuring probing pocket depths (PD), clinical attachment loss (CAL), plaque scores, and bleeding on probing (BOP). Five patients with gingivitis (CAL ≤ 3 mm, PD ≤ 4 mm, BOP > 10%) and seven patients with mild-moderate periodontitis (CAL ≥ 4 mm, PD ≥ 5 mm, BOP ≥ 30 all aged 21-40 were enrolled in the study. Following completion of periodontal treatment (at least 6 weeks later), patients returned for a follow-up visit.

Patients received a baseline dental examination which included a full dental screening and measurement of periodontal pocket depths of all teeth. Following the clinical examination, microbial samples were collected from the two deepest periodontal pockets of the dentition using a periodontal scaler. The sample material was wiped onto sterile Whatman filters and submerged into 10 mL of sterile Sodium-Magnesium buffer (SM buffer) and kept at 4^{o}C. DNA was extracted with the NucleoSpin Tissue Nucleic Acid and Protein Purification Kit

(Macherey-Nagel GmbH & Co, Germany) from the supernatant after vigorous vortexing. The same procedure was repeated at least six weeks following completion of standard periodontal disease treatment. Patients were classified as improved if their average pocket depth decreased (six patients) or worsened if their average pocket depth increased (six patients) (Badersten, Nilvéus et al. 1981; Cobb 2002).

Sequencing and Data Analysis

Extracted DNA samples were submitted to the core sequencing facility at The Scripps Research Institute in San Diego, California for purification, equimolar dilution and sequencing on an Illumina Hi-Seq instrument.

Sequencing data were analyzed using MG-RAST (Meyer, Paarmann et al. 2008) for taxonomic and metabolic profiling. Sequences were also analyzed using MetaPhlAn (Segata, Waldron et al. 2012) for taxonomic profiling. Numbers of functional gene hits were exported from MG-RAST for statistical analyses in R version 2.15.1 (Team 2008).

RESULTS

A total of 24 periodontal pocket microbial community samples from 12 patients were analyzed on the Illumina Hi-Seq instrument. Using MG-RAST, we found that the sequencing yielded a range of $1.09x10^9$ – $1.86\ x10^9$ base pairs (bp) per sample with an average of $1.41\ x10^9$ bp. The sequences per sample ranged between $1.02\ x10^7$ and $1.84\ x10^7$ with an average of $1.40\ x10^7$ bp. The DRISEE score (Keegan, Trimble et al. 2012) for sequencing error estimation ranged between 0.724% and 34.477% with a mean score of 20.83%. The alpha diversity score, which is a summary of distinct species found in each sample file, ranged between 136.74 and 369.13 with an average of 227.24.

The top 25 bacterial genera in the samples as identified by MetaPhlAn can be seen in Figure 3.1. The abundance heatmap shows that the most abundant genera are: *Prevotella, Streptococcus, Capnocytophaga, Veillonella* and *Leptotrichia*. The phylogenetic cladogram of sample similarity indicates that the microbial composition of most patients after periodontal treatment did not group with their initial microbial composition. Only two patient samples grouped together: 057 and 015. While not most similar to each other, samples from patients 035 and 068 cluster together in the center clade (Figure 3.1).

Figure 3.2 depicts a heatmap of functional gene categories for all samples. Most samples appear to have high levels of hits to housekeeping genes such as ones for RNA metabolism and respiration. Most samples appear to have relatively high levels of genes related to aromatic acid metabolism, which is

associated with a virulent lifestyle in pathogenic periodontal bacteria (Barnes, Teles et al. 2009; Liu, Faller et al. 2012) (Figure 3.2).

When examining a principal coordinates analysis (PCoA) of the samples based on functional gene categories, there is no apparent clustering of samples based on disease state (gingivitis or mild-moderate periodontitis), time of sampling (before of after treatment) or whether or not the samples belonged to a patient who improved or worsened (Figure 3.3).

Further investigation into the functional gene categories was conducted in R version 2.15.1 (Team 2008) and can be seen in Figure 3.4. When singling out bacterial functions that are associated with a virulent lifestyle, it is apparent that there are some differences within the samples. There is a trend of higher levels of all virulence factors after periodontal treatment. Fatty acid metabolism is significantly reduced in samples from patients that improved after treatment ($P = 0.027$). There are significantly more sequences that match type IV secretion systems in patients after periodontal treatment ($P = 0.017$).

DISCUSSION

A metagenome of a sample contains sequences from all of the genomes that the sample contains. The massive amount of information produced from the whole-genome shotgun sequencing technology is difficult to navigate and there still aren't any analysis tools that are considered the "gold standard" for interpreting the data. In this study, we used two popular tools for analysis – MG-RAST (Meyer, Paarmann et al. 2008) and MetaPhlAn (Segata, Waldron et al. 2012), which can provide data about taxonomy and metabolic information based on gene sequences. These tools use reference databases to identify the sequences, thus causing two potential issues: the reads are very short and could cause inaccurate matches, and there may not be sequences in the database that match novel genes in the samples. However, all analysis tools introduce some bias, and the field of metagenomics is advancing rapidly, thus adding to the information we have in these reference databases and increasing the accuracy of their results.

Metagenome shotgun sequencing provides sequencing depth that is greater than 454-pyrosequencing of the 16S rRNA gene. The samples from this study are a subset of samples that were investigated in a 454-pyrosequencing study (Schwarzberg *et al.*, submitted for publication). Comparing those results to the top 25 genera identified in the samples by Illumina sequencing (Figure 3.1) yields similar results. Interestingly, 19 of the genera were identical. The genera that were present in the top 25 genera identified by 16S that were not in the

metagenomic top 25 were: *Tannerella, Granulicatella,* TM7, *Actinomyces, Gemella and Olsonella*. All of these genera were identified in the samples at a lower ranking. Of the 6 genera that were identified as the top 25 by MetaPhlAn that were different from the top 25 from 16S, 4 were present in lower rankings in the 16S data: *Cardiobacterium, Aggregatibacter, Peptoniphilus* and *Rothia.* Only two were not present in the 16S data at all: *Haemophilus* and *Eikenella*. However, it is worth noting that based on the Human Oral Microbiome Database (Chen, Yu et al. 2010) these two genera have been identified previously in the oral cavity.

Metagenomes do not rely on one gene, and therefore contain information that 16S can not provide, such as: sequences matching functional gene categories (Figure 3.2), including metabolic genes. Our samples here did not group in any way based on the hits to known functional gene categories, as seen in Figure 3.3. There is a large cluster that includes most of the samples, 19 out of the 24. The 5 samples that did not cluster as strongly should be investigated in order to understand what differentiates them from the rest.

Certain functional gene categories are especially interesting in the case of periodontal disease, which is characterized by inflammation caused by bacteria (Dentino, Lee et al. 2013). Liu *et al.* found that the microbiome in periodontal pocket samples was enriched for virulence factors such as biosynthesis of toxic factors (e.g., acetone) and Lipid-A of the lipopolysaccharide (LPS). In addition, they found that the microbiome was adapted to a parasitic lifestyle, for example by degrading host tissue and other bacterial cells for their own benefit, by being

enriched for aromatic amino acid degradation, fatty acid metabolism, energy-coupling factor (ECF) class transporters and resistance to drugs and metals. This builds upon other studies that have found similar findings in non-metagenomic methods. For example, Pike *et al.* found that mercury-resistant bacteria were present in children with and without amalgam fillings and that many of the organisms they identified were also resistant to antibiotics (Pike, Lucas et al. 2002). Many studies have investigated the connection between fatty acids and gingival tissues, and have even linked an increase in unsaturated fatty acids to inflamed periodontal tissues (Campan, Planchand et al. 1997; Rosenstein, Kushner et al. 2003; Cicek, Ozmen et al. 2005; Kesavalu, Bakthavatchalu et al. 2007). In our samples, we observed a trend of increased hits to virulence factors after treatment (Figure 3.4). For example, fatty acid metabolism was significantly lower in patients that worsened compared to patients that improved. This is the opposite of what was expected; that virulence factors would be reduced in cases that responded well to treatment. Another finding that was surprising is the significant increase in type IV secretion system hits found in samples after treatment. This could also be explained by the need to re-investigate the microbial communities at a later time after the perturbation caused by the periodontal treatment, giving the oral biofilms time to reach a new steady state and see long-term effects of treatment.

The data in these 24 metagenome files is immense and could be studied further. It would be worthwhile to conduct a follow up study investigating the differences in data interpretation when using additional metagenomics tools and

comparing these files to the human oral microbiome data in greater depth. In addition, it is necessary to verify that these results are repeatable and to track the gene categories over a longer period of time as potential indicators of health or disease.

In addition, obtaining the sequences of all of the genes present in each one of the gene categories mentioned in this study that were identified by Liu *et al*. would be highly informative. By doing so, it would be possible to examine which bacteria these hits came from, and then by comparing samples from before and after treatment it would be possible to see which one of these bacteria changed in response to the treatment. This analysis would also be worthwhile repeating in a study that follows patients during a longer time period.

Another bioinformatics method that would be a valuable pursuit is the creation of genome assemblies from these data files. Creating genome assemblies could identify novel genera and species not yet identified in the human periodontal pocket. This method compliments the work done in this study, as it does not rely on matching short reads to known sequences in databases that might not contain all of the genes found in the samples.

Aside from information that can be inferred from the sequences that match known genes, there is still information that cannot be gleaned from metagenome sequencing. Proteomics and metabolomics studies would be a worthwhile follow up to this study as well. These approaches would be able to gather information about what genes are actually being expressed in the samples at different time points and in relation to different treatments.

This study investigated the effects of periodontal treatment on the microbial communities in the human periodontal pocket using the whole-genome shotgun sequencing on the Illumina Hi-Seq platform. The effects of treatment have not been studied before using metagenomics, making this a unique dataset that sheds light on the content of the bacterial population in the periodontium before and after periodontal treatment. Our findings indicate that a longer period of time needs to pass before drawing more conclusions regarding the bacterial populations after treatment, allowing the bacteria in the biofilm time to reach a new steady state after perturbation.

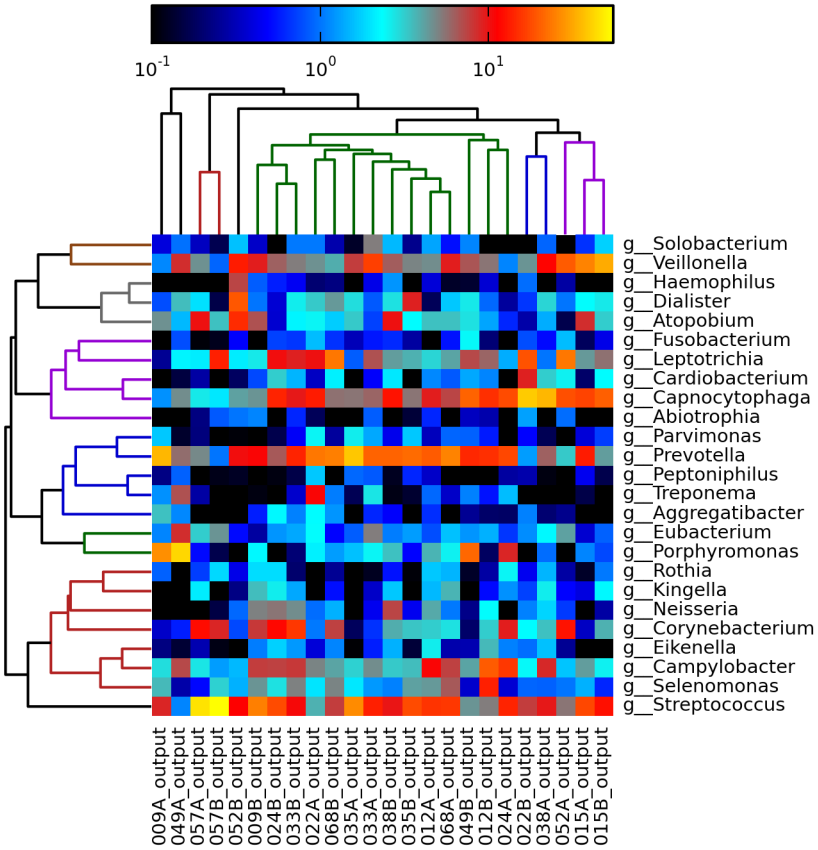

Figure 3.1: Heatmap of top 25 genera identified in samples. Depiction of the taxonomic distribution of the top 25 genera identified by MetaPhlAn (on the right) in the 24 samples (sample names on the bottom). The top cladogram represents the taxonomic closeness of the samples based on their phylogenetic diversity, while the cladogram on the right represents the phylogenetic relationships between the genera.

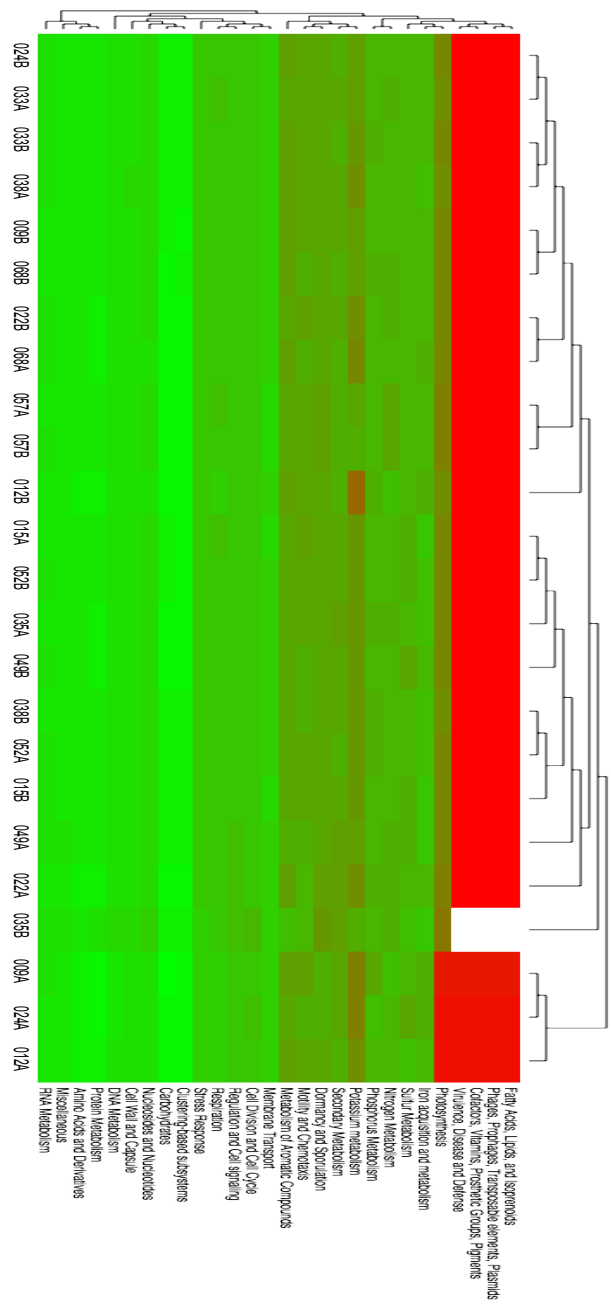

Figure 3.2: Heatmap representing different functional gene categories in all samples. In the heatmap, green shows stronger representation while red shows weaker representation. The cladogram on the top represents the relation of the samples to each other based on the prevalence of gene categories, while the cladogram on the left represents the phylogenetic relationships of the gene categories.

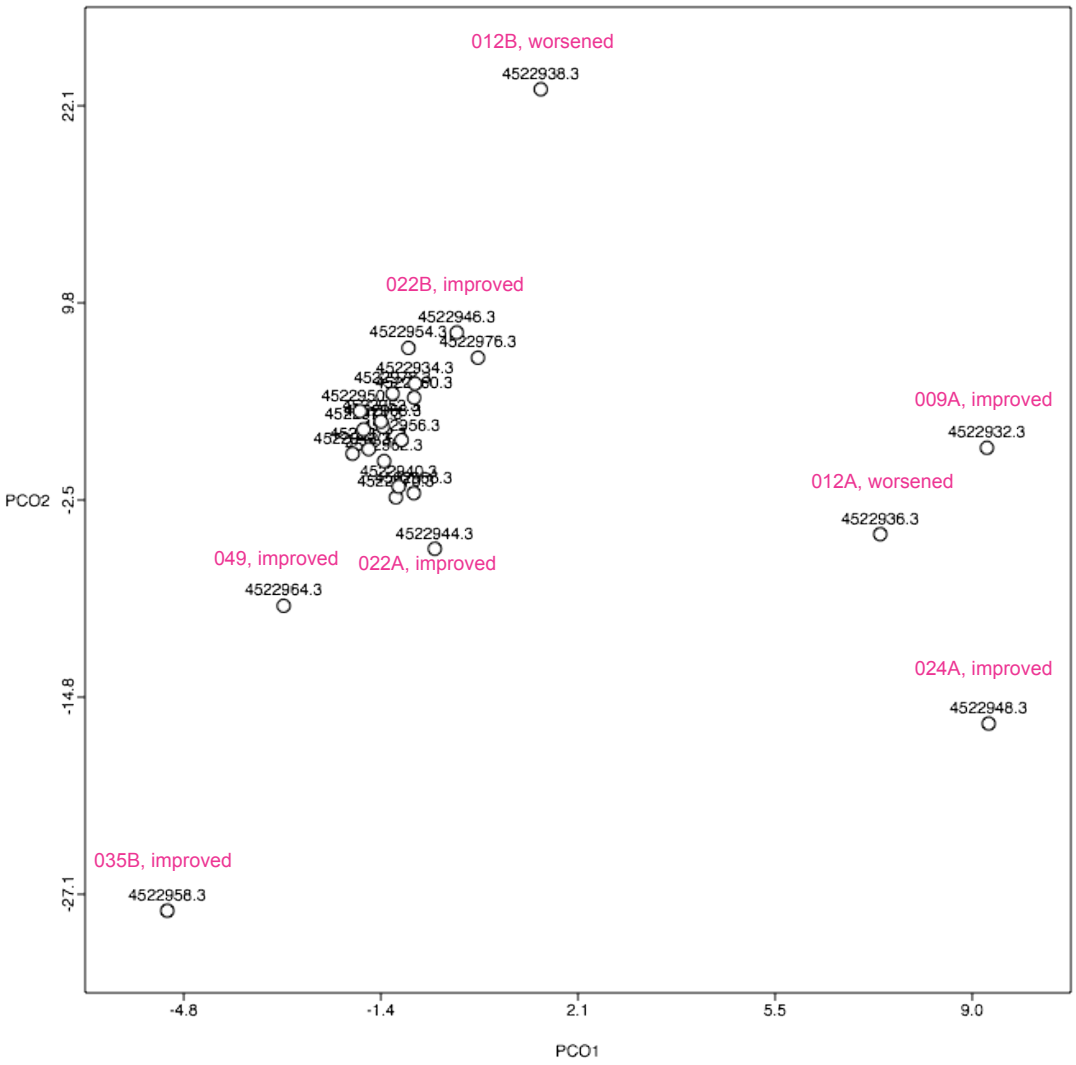

Figure 3.3: PCoA plot of the samples based on functional gene categories. Sample names are as given in MG-RAST. Samples that are sufficiently separated out include the identifiers used in this study along with information on whether that patient was classified as improved or worsened in pink.

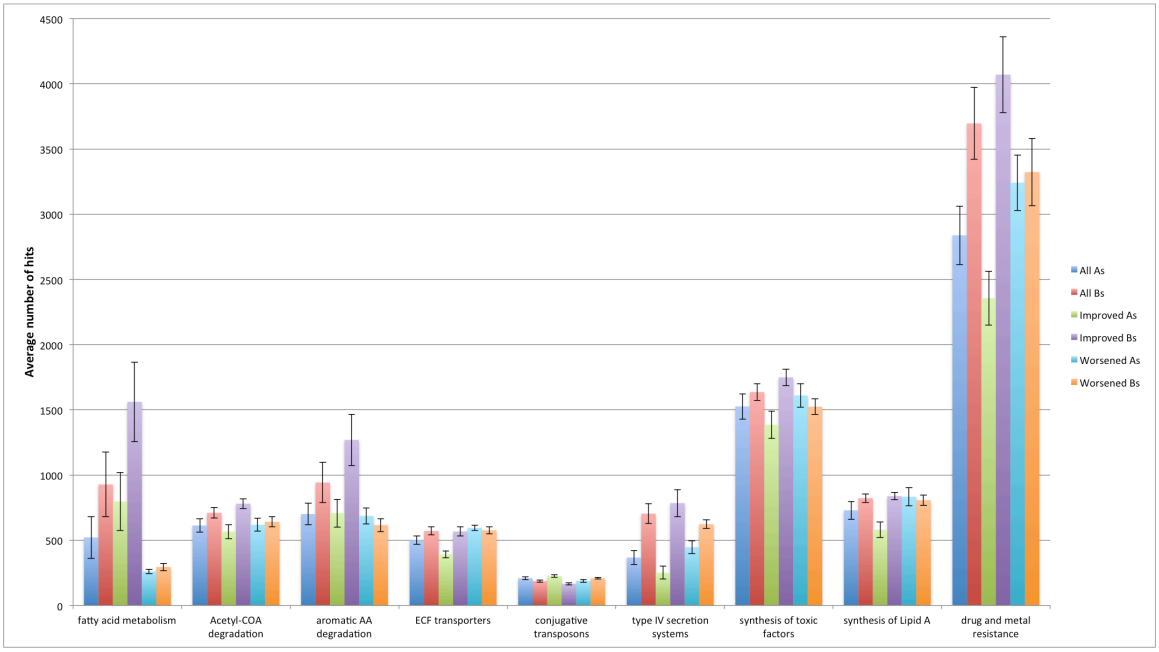

Figure 3.4: Number of hits for virulence gene categories in samples. Samples are grouped by categories: all As (before treatment) in blue, all Bs (after treatment) in red, all As that were classified as improved in green, all Bs that were classified as improved in purple, all As that were classified as worsened in teal and all Bs that were classified as worsened in orange. The Y-axis is the average number of hits for each category. The standard error of the mean is noted in the error bars.

REFERENCES

Badersten, A., R. Nilvéus, et al. (1981). "Effect of nonsurgical periodontal therapy." Journal of Clinical Periodontology **8**(1): 57-72.

Barnes, V. M., R. Teles, et al. (2009). "Acceleration of Purine Degradation by Periodontal Diseases." Journal of Dental Research **88**(9): 851-855.

Belda-Ferre, P., L. D. Alcaraz, et al. (2011). "The oral metagenome in health and disease." The ISME journal **6**(1): 46-56.

Campan, P., P.-O. Planchand, et al. (1997). "Pilot study on n-3 polyunsaturated fatty acids in the treatment of human experimental gingivitis." Journal of Clinical Periodontology **24**(12): 907-913.

Chen, T., W.-H. Yu, et al. (2010). "The Human Oral Microbiome Database: a web accessible resource for investigating oral microbe taxonomic and genomic information." Database: the journal of biological databases and curation **2010**.

Cicek, Y., I. Ozmen, et al. (2005). "Content and composition of fatty acids in normal and inflamed gingival tissues." Prostaglandins, Leukotrienes and Essential Fatty Acids **72**(3): 147-151.

Cobb, C. M. (2002). "Clinical significance of non-surgical periodontal therapy: an evidence-based perspective of scaling and root planing." Journal of Clinical Periodontology **29**: 22-32.

de Lillo, A., F. P. Ashley, et al. (2006). "Novel subgingival bacterial phylotypes detected using multiple universal polymerase chain reaction primer sets." Oral Microbiol Immunol **21**(1): 61-68.

Dentino, A., S. Lee, et al. (2013). "Principles of periodontology." Periodontology 2000 **61**(1): 16-53.

Keegan, K. P., W. L. Trimble, et al. (2012). "A platform-independent method for detecting errors in metagenomic sequencing data: DRISEE." PLoS computational biology **8**(6): e1002541.

Kesavalu, L., V. Bakthavatchalu, et al. (2007). "Omega-3 fatty acid regulates inflammatory cytokine/mediator messenger RNA expression in Porphyromonas gingivalis-induced experimental periodontal disease." Oral Microbiology and Immunology **22**(4): 232-239.

Kuramitsu, H. K., X. He, et al. (2007). "Interspecies interactions within oral microbial communities." Microbiology and Molecular Biology Reviews **71**(4): 653-670.

Ley, R. E., D. A. Peterson, et al. (2006). "Ecological and evolutionary forces shaping microbial diversity in the human intestine." Cell **124**(4): 837-848.

Liu, B., L. L. Faller, et al. (2012). "Deep sequencing of the oral microbiome reveals signatures of periodontal disease." Plos One **7**(6).

Methe, B. A., K. E. Nelson, et al. (2012). "A framework for human microbiome research." Nature **486**(7402): 215-221.

Meyer, F., D. Paarmann, et al. (2008). "The metagenomics RAST server - a public resource for the automatic phylogenetic and functional analysis of metagenomes." BMC Bioinformatics **9**: 386.

Pike, R., V. Lucas, et al. (2002). "Prevalence and antibiotic resistance profile of mercury-resistant oral bacteria from children with and without mercury amalgam fillings." Journal of Antimicrobial Chemotherapy **49**(5): 777-783.

Rosenstein, E. D., L. J. Kushner, et al. (2003). "Pilot study of dietary fatty acid supplementation in the treatment of adult periodontitis." Prostaglandins, Leukotrienes and Essential Fatty Acids **68**(3): 213-218.

Segata, N., L. Waldron, et al. (2012). "Metagenomic microbial community profiling using unique clade-specific marker genes." Nature Methods.

Skrepcinski, F. B. and W. J. Niendorff (2000). "Periodontal Disease in American Indians and Alaska Natives." Journal of Public Health Dentistry **60**: 261-266.

Team, R. C. (2008). "R: A language and environment for statistical computing." R Foundation Statistical Computing, Vienna, Austria.

Turnbaugh, P. J., R. E. Ley, et al. (2007). "The human microbiome project." Nature **449**(7164): 804-810.

Venter, J. C., K. Remington, et al. (2004). "Environmental genome shotgun sequencing of the Sargasso Sea." science **304**(5667): 66-74.

CONCLUSION OF THE DISSERTATION

The oral cavity is densely populated with microbial communities that often aggregate into biofilms (Socransky and Haffajee 1994; Lepp, Brinig et al. 2004; Sabeti and Slots 2004; Kolenbrander, Palmer et al. 2006; Paster, Olsen et al. 2006; Naglik, Tang et al. 2013). Our oral health can be greatly affected by these communities and the diseases that they create, such as dental caries (Ling, Kong et al. 2010), periodontal disease (Moore and Moore 1994; Pihlstrom, Michalowicz et al. 2005; Paster, Olsen et al. 2006; Wang, Qi et al. 2013) and endodontic lesions (Abou-Rass and Bogen 1998; Li, Hsiao et al. 2010; Ribeiro, Matarazzo et al. 2011; Saber, Schwarzberg et al. 2012). Recent research has also implicated these microbial communities with systemic diseases, such as certain types of cancer (Soder, Yakob et al. 2011; Ahn, Chen et al. 2012; Farrell, Zhang et al. 2012), rheumatoid arthritis (Detert, Pischon et al. 2010), cardiovascular disease (Gaetti-Jardim, Marcelino et al. 2009; Koren, Spor et al. 2011; das Merces Batista, Rosetti et al. 2012; Lockhart, Bolger et al. 2012; Yakob, Meurman et al. 2012), ulcerative colitis (Kojima, Nakano et al. 2012) and more. A better understanding of the microbial inhabitants in the oral cavity and how their composition changes can have serious implications for human health. If we know the culprits of certain diseases and the best methods to reduce their prevalence, we could potentially lower the chances of these diseases, some of which are life-threatening.

Endodontic lesions are infections (Eriksen 1991) that originate in infected pulp tissue in the root canal of the tooth and progress into an infected lesion in the upper or lower jawbone (Kakehashi, Stanley et al. 1965; Seltzer and Farber

1994). Until recently, there was a commonly held belief that these lesions do not contain bacteria or that they are sparsely populated (Andreasen and Rud 1972; Langeland, Conn et al. 1977). This led us to investigate the surgically removed lesions using fluorescence microscopy to visually verify the presence of microorganisms. Using SYBR Gold, we were able to detect single- or double-stranded DNA or RNA, allowing identification of bacterial cells and virus-like particles based on cell size (Figure 1.1).

Our recovery rate of bacterial DNA from the lesions was 54% - 7 of 11 symptomatic lesions, but not from 4 symptomatic and 2 asymptomatic lesions. The 6 lesions that did not yield bacterial DNA may not have contained bacteria or, less likely, were the result of a failure to sample bacteria-positive areas within the lesions or technical problems with the PCR/pyrosequencing procedures. Our findings are consistent with other studies that used different methods but encountered similar recovery rates (Sunde, Olsen et al. 2003; Desai, Love et al. 2011).

This study demonstrated that most bacteria found in the surgically removed lesions belonged to the genera *Fusobacterium*, *Prevotella, Porphyromonas*, *Streptococcus* and *Actinomyces* (Figure 1.2). Increased insights into the microbiota of endodontic infections will enhance our understanding of endodontic diseases. This will hopefully lead to improved endodontic treatment regimens based on better understanding of the microbial communities that cause these infections and inflammation in the bone. Repeating this study with a greater sample size could broaden our understanding of the taxonomic

composition of these lesions. However, more interesting information may be found in a metagenomics study of endodontic lesions. The key to the problem may lie in the microorganisms that cannot be amplified using common PCR primers.

Periodontal disease is a polymicrobial inflammation of the periodontium that can lead to edentulism (tooth loss) from loss of connective tissue and bone support (Pihlstrom, Michalowicz et al. 2005). Over 700 species of bacteria have been identified in the human mouth (Paster, Olsen et al. 2006), including periodontal pathogens such as *Porphyromonas gingivalis, Treponema denticola,* and *Tannerella forsythia,* whose integration into the biofilm on teeth is due to the prior addition of *Fusobacterium nucleatum* (Kolenbrander, Palmer et al. 2006). Many previous studies focused on defining the typical microbiome in health and disease (Ximenez-Fyvie, Haffajee et al. 2000; Kumar, Griffen et al. 2005; Griffen, Beall et al. 2012; Liu, Faller et al. 2012; Abusleme, Dupuy et al. 2013; Wang, Qi et al. 2013). These studies were often limited in sample size and did not examine the effects of treatment on these communities, which has critical implications in cases of non-responders. A better understanding of the factors that change in responders and non-responders could change the approach used with non-responders. A different treatment regimen could address the microorganisms that need to be dealt with differently in non-responders.

In our study, we identified 87 bacterial genera belonging to 12 different divisions, the majority of which were common members of periodontal pocket microbiota. Contrary to what we expected to find, we did not uncover clear

differences between samples collected prior to treatment with those collected post-treatment, even after accounting for the treatment effectiveness. Interestingly, post-treatment samples remained most similar to pre-treatment samples from the same individual (Figure 2.1).

Prior studies found certain bacteria associated with health (*Streptococcus*, *Veillonella*) and disease (*Fusobacterium*, *Prevotella* and *Leptotrichia*) (Griffen, Beall et al. 2012; Liu, Faller et al. 2012; Abusleme, Dupuy et al. 2013). We sought to delve deeper into these bacteria to see their behavior in our samples, and found only *Fusobacterium* to be significantly correlated with pocket depth over all samples (Figure 2.2a). We found an inverse correlation between the abundance of *Fusobacterium* and *Streptococcus* (data not shown) and between *Streptococcus* and *Prevotella* (Figure 2.2b). This finding was expected: bacteria associated with disease were inversely correlated with bacteria associated with health. Our deeper phylogenetic analyses allowed us to see that these correlations were primarily driven by the negative correlation between *Streptococcus* and *P. loescheii* (Figure 2.2c). *Fusobacterium*, especially *F. nucleatum*, plays a key role in periodontal biofilm development by bridging early and late colonizers, according to the successional integration theory that explains the dynamics of the periodontal biofilm (Kolenbrander, Palmer et al. 2006). According to this theory, *Streptococcus* species establish the biofilm and *P. loescheii* attaches directly to *Streptococcus*, unlike the other *Prevotella* species. The roles played by these bacterial genera may make them particularly

responsive to biofilm disturbance, making them useful indicators of the efficacy of periodontal treatment.

We observed consistent changes associated with recovery from periodontal disease (e.g., a decrease in *Prevotella* abundance). However, the "healthy" amount of *Prevotella* differs on an individual basis. In addition, we found that the flora of some individuals changed contrary to the prevailing trends, notably in the *Fusobacterium* and *Prevotella*. *Streptococcus* remained steady or slightly increased in patients that improved after treatment, except in the case of two individuals who experienced dramatic declines post-treatment (Figure 2.3c). We expected to see an increase in *Veillonella* in improving individuals post-treatment since it is associated with periodontal health, however we did not observe this expected increase (Figure 2.3d). Tracking the changes post-treatment for a longer time period may lead to a better understanding of the behavior of *Veillonella* in the periodontal pocket.

In order to better understand the biofilm response, we analyzed the *Prevotella* and *Fusobacterium* OTUs from our study to acquire more species-specific knowledge. *Prevotella* were successfully differentiated in a phylogenetic tree that clustered by species (Figure 2.6). When examining specific *Prevotella* species, we found the abundance of *P. melaninogenica* and *P. loescheii* changed in opposite directions, while other *Prevotella* showed highly variable response post-treatment (Figure 2.4). A closer examination of *Fusobacterium* diversity also provided intriguing insight into periodontal biofilms. OTU clustering and phylogenetic analysis determined as many as 73 different species (Figure 2.5;

Figure 2.7). Only four of these OTUs were abundant across all of our samples, and only two were found in every sample (Figure 2.5). This supports recent findings that the core human microbiome in unrelated individuals tends to be minimal at lower taxonomic levels (Faith, Guruge et al. 2013). These less frequently found species might increase the overall host immune response and metabolic activity of the biofilm. This could be interpreted as bacteria in the biofilm who contribute little to actual biofilm stability and rely on the other bacterial components of the biofilm for nutrients, but may alter the biofilm immunogenicity.

In the past, it was common to focus on the presence or absence of the bacteria that comprise a group known as the “red complex” (*Porphyromonas gingivalis, Tannerella forsythia* and *Treponema denticola*), which were implicated in samples from diseased patients (Socransky, Haffajee et al. 1998). However, it is clear from recent studies that culturing and emphasis on specific bacteria will not capture all the variability in the diseased periodontium (Griffen, Beall et al. 2012; Liu, Faller et al. 2012; Abusleme, Dupuy et al. 2013). Combination antibiotic therapy is frequently employed to cover a broader spectrum of pathogens (Slots 2012). This leads us to question the choice and use of antibiotics in treatment of periodontal disease due to the variability of bacteria found in different diseased patients and the varied susceptibility of bacteria to different kinds of antibiotics. In addition, as we enter an era of awareness of the production of antibiotic-resistant pathogens, the practice of systemic antibiotic

therapy in periodontics should be questioned. Misuse of unnecessary antibiotics could lead to disease that is harder to treat and eradicate.

Our results highlight the importance of understanding the personal oral microbiome, a goal achievable by collecting and analyzing pre- and post-treatment samples. Furthermore, they lead us to believe that there is not a single composition that represents a healthy periodontal state or a single composition that represents a diseased periodontal state. Recovery from periodontal disease appears to reflect a shift from a personalized disease state to a personalized healthy state. While there is consensus that particular communities should shift with response to disease, there may not be consistent levels of these bacteria across individuals, both before treatment and after treatment. Further research with a larger patient sample size and more sampling over a longer time period will be necessary to confirm these hypotheses. This proposed study would help gather more information about the predictive value of specific disease-related species such as *Prevotella* and *Fusobacterium*.

In addition to the study design we used, a study that examines the different microbial communities in different locations of the mouth. Bacterial communities in periodontal pockets in different teeth may change based on factors such as exposure to different temperatures or proximity to salivary glands.

Another worthwhile study would involve comparing our results and sequences to those from the human oral microbiome database, which is a

database of oral bacterial species identified by 16S rRNA research that is curated by experts in the field (Chen, Yu et al. 2010).

Studies based on 16S rRNA give us a significant amount of information on the microbial community in a given sample, but the bias introduced by 16S cannot be overlooked. It has been shown that using metagenomics approaches instead of PCR studies bypasses biases introduced into the estimates of microbial diversity (de Lillo, Ashley et al. 2006). Using a genomic approach through whole-genome shotgun sequencing allows for better overall environment characterization and an understanding of complex microbial interactions in environmental samples (Venter, Remington et al. 2004).

Few studies have used metagenomics approaches to investigate diseases in the mouth, such as dental caries and periodontal pocket microbial communities (Belda-Ferre, Alcaraz et al. 2011; Liu, Faller et al. 2012). The study focusing on periodontal pockets by Liu *et al.* investigated the microbial communities in health and disease; however, they had a limited number of samples for each disease class (3 healthy controls and 2 periodontal patients) (Liu, Faller et al. 2012). In addition to microbial diversity, metagenomics studies are able to provide some information on the metabolic profiles of the microbial communities since they are not based on only one gene.

Our whole-genome shotgun sequencing study investigated the metagenomes from 12 periodontal patients (a subset of the 16S rRNA study) to determine how standard periodontal treatment, scaling and root planing and oral

hygiene instruction altered the polymicrobial diversity and metabolic profiles in periodontal pockets.

Whole-genome shotgun sequencing produces very large datasets that are difficult to navigate. There still is not a consensus regarding analysis tools for interpreting the data in the most effective way with minimal biases and maximum information gleaned from the massive dataset. We used two relatively popular tools for analysis – MG-RAST (Meyer, Paarmann et al. 2008) and MetaPhlAn (Segata, Waldron et al. 2012), which provide data about taxonomy and metabolic information from the gene sequences supplied. These tools use reference databases to identify the sequences, thus causing two potential biases: the reads are very short which could cause inaccurate matches in the databases, and there may not be sequences in the database that match novel genes in the samples. However, all analysis tools introduce some bias, and the field of metagenomics is advancing rapidly, thus adding to the information we have in these reference databases and increasing the accuracy of their results.

Comparing our 16S rRNA results to the top 25 genera identified in the samples by Illumina sequencing (Figure 3.1) yielded similar results. Interestingly, 19 of the genera were identical. The genera that were present in the top 25 genera identified by 16S that were not in the metagenomic top 25 were: *Tannerella, Granulicatella,* TM7, *Actinomyces, Gemella and Olsonella*. All of these genera were identified in the Illumina samples at a lower ranking than the top 25. Of the 6 genera that were identified as the top 25 by MetaPhlAn that were different than the top 25 from 16S, 4 were present in lower rankings in the 16S

data: *Cardiobacterium, Aggregatibacter, Peptoniphilus* and *Rothia.* Only two were not present in the 16S data at all: *Haemophilus* and *Eikenella*. However, it is worth noting that based on the Human Oral Microbiome Database (Chen, Yu et al. 2010) these two genera have been identified previously in the oral cavity.

Metagenomes contain information not available from 16S rRNA gene sequencing since it does not rely on only one gene: sequences matching functional gene categories (Figure 3.2), including metabolic genes. Our samples here did not group in any way based on the hits to known functional gene categories, as seen in Figure 3.3. There is a large cluster that includes most of the samples, 19 out of the 24. The 5 samples that did not cluster as strongly warrant further investigation to better understand what makes them so different.

Certain functional gene categories are especially interesting in the case of periodontal disease, which is characterized by inflammation caused by bacteria (Dentino, Lee et al. 2013). In the study by Liu *et al.,* the authors identified enrichment in the periodontal pocket samples for virulence factors such as biosynthesis of toxic factors (such as acetone) and Lipid-A of the lipopolysaccharide (LPS). In addition, they found that the microbiome was adapted to a parasitic lifestyle, for example by degrading host tissue and other bacterial cells for their own benefit, by being enriched for aromatic amino acid degradation, fatty acid metabolism, energy-coupling factor (ECF) class transporters and resistance to drugs and metals. This builds upon other studies that have found similar findings in non-metagenomic methods. For example, Pike *et al.* found that mercury-resistant bacteria were present in children with and

without amalgam fillings and that many of the organisms they identified were also resistant to antibiotics (Pike, Lucas et al. 2002). Many studies have investigated the connection between fatty acids and gingival tissues, and have even linked an increase in unsaturated fatty acids to inflamed periodontal tissues (Campan, Planchand et al. 1997; Rosenstein, Kushner et al. 2003; Cicek, Ozmen et al. 2005; Kesavalu, Bakthavatchalu et al. 2007).

In our samples, we saw trends including an increase in all the virulence factors after treatment. This could indicate that more time was needed after perturbation of the periodontal microbiome to see long-term effects of the treatment. Some of our findings were the opposite of what we had expected to see. We saw a decrease in virulence factors hits in patients that responded well to treatment, such as in the case of conjugative transposons. A surprising finding was the significant increase in type IV secretion system hits found in samples after treatment, both in improved and in worsened individuals. This could also highlight the need to re-investigate the microbial communities at a later time after the perturbation caused by the periodontal treatment, giving the oral biofilms time to reach a new steady state.

The data in these 24 metagenome files is immense and could be studied more in depth. It would be worthwhile to conduct a follow up study investigating the differences in data interpretation when using additional metagenomics tools and comparing these files to the human oral microbiome data in greater depth.

Aside from information from the sequences that match known genes, such as metabolic gene hits, there is still information that cannot be inferred from

metagenome sequencing. Proteomics and metabolomics studies would be a worth follow-up to this study as well. These approaches would be able to gather information about what genes are actually being expressed in the samples at different time points and in relation to different treatments. Presence of the genes in the organisms does not mean that they are actively being expressed. It would be worthwhile to see if those genes are expressed, at what level and at what time point before or after treatment. This would truly give us a more complete picture of the biofilm metabolic activity, which could explain the severity of disease in some cases. It could also explain why some patients do not improve or in fact worsen even though they are being treated in the same way that people who respond well are being treated. If there is an increase in virulence in the biofilm due to a shift of the bacteria in a way that is not conducive to healing, it would warrant a different approach, potentially based on the pattern of virulence observed.

Another bioinformatics method worth exploring is the creation of genome assemblies from these data files. These assemblies could identify novel genera and species not yet identified in the human periodontal pocket. This method complements the work done in this study, as it does not rely on matching short reads to known sequences in databases that might not contain all of the genes found in the samples. This would eliminate that bias, but add in the bias of the tools used to create the assemblies.

All three studies in this body of work investigated polymicrobial communities in the human oral cavity that are associated with specific diseases –

endodontic infections and periodontal disease. Modern disease etiology no longer relies on one microorganism as the root cause of problems in the human body, but recognizes that often it is a community that causes the imbalance and change that characterize the disease. Illness in the human body might not be isolated only to the region where it is directly observed, especially when the cause of illness is microbial. It is possible that local imbalance in an area of the body indicates that there is a systemic imbalance, microbial or otherwise. As the field of human microbiomics develops, it is anticipated that many of our questions regarding human health and disease will be answered. Most likely these answers will confirm what the current data suggests: it’s all in the microbes.

REFERENCES

Abou-Rass, M. and G. Bogen (1998). "Microorganisms in closed periapical lesions." International endodontic journal **31**(1): 39-47.

Abusleme, L., A. K. Dupuy, et al. (2013). "The subgingival microbiome in health and periodontitis and its relationship with community biomass and inflammation." ISME J. 2013 Jan 10. doi: 10.1038/ismej.2012.174.(1751-7370 (Electronic)): T - aheadofprint.

Ahn, J., C. Y. Chen, et al. (2012). "Oral microbiome and oral and gastrointestinal cancer risk." Cancer Causes & Control **23**(3): 399-404.

Andreasen, J. and J. Rud (1972). "A histobacteriologic study of dental and periapical structures after endodontic surgery." International journal of oral surgery **1**(5): 272-281.

Belda-Ferre, P., L. D. Alcaraz, et al. (2011). "The oral metagenome in health and disease." The ISME journal **6**(1): 46-56.

Campan, P., P.-O. Planchand, et al. (1997). "Pilot study on n-3 polyunsaturated fatty acids in the treatment of human experimental gingivitis." Journal of Clinical Periodontology **24**(12): 907-913.

Chen, T., W.-H. Yu, et al. (2010). "The Human Oral Microbiome Database: a web accessible resource for investigating oral microbe taxonomic and genomic information." Database: the journal of biological databases and curation **2010**.

Cicek, Y., I. Ozmen, et al. (2005). "Content and composition of fatty acids in normal and inflamed gingival tissues." Prostaglandins, Leukotrienes and Essential Fatty Acids **72**(3): 147-151.

das Merces Batista, R., E. P. Rosetti, et al. (2012). "Association between periodontal disease and subclinical atherosclerosis: the ELSA-Brasil study." Cad. Saude Publica **28**(5): 965-976.

de Lillo, A., F. P. Ashley, et al. (2006). "Novel subgingival bacterial phylotypes detected using multiple universal polymerase chain reaction primer sets." Oral Microbiol Immunol **21**(1): 61-68.

Dentino, A., S. Lee, et al. (2013). "Principles of periodontology." Periodontology 2000 **61**(1): 16-53.

Desai, S., R. Love, et al. (2011). "Toll-like receptor 2 expression in refractory periapical lesions." International endodontic journal **44**(10): 907-916.

Detert, J., N. Pischon, et al. (2010). "The association between rheumatoid arthritis and periodontal disease." Arthritis Research and Therapy **12**(5): 218.

Eriksen, H. M. (1991). "Endodontology: epidemiologic considerations." Dental Traumatology **7**(5): 189-195.

Faith, J. J., J. L. Guruge, et al. (2013). "The Long-Term Stability of the Human Gut Microbiota." Science **341**(6141).

Farrell, J. J., L. Zhang, et al. (2012). "Variations of oral microbiota are associated with pancreatic diseases including pancreatic cancer." Gut **61**(4): 582-588.

Gaetti-Jardim, E., S. L. Marcelino, et al. (2009). "Quantitative detection of periodontopathic bacteria in atherosclerotic plaques from coronary arteries." Journal of medical microbiology **58**(12): 1568-1575.

Griffen, A. L., C. J. Beall, et al. (2012). "Distinct and complex bacterial profiles in human periodontitis and health revealed by 16S pyrosequencing." Isme Journal **6**(6): 1176-1185.

Kakehashi, S., H. Stanley, et al. (1965). "The effects of surgical exposures of dental pulps in germ-free and conventional laboratory rats." Oral Surgery, Oral Medicine, Oral Pathology **20**(3): 340-349.

Kesavalu, L., V. Bakthavatchalu, et al. (2007). "Omega-3 fatty acid regulates inflammatory cytokine/mediator messenger RNA expression in Porphyromonas gingivalis-induced experimental periodontal disease." Oral Microbiology and Immunology **22**(4): 232-239.

Kojima, A., K. Nakano, et al. (2012). "Infection of specific strains of Streptococcus mutans, oral bacteria, confers a risk of ulcerative colitis." Scientific reports **2**.

Kolenbrander, P. E., R. J. Palmer, Jr., et al. (2006). "Bacterial interactions and successions during plaque development." Periodontology 2000 **42**: 47-79.

Koren, O., A. Spor, et al. (2011). "Human oral, gut, and plaque microbiota in patients with atherosclerosis." Proceedings of the National Academy of Sciences **108**(Supplement 1): 4592-4598.

Kumar, P. S., A. L. Griffen, et al. (2005). "Identification of candidate periodontal pathogens and beneficial species by quantitative 16S clonal analysis." Journal of clinical microbiology **43**(8): 3944-3955.

Langeland, K., F. Conn, et al. (1977). "A histopathologic and histobacteriologic study of 35 periapical endodontic surgical specimens." Journal of endodontics **3**(1): 8-23.

Lepp, P. W., M. M. Brinig, et al. (2004). "Methanogenic Archaea and human periodontal disease." Proceedings of the National Academy of Sciences of the United States of America **101**(16): 6176-6181.

Li, L., W. Hsiao, et al. (2010). "Analyzing endodontic infections by deep coverage pyrosequencing." Journal of dental research **89**(9): 980-984.

Ling, Z., J. Kong, et al. (2010). "Analysis of oral microbiota in children with dental caries by PCR-DGGE and barcoded pyrosequencing." Microbial ecology **60**(3): 677-690.

Liu, B., L. L. Faller, et al. (2012). "Deep sequencing of the oral microbiome reveals signatures of periodontal disease." Plos One **7**(6).

Lockhart, P. B., A. F. Bolger, et al. (2012). "Periodontal disease and atherosclerotic vascular disease: does the evidence support an independent association? A scientific statement from the American Heart Association." Circulation **125**(20): 2520-2544.

Meyer, F., D. Paarmann, et al. (2008). "The metagenomics RAST server - a public resource for the automatic phylogenetic and functional analysis of metagenomes." BMC Bioinformatics **9**: 386.

Moore, W. E. C. and L. V. H. Moore (1994). "The bacteria of periodontal diseases." Periodontology 2000 **5**(1): 66-77.

Naglik, J. R., S. X. Tang, et al. (2013). "Oral Colonization of Fungi." Current Fungal Infection Reports: 1-8.

Paster, B. J., I. Olsen, et al. (2006). "The breadth of bacterial diversity in the human periodontal pocket and other oral sites." Periodontology 2000 **42**: 80-87.

Pihlstrom, B. L., B. S. Michalowicz, et al. (2005). "Periodontal diseases." Lancet **366**(9499): 1809-1820.

Pike, R., V. Lucas, et al. (2002). "Prevalence and antibiotic resistance profile of mercury-resistant oral bacteria from children with and without mercury amalgam fillings." Journal of Antimicrobial Chemotherapy **49**(5): 777-783.

Ribeiro, A. C., F. Matarazzo, et al. (2011). "Exploring bacterial diversity of endodontic microbiota by cloning and sequencing 16S rRNA." Journal of endodontics **37**(7): 922-926.

Rosenstein, E. D., L. J. Kushner, et al. (2003). "Pilot study of dietary fatty acid supplementation in the treatment of adult periodontitis." Prostaglandins, Leukotrienes and Essential Fatty Acids **68**(3): 213-218.

Saber, M. H., K. Schwarzberg, et al. (2012). "Bacterial flora of dental periradicular lesions analyzed by the 454-pyrosequencing technology." Journal of Endodontics **38**(11): 1484-1488.

Sabeti, M. and J. Slots (2004). "Herpesviral-bacterial coinfection in periapical pathosis." Journal of endodontics **30**(2): 69-72.

Segata, N., L. Waldron, et al. (2012). "Metagenomic microbial community profiling using unique clade-specific marker genes." Nature Methods.

Seltzer, S. and P. A. Farber (1994). "Microbiologic factors in endodontology." Oral Surgery, Oral Medicine, Oral Pathology **78**(5): 634-645.

Slots, J. (2012). "Low-cost periodontal therapy." Periodontology 2000 **60**(1): 110-137.

Socransky, S., A. Haffajee, et al. (1998). "Microbial complexes in subgingival plaque." Journal of Clinical Periodontology **25**(2): 134-144.

Socransky, S. S. and A. D. Haffajee (1994). "Evidence of bacterial etiology: a historical perspective." Periodontology 2000 **5**(1): 7-25.

Soder, B., M. Yakob, et al. (2011). "Periodontal disease may associate with breast cancer." Breast cancer research and treatment **127**(2): 497-502.

Sunde, P. T., I. Olsen, et al. (2003). "Fluorescence in situ hybridization (FISH) for direct visualization of bacteria in periapical lesions of asymptomatic root-filled teeth." Microbiology **149**(5): 1095-1102.

Venter, J. C., K. Remington, et al. (2004). "Environmental genome shotgun sequencing of the Sargasso Sea." science **304**(5667): 66-74.

Wang, J., J. Qi, et al. (2013). "Metagenomic sequencing reveals microbiota and its functional potential associated with periodontal disease." Scientific reports **3**.

Ximenez-Fyvie, L. A., A. D. Haffajee, et al. (2000). "Comparison of the microbiota of supra- and subgingival plaque in health and periodontitis." Journal of Clinical Periodontology **27**(9): 648-657.

Yakob, M., J. H. Meurman, et al. (2012). "C-reactive protein in relation to early atherosclerosis and periodontitis." Clinical oral investigations **16**(1): 259-265.