EVALUATION OF THE ROOT CANAL MICROBIOME IN PRIMARY ENDODONTIC INFECTIONS AND ASSOCIATED PATIENT-RELATED FACTORS

A THESIS
SUBMITTED TO THE FACULTY OF THE UNIVERSITY OF MINNESOTA
BY

DAVID R. SCHUWEILER

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
MASTER OF SCIENCE

DR. RONALD ORDINOLA-ZAPATA

DR. W. CRAIG NOBLETT

DR. LARRY F. WOLFF

AUGUST 2022

ACKNOWLEDGEMENTS

- Dr. Ronald Ordinola-Zapata of the University of Minnesota School of Dentistry Division of Endodontics for unconditional support, encouragement, education, and mentorship.
- Dr. W. Craig Noblett of the University of Minnesota School of Dentistry Division of Endodontics for my endodontic education, assistance with thesis revisions and defense.
- Dr. Scott B. McClanahan of the University of Minnesota School of Dentistry Division of Endodontics for being integral in my endodontic journey and education.
- Christopher Staley PhD and Matthew Dietz PhD student for Bioinformatic analysis.
- Ruoqiong Chen for laboratory assistance with DNA extraction.
- The mentorship and support of Dr. Thomas Karn, Dr. Carolina Rodriguez-Figueroa, Dr. Omar Abusteit, Dr. Jeffrey Ryan, Dr. Scott Doyle, Dr. Dan Ang, Dr. Robert Kaufman, Dr. Ramon Aguirre.
- Dr. Jim Spitzmueller, Dr. R. Bruce Templeton, Dr. Aram Mazhary, Dr. Brian Barsness for encouraging me to pursue my ambition of endodontics.
- The clinical support of Ling Hui Anderson, Lisa Loven, Karen Demonakos.
- My amazing co-residents: Dr. Mallory Hackbarth, Dr. Meghan Montoya, Dr. Ki Hong Park.
- Class of 2021: Dr. Ashley Asano, Dr. Jonas Dale, Dr. Michael Pham, Dr. Lisa Kandella.
- Research reported in this publication was supported by the National Center for Advancing Translational Sciences of the National Institutes of Health Award Number UL1-TR002494

DEDICATION

I dedicate this thesis to my wife Elizabeth for her unconditional love, sacrifice, and encouragement throughout my educational endeavors. I would also like to dedicate this thesis to my loving parents Stephen and JoAnn for their wholehearted support and encouragement throughout my life. The support of my wife and parents has truly made my journey an achievable reality. I am truly blessed and forever grateful.

DISCLAIMER

The opinions or assertions contained herein are those of the author(s) and are not to be construed as official or reflecting the view of the University of Minnesota.

ABSTRACT

PURPOSE: The aim of this study was to characterize the microbiome of primary endodontic infections using 16S ribosomal RNA next-generation sequencing (NGS) technology and evaluate for differences among various demographic and radiographic factors.

MATERIALS AND METHODS: 71 human participants with primary endodontic infections were evaluated for percussion tenderness, sinus tract presence, active caries, gender, probing depth >4mm, and age. Pre-operative radiographic lesion size was assessed with periapical index (PAI) as either PAI ≤ 2 (n = 12) or PAI = 5 (n = 19). Samples from the root canal were obtained and subsequent microbiome data was constructed. Differences in abundance of genera were evaluated using the Kruskal-Wallis test. Alpha and beta-diversity indices were calculated using Mothur. The Shannon and Chao1 indices were used to measure alpha diversity. Differences in community composition were evaluated using analysis of similarity (ANOSIM) with Bonferroni correction for multiple comparisons.

RESULTS: No significant differences in microbiome relative to clinical factors were observed. A significant difference in beta-diversity was noted between PAI 2 and 5. Larger radiographic lesions demonstrated significant increase in *Oribacterium*, *Phocaeicola*, *Lachnospiraceae uncl*, *Prevotellaceae*, *Selenomonadaceae uncl*., *Treponema*, and *Olsenella.*

CONCLUSION: No correlations were found between the microbial composition and the studied clinical factors associated with apical periodontitis. Differences in community composition were found in teeth with large radiolucencies.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

The human body is host to a vast and complex diversity of microbial organisms. Diversity and composition of microbial species is known to have a significant role in human nutrition, health, and development of disease (Durack & Lynch, 2019). Investigation into the diversity, abundance, and composition of the human microbiome has been a considerable topic of interest with the goal of understanding disease progression, risk factors, and potential therapeutic strategies (Mimee et al. 2016). The use of microscopy, staining, and culturing techniques have paved the way for understanding the bacteria that are present in humans. Unfortunately, these techniques fall short of identifying all species present in any given sample.

Microscopic evaluation and staining can only provide limited phenotypic information and culturing techniques are not always feasible for environmentally sensitive species. Having entered the era of molecular techniques, the ability to recognize, identify, and quantify bacteria in situ has expanded our understanding exponentially. Modern molecular sequencing techniques have allowed for identification of uncultivable and cultivable bacterial species. Coupled with The Human Microbiome Project (HMP) which has developed data bases of cataloged bacterial genetic profiles, sample sequences can be compared, identified, and quantified with relative ease.

Previous studies have attempted to correlate bacterial species to the specific characteristics of the endodontic infection. Culturing techniques have associated *Prevotella, Peptostreptococcus,* and *Eubacterium* with pain and swelling (Gomes et al., 1996). Polymerase chain reaction (PCR) studies have previously linked *P. endodontalis*,

T. denticola, and P. gingivalis to acute periradicular abscesses (Siqueira et al., 2001). Reverse transcriptase-polymerase chain reaction and reverse-capture checkerboard assays have linked *O. uli, P. acnes,* and *F.* nucleatum to be risk factors in endodontic infections (Rocas & Siqueira, 2010). Many of these studies relied on techniques that could not identify all species, lacked controls, or had samples sizes lacking adequate power.

Sequences and subsequent taxonomic profiles acquired through 16S ribosomal RNA combined with comparison to microbial genomes from the human body has proven to be a reliable and accurate method of sample analysis without prior knowledge of what a sample contains. A variety of sequences and taxonomic profiles can be studied simultaneously to understand the profile of a whole community rather than one or so species at a time. It is important to note that the sequences and taxonomic profiles generated during analysis provide only a snapshot in time of the dynamic ecology of the microbiome sampled. However, such snapshots can be used to construct inferences or correlations between other variables such as disease process, symptomatology, or even a healthy environment. The technology relies on sequencing a specific marker portion of the genome which is typically 16S ribosomal RNA.

The 16S ribosomal RNA is amplified with polymerase chain reaction (PCR). These 16S ribosomal RNA gene sequences contain hyper-variable regions (HVR) that provide signatures that are bacterial species specific. Because the sequences can be very diverse, operational taxonomic units (OTU) are utilized to categorize closely related groups of sequences which can allow for data to be organized into meaningful groupings that can be referenced with the established databases. Stratifying 16S ribosomal RNA data into OTUs permits the understanding of the genus level microbiome present in any given sample.

It is of relevance to note that data derived from 16S ribosomal RNA sequencing can yield information regarding microbial abundance, species richness, and ultimately diversity. Abundance refers to the total number of microorganisms detected, whereas richness refers to variety of species present. Utilizing the data from both abundance and richness allows for computation of species diversity present in a sample. Diversity represents the total population number and the magnitude of each individual species present.

The purpose of this study is to characterize the composition of a primary endodontic infection and associated clinical and radiographic characteristics utilizing next generation sequencing 16S ribosomal RNA sequencing. Furthermore, analysis of sample sizes of adequate power to draw significant conclusions relating to demographic, clinical, and radiographic factors.

BACKGROUND

EVALUATING SAMPLING DATA

The Shannon Index allows a value assignment to quantify species diversity by utilizing the abundance and richness of species detected. Higher Shannon Index values indicate greater population diversity. Chao Index is a statistical method to predict the number of species in a population given a finite sample. In essence, the Shannon Index and Chao Index allow for statistically evaluating sample diversity and comparing different communities to one another.

Additional ways of evaluating species are calculating alpha-diversity and beta-diversity indexes. The alpha-diversity index is a means of demonstrating the richness or diversity present in any given sample; what species are present and how many species are present in a community. Beta-diversity index provides a method to identify the types of species present and how it compares to other populations; how do different populations contrast in diversity relative to one another. Beta-diversity focuses on the extent of microbiome dissimilarity of a sample compared to other populations. Therefore, alpha and beta-diversity analysis provides information about microbiome assemblage which can then be compared with another population, respectively.

Understanding which microbiomes are of healthy composition and which are dystopian are also of importance in understanding their impact on host health. While alpha and beta-diversity provides information about microbiome composition, additional efforts have been undertaken to reveal which species may be drivers that modulate the bacterial community and influence host response. Such modulatory species are described as Keystone pathogens, or 'alpha-bugs' that can have a disproportionate influence on the community to which they belong (Hajishengallis et al., 2016).

KEYSTONE SPECIES CONCEPT

There may also be bacterial species that exploit a disrupted homeostasis that can flourish and further contribute to a dystopian bacterial community; these species may be referred to as Pathobionts. These pathobionts may be indigenous to the environment and generally benign until they are introduced into an environment that is not under a normal

and healthy homeostasis. It is important to note that these influential keystone species may not always be present as the majority of the population.

Keystone species may not directly be the cause of a disease process but may be considered as an environmental stabilizer. In other words, they provide stability for other microbial species to flourish that cause disease in the host. Such keystone species may undermine the effectiveness of a host's immune system, only to subsequently allow pathobionts to flourish and cause an overreaction of the host immune response.

By having the 16S ribosomal RNA technology paired with the HMP database, a multitude of human tissues can be studied in relation to their microbial composition. Of particular interest in this study is a more in-depth understanding of the microbiome associated with pulp necrosis and apical periodontitis. Further understanding may elucidate and confirm the main causes of pathogenesis, symptoms, and disease progression. In addition, greater understanding may promote better therapeutics and understanding of prognosis when evaluating and treating pulp necrosis and apical periodontitis.

BACTERIA IN ROOT CANAL INFECTIONS AND APICAL PERIODONTITIS

Studies dating back to the late 1800s had identified the presence of microorganisms in necrotic human pulp using staining and microscopy (Miller 1884). In the 1950s teeth demonstrating periapical pathosis were shown to have similar bacteria intra and extra-radicular using culturing techniques (Hedman 1951). It was not until 1965 that Kakehashi et al. (1965) published their landmark study demonstrating a direct causal relationship of bacteria causing pulp and periapical disease in an in-vivo animal study.

Understanding which bacterial species contribute to the disease process had inherent limitations in historical studies. Very early studies relying on visual identification with staining lacked the ability to identify the diversity of bacteria on a genus and species level. Furthermore, culturing has been shown to be very technique sensitive with several bacteria being uncultivable. The stringent requirements for culturing were exemplified by Melville and Birch in 1967 whereby bacteria were obtained and cultured utilizing anaerobic and aerobic environments. Because of the demanding conditions required for culturing bacteria of the oral cavity, it has been estimated that over 50% of oral bacteria are uncultivable (Paster et al., 2001).

Although fungi, archaea, and viruses have been implicated, the largest body of evidence suggests bacteria are primarily responsible for apical periodontitis (Siqueira & Rocas 2009, Moller et al., 1981, Lin et al., 2006). Bacteria appear to be the primary source of pulp and periapical disease; studies have shown a positive correlation between duration and quantity of microorganisms influencing the degree of disease (Korzen et al., 1974). Under normal healthy conditions, the pulpodentin complex is an environment devoid of viable bacteria, in essence a sterile environment. It is not until the overlying cementum, dentin, or enamel is breached that pathosis begins to take place (Love & Jenkinson, 2002). A breach of the pulp space to the external environment could include caries, periodontal disease, or trauma. In some instances, the phenomenon of anachoresis has been implicated (Robinson & Boling, 1941), although this theory is controversial.

Primary infections appear to represent more mixed gram-positive and gram negative anaerobes, whereas secondary infections are more characterized by mostly gram-positive anaerobes (Gomes 2004). Prominent species presenting in primary endodontic

infections have been shown to include *E. corodens, V. parvula* (Rocas & Siqueira 2006), *Treponema* species (Leite et al., 2015), and P. gingivalis (Siqueira et al., 2008). On the other hand, considerable literature has noted that secondary infections primarily consist of gram positive facultative anaerobes, specifically *E. faecalis* (Gomes et al. 2004, Sundqvist et al. 1998). It had been suggested that persistent primary bacteria that continue to cause pathosis after endodontic treatment may be species that have a tendency to be more alkaline, which may include *Streptococcus, E. faecalis, E. cancerogenus,* and *F. nucleatum* (Lew et al., 2015).

With the advent of molecular techniques, identification of bacterial species present in samples has increased significantly compared to previous investigations (Rolph et al., 2001). Over 460 bacterial taxa have been identified and associated with endodontic infection (Siquera & Rocas 2009). Historically, primary apical periodontitis was significantly more diverse relative to secondary apical periodontitis (Sundqvist et al., 1998). Molecular techniques have demonstrated that secondary infections may in fact have significantly more diversity than previously thought (Hong et al., 2013). Molecular techniques have allowed the discovery of unculturable species, but the contribution and dominance of such species has been a topic in need of elucidation (Nair 2007). Furthermore, additional research is needed regarding the interplay of species within the root canal space (Ozok et al., 2012). The environment of the endodontic space provides a distinctly different environment from the oral cavity which may include an abundance of necrotic tissue and differences in oxygen gradients which may favor different taxa compositions (Ozok et al., 2012). Because several bacterial species are present and function as communities that are functionally organized with surface-associated biofilms, apical

periodontitis is now considered a biofilm-mediated disease (Siqueira & Rocas 2009, Ricucci & Siqueira 2010, Chavez de Paz 2007).

Bacteria must be present in sufficient numbers, have access to the periradicular tissues and have an array of virulence factors that result in periapical disease (Siqueira 2002). By utilizing Next Generation Sequencing (NGS) 16S rRNA technology it is expected that predominant bacteria present may illuminate key bacteria responsible for periapical pathosis progression, while simultaneously affirming previous culturing and molecular studies. NGS technology allows for a correlation of demographic data along with microbiota profiles providing an avenue in understanding the pathogenesis of apical periodontitis and its symptoms. Elucidating the role that bacteria play in endodontic disease is essential for guiding treatment (Haapasalo et al., 2003), predicting outcome, and understanding why some bacterial profiles may have a greater impact than others in different individuals (Provenzano et al., 2016).

Current molecular techniques are not without their drawbacks, however. One major shortcoming of DNA molecular techniques is the inability to discriminate between live and dead microorganisms; dead and damaged cells can be easily amplified and incorporated into assessments (Ozok et al., 2012). While 16S ribosomal RNA sequencing improves upon DNA sequencing methods and is incredibly sensitive, there also exists the possibility that some amplified bacteria are not actively contributing to pulp and periapical disease. In fact, the bacteria detected with 16S ribosomal RNA sequencing may have been involved in disease progression or may have been an insignificant bystander.

Because 16S ribosomal RNA sequencing does not consider metabolic function or bacterial community contribution, it may be considered more speculative to the given

snapshot in time that the sample was collected. In addition, while 16S ribosomal RNA sequencing is accurate in detecting the genus level of bacteria, it is unable to detect species level taxonomy. Such sequencing techniques can assist in confirmation of previous microscopic, culture, and molecular research to further understand the pathologic progression of pulp and periapical disease, but limitations still exist.

SPECIFIC AIMS

The purpose of this study is to characterize the microbiome of primary endodontic infections. A secondary objective is to evaluate the effect of demographic and radiographic factors on the root canal microbiome composition.

HYPOTHESIS

1) Null hypothesis: There is no significant difference in the microbiome composition between the demographic factors of age, gender, pre-operative pain presence/absence, sinus tract presence/absence, pre-operative caries presence/absence, or radiographic lesion size.

   General research hypothesis: There are significant differences in microbiome composition with respect to various demographic and clinical factors such as age, gender, presence of pain, sinus tract presence, or radiographic lesion size.

MATERIALS AND METHODS

The study protocol was approved by this institutional board of the University of Minnesota (IRB protocol # 00011937).

POWER ANALYSIS

A power calculation was done to determine the sample size necessary to distinguish differences between binary variables measured (taken as observed abundance of genera). For this purpose, the HMP package in R software (la Rosa et al., 2012) was used. A sample size of 10 per variable was established to find differences in genus-level composition among groups at $\alpha = 0.05$.

PATIENT SELECTION

Seventy-one patients were enrolled in this study. Inclusion criteria included the following:

1. Age of patient, older than 18 years.
2. Patient diagnosed with pulp necrosis and apical periodontitis confirmed by cold testing and radiographic evaluation.
3. Pulp and periapical disease must be a primary endodontic infection.
4. Teeth were considered to have favorable restorative prognosis at time of treatment.

Exclusion criteria were the following:

1. Patients who were currently pregnant.
2. Inability to sign the informed consent.
3. Teeth with extensive coronal tooth structure loss that compromised aseptic sampling of the root canal space.

The following clinical variables were recorded during clinical examination: gender, age, mechanical allodynia, presence of sinus tract, periodontal disease, and presence of frank caries. Patient's gender was recorded as either male or female. Patients were recorded as either adults or older adults, with older patients being considered ≥65 years of age according to the Centers for Disease Control guidelines. Mechanical allodynia was evaluated by striking a blunt metallic instrument (mouth mirror) on the affected tooth. Teeth responding with mechanical allodynia relative to control teeth were recorded as sensitive. Intra-oral sinus tracts were recorded as either being present or absent based upon intraoral clinical examination at time of treatment. Periodontal disease was determined by placing a metallic periodontal probe into the sulcus and recording the distance from the free gingival margin to the base of the sulcus at six points around the tooth. Periodontal status was assessed as either being present or absent of periodontal disease. Presence of periodontal disease was classified as a probe measurement greater than or equal to 4mm according to the Community Periodontal Index for Treatment Needs (CPITN). Primary or recurrent caries observed clinically or by radiographic examination prior to endodontic treatment, were noted as either present or absent.

RADIOGRAPHIC ASSESSMENT

Preoperative periapical radiographs were obtained of teeth in need of endodontic treatment that qualified for the study using CareStream RVG6200 digital sensors and CareStream imaging software. The severity of apical pathosis was evaluated using the Periapical Index score (PAI) proposed by Orstavik et al., (1986). According to the PAI, a score of 1 indicates normal periapical tissues, 2 indicates small changes in bone structure with no demineralization, 3 indicates changes in bone structure with some diffuse mineral loss, 4 indicates apical periodontitis with well-defined radiolucent area, 5 indicates severe apical periodontitis with exacerbating features and bone expansion. Due to the known limitations of bidimensional periapical images, only teeth that were scored with a PAI of either 2 (small changes, N=12) or 5 (apical periodontitis with exacerbating features, N=19) were included in the radiographic analysis.

SAMPLING PROCEDURE

Teeth with primary endodontic infections selected for sampling were first isolated with a non-latex rubber dam. Following caries excavation, the tooth and rubber dam field was scrubbed with sterile cotton saturated with 3% hydrogen peroxide for 1 minute. Then, the tooth and rubber dam field were scrubbed with sterile cotton saturated with 5.25% sodium hypochlorite. The sodium hypochlorite was then inactivated by scrubbing the surfaces with sterile cotton saturated with sodium thiosulfate. A surface control was then obtained by sampling the tooth surface with a sterile cotton pellet, and immediate

placement of the cotton pellet into a sterile Eppendorf tube with 1mL of tris-HCl. To ensure decontamination, random samples of the specimen surface (N=20) were taken and processed using qPCR analysis. The root canal space was then sampled by instrumenting to the middle-apical third of the root canal using a sterile Vortex Blue 30.04 rotary file in a sterile handpiece. The rotary file with debris in flutes was then removed from the handpiece using sterile instruments and placed in a sterile Eppendorf tube with 1mL of tris-HCl. The Eppendorf tubes with surface controls and root canal samples were placed in -80C until processing. To decrease the influence of anatomy, the largest root with a single canal was sampled in molars, specifically the palatal root of maxillary molars and distal root of mandibular molars.

DNA EXTRACTION AND SEQUENCING ANALYSIS

The DNeasy PowerLyzer PowerSoil Kit (Qiagen, Hilden, Germany) was used to recover bacterial DNA from the samples. Clinical samples were processed by the University of Minnesota genomic center for qPCR quality control and sequencing analysis. For characterization of bacteria in the root canal, the V3 and V4 hypervariable regions of the 16S rRNA gene were amplified and sequenced using the S-D.Bact-0341.b-S-17/S-D-Bact-0785-a-A-21 primer set (Klindworth et al., 2013). Pair-end sequencing was completed by the University of Minnesota Genomics Center at a read length of 301 nucleotides (nt) on the Illumina MiSeq platform using their previously described dual-index method (Gohl et al., 2016). Negative control (blank) samples were included in each run.

AMPLICON PROCESSING AND ANALYSIS

Amplicon Processing and Analysis was completed by a specialist in bioinformatics. Sequence data were processed using Mothur ver. 1.41.1. (Schloss et al., 2009), 16S rRNA amplicons were first trimmed to 250 nt and pair-end joined using fastq-join software (Aronesty 2013). Quality trimming at a threshold of 35 was then performed with a sliding window of 50 nt. Sequences with homopolymers of >6 nt, ambiguous bases, or >2 mismatches from primer sequences were also removed. High-quality sequences were aligned against the SILVA database ver. 138, chimeras were removed using UCHIME ver. 4.2.40 (Edgar et al., 2011) and sequencing errors were removed using a 2% pre-clustering step (Huse et al., 2010). Operational taxonomic units (OTUs) were binned at 99% similarity using the furthest-neighbor algorithm and classified against the version 18 database from the Ribosomal Database Project. Different databases were used for alignment and classification due to processing considerations described previously (Schloss et al., 2011).

STATISTICAL ANALYSIS

Differences in genera abundance were evaluated using a Kruskal-Wallis test in XLSTAT ver. 2020.2.3 (Addinsoft; Belmont, MA, USA). Alpha diversity indices were calculated in Mothur. The Shannon (Shannon & Weaver 1949) and Chao1 (Chao 1984) indices were employed for this analysis. The Bray-Curtis dissimilarity (Bray & Curtis 1957) was used to measure beta (between-sample) diversity and was visualized by ordination using principal coordinate analysis (Anderson & Willis 2003) Analyses of similarity (ANOSIM) to determine differences in community composition (gender, age,

percussion sensitivity, probing depth presence of sinus tract, and caries) were done in Mothur, with Bonferroni correction for multiple comparisons (Clarke 2003).

RESULTS

Sixty-five out of 71 total samples yielded a successful PCR amplification of V3 and V4 16S rRNA gene sequences. Six samples were excluded from final analysis because of low quality DNA, paucity of reads after sequencing, or obvious sample contamination as determined by qPCR. Control samples gave on average <10 copies/µl for non-surgical samples. All teeth sampled had pulp necrosis as demonstrated by lack of bleeding from the pulp chamber or canal space upon access and instrumentation. Demographic characteristics of the sampled population including age, gender, caries, presence of percussion pain, presence of sinus tract, and periodontal probing greater than 4mm can be found in Table 1**.**

The total number of OTUs obtained was 5,937. The average number of OTUs identified in samples was 143.6 with a standard deviation of 94.44. The number of reads, Shannon and Chao1 alpha diversity values can be found in **(Table 2 and 3)**. The most common dominant taxa observed among all samples (>3%) of relative abundance were predominantly classified to of nine microbial genera including *Parvimonas, Fusobacterium, Prevotella, Eubacterium, Peptostreptococcus, Arachnia, Sphingomonadaceae, Fretibacterirum, and Campylobacter* **(Figure 1 and 7)**. Only 1 non-oral bacteria genera was identified in the samples (*Sphingomonadaceae*). Among all samples, gender, age (adult vs older adult), percussion sensitivity, sinus tract presence, caries, and probing depths >4mm showed no significant effects on community composition via an ANOSIM **(See Table 4)**. However, community composition was significantly

different depending on whether the sample had a PAI score of 2 or 5 (R = 0.104, *P* = 0.039). **(See Figure 2 - 5)**. Additional statistical analysis was performed to know if the presence of *Sphingomonadaceae* affected the results. The data revealed that there was no change in data significance **(See Table 4)**.

Samples with small radiographic lesions (PAI-2) contained a significantly different community composition (Kruskal-Wallis analysis). The bacterial taxa *Oribacterium* (P = 0.018), *Phocaeicola* (P = 0.020), *Lachnospiracrae uncl.* (P = 0.022), *Prevotellaceae uncl.* (P = 0.027), *Selenomonadaceae uncl.* (P = 0.027), *Treponema* (P = 0.030), and *Olsenella* (P = 0.039) were shown to be significantly increased in samples with larger radiographic lesion size **(See Figure 6)**. Samples with small radiographic lesions had significantly higher levels of *Cutibacterium* (P = 0.014) and *Lacticaseibacilus* (P = 0.019).

DISCUSSION

Understanding the bacterial composition associated with pulpal necrosis and apical periodontitis has evolved significantly since the advent of open-end molecular techniques. The diversity of species known to exist has increased exponentially. Few studies have focused on clinical findings and demographic factors that coincide with pulp necrosis and periapical disease, let alone have sample sizes of enough magnitude to allow for data of significant power. In many cases the number of patients studied was equal or less than 15 (Li et al., 2010; Santos et al., 2011; Siqueira et al., 2010; Hsiao et al., 2012; Siqueira et al., 2016; Zandi et al., 2018; de Brito et al., 2020; Nardello et al., 2020), or between 18 and 23 (Ozok et al., 2012; Hong et al., 2013; Gomes et al., 2015; Iriboz et al., 2018).

The findings of this study identified several anaerobic bacteria in the root canal space of teeth demonstrating pulp necrosis and apical periodontitis. Data revealed that bacteria of the oral cavity are present in the root canal space, although their distributions and population compositions are distinctly different (Costalonga & Herzberg, 2014). Previous studies have noted relatively higher amounts of *Prevotella, Streptococcus* and *Veillonella* in plaque and saliva (Costalonga & Herzberg 2014). In addition, periodontal pockets appear to present a higher abundance of *Tannerella, Porphyromonas* and *Treponema* species (Lenartova 2021). Differences in microbiome composition in the root canal space compared to other oral niches may be explained by the anaerobic environment, lack of replenishable nutrients, presence of necrotic pulp tissue, and accumulation of bacterial byproducts.

The notion that apical periodontitis can correspond to a myriad of different bacterial species compositions has been suggested in the literature (Amaral et al., 2022). In fact, because endodontic infections largely seem to arise from oral bacteria, it would seem reasonable that apical periodontitis may take on oral bacterial compositions that are shaped by diet, genetics, habits, and systemic conditions (Xu et al. 2015, Mason et al. 2013, Belstrom et al. 2014). The findings reported in this study suggest that when ecological parameters are used the bacterial composition is not really affected by several clinical parameters such as gender, age, caries or periodontal disease. Furthermore, the microbial composition did not affect important diagnostic descriptors of apical periodontitis such as percussion tenderness or presence of sinus tract. It can be assumed that the root canal space produces environmental pressures to reliably and consistently select for a few oral bacteria; at least those oral bacteria in abundances greater than 3%.

Some of the main genera found in this study (*Parvimonas, Peptostreptococcus and Eubacterirum*) belong to the order of *Clostridiales*. Of note, *Clostridiales* produce butyrate, hydrogen and carbon dioxide by anaerobic fermentation leading to an acidic environment (Liu et al., 2005). Similarly, microbial composition of the root canal space may be influenced by proteolytic bacteria that produce sulfur and aromatic compounds in their metabolic process. Through methionine and cysteine metabolism, *Prevotella* has been noted to produce toxic sulfur compounds (Foo et al., 2021) which can limit the growth of other bacteria and cause human tissue damage. It seems reasonable that while the root canal space shares bacterial species with other areas of the oral cavity, the endodontic environment would provide pressures that would select for different bacterial proportions.

Sphingomonadaceae was detected in high proportions in 4 samples. Previous studies have shown *Sphingomonadaceae* species to be a prevalent contaminant in dental water unit systems (Singh et al., 2003). It could be suspected that the samples exhibiting high proportions of *Sphingomonadaveae* could be attributed to contamination during the sampling or processing protocol, and thus these results should be considered outliers in the data set. However, upon statistical analysis excluding *Sphingomonadaceae*, there was no change in data significance.

While it seems that different bacterial compositions may result in different clinical findings, it was interesting to note in this study that the analysis of clinical factors resulted in no significant difference in microbiome composition. However, radiographic lesion size was demonstrated to have significant differences in microbiome composition. Bacterial taxa including *Oribacterium*, *Phocaeicola*, *Lachnospiraceae uncl*, *Prevotellaceae*, *Selenomonadaceae*, *Treponema*, and *Olsenella* were all significantly elevated in samples demonstrating large radiographic lesion size. Interestingly, *Phocaeicola*, *Selenomonadaceae*, *Lachnospiraceae*, *Prevotellaceae*, and *Treponema* and have been positively associated with development of periodontal disease and bone loss (Costalonga & Herzberg 2014, Socransky et al., 1998, Chen et al., 2018). The findings of this study suggest there may be elevated host response and subsequent osteolysis associated with these bacterial pathogens.

Radiographic lesion size was demonstrated to have significant differences in microbiome composition. Bacterial taxa including *Oribacterium*, *Phocaeicola*, *Lachnospiraceae uncl*, *Prevotellaceae*, *Selenomonadaceae*, *Treponema*, and *Olsenella* were all significantly elevated in samples demonstrating large radiographic lesion size.

Interestingly, *Phocaeicola, Selenomonadaceae*, *Lachnospiraceae*, *Prevotellaceae*, and *Treponema* and have been positively associated with development of periodontal disease and bone loss (Costalonga & Herzberg 2014, Socransky et al., 1998, Chen et al., 2018). The *Treponema* phylotypes generally show a stronger association with disease (Costalonga & Herzberg 2014).

Treponema, *Oribacterium*, *Phocaeicola*, and *Selenomonadae* species share the characteristic of motility (Ng et al., 2016, Sizova et al. 2014, Roux et al., 2013, Kingsley & Hoeniger, 1973). In addition, it has also been demonstrated that bacterial flagella contain the protein flagellin that is responsible for Toll-Like Receptor 5 activation and immune response (Hayashi et al., 2001, Yang & Yan., 2017). *Treponema* species have also been noted to attach to laminin, a major basement membrane protein, thus lending itself to another avenue of virulence and host disease (Haapasalo et al., 1991). Furthermore, *Treponema* has been noted to demonstrate virulence factors such as toxin-antitoxin, transposases, outer sheath proteins, dentilisin, trypsin-like protease activity, and outer sheath vesicles (Dashper et al., 2011).

In murine models, *Treponema* infection has been shown to elicit host humoral immune activity by increasing serum immunoglobulin G levels and antigenic specificity, but interestingly these responses were not capable of resolving *Treponema* infection (Kesavalu et al. 1999). Spirochetes, such as *Treponema*, have developed specialized mechanisms to evade the complement system that contribute to their survival in a host (Barbosa and Isaac 2018). In addition, *Treponema* species have been shown to contain conserved regulatory genes such as LytTR DNA binding domain that control critical transcriptional responses required for environmental adaptation (Miller et al. 2014).

Bacteria present in samples with small radiographic lesion size showed significant increases in *Cutibacterium*, a gram-positive bacterium of the skin, oral cavity, large intestine, conjunctiva, and external ear canal (Achermann et al., 2014). Interestingly, *Cutibacterium* has the ability to form biofilms and has been recognized as a pathogen that causes low-grade clinical symptoms (Mongaret et al., 2021) and rarely any increase in systemic biological markers of inflammation (Dramis et al., 2009).

Lacticaseibacilus is often isolated from intestinal, oral, and urogenital tracts. Of interest, this bacterial taxa has been extensively studied and has been implicated as a probiotic of therapeutic benefit for urogenital, gastro-intestinal, and cardiovascular health (Petrova et al., 2021). *Lacticaseibacilus* was noted in only one sample and represented more than 90% of the bacterial composition of that sample. Because that one sample was an outlier relative to the other samples, it may be speculated that the sample was contaminated.

Since larger periapical radiolucencies secondary to pulp necrosis represent an advanced disease process, it is relevant to note that more virulent bacterial taxa are present in significantly increased proportions. While it appears that increased *Oribacterium*, *Phocaeicola*, *Lachnospiraceae uncl*, *Prevotellaceae*, *Selenomonadaceae uncl*., *Treponema*, *Olsenella* lead to increased periapical rarefaction the finding can only be considered an association at this time. As previously mentioned, perhaps these bacterial taxa are actually pathobionts, with a keystone species lurking amongst them. It would be advantageous to further attempt to identify the keystone species which may help in developing a therapeutic protocol.

It could be hypothesized that the significant results derived from this study were merely due to random variability in individual samples. Although common sense may suggest that larger radiolucencies are indicative of longer standing disease, one would need to perform a longitudinal study to observe for bacterial composition shifts overtime; a study that seems virtually impossible. One limitation of this study was obtaining samples primarily from the main canal, potentially leaving apical bacteria, complex ramifications, and anatomical irregularities unsampled. A method to overcome this sampling bias would entail more invasive methods such as root end resection or extraction and cryopulverization methods (Alves et al., 2009). Regarding evaluation of radiographic lesion size, measurements could be more accurate with cone-beam computed tomography evaluation (Patel et al., 2019), especially considering that varying anatomic features may obstruct true radiographic radiolucent areas.

The findings of this study only represent a snapshot in time of the microbiome of endodontic disease. The bacterial community within the root canal space, like any environment, can vary based upon metabolic byproduct composition, upregulation, or downregulation of mediators during the disease process (Costalonga & Herzberg 2012). Overall, the hypothesis of this study was partially accepted with respect to radiographic lesion size having a significant influence on the composition of the endodontic microbiome in primary endodontic infections.

CONCLUSION

No correlations were found between the microbial composition and the studied clinical factors associated with apical periodontitis. Differences in community composition were found in teeth with large radiolucencies.

REFERENCES

Achermann Y, Goldstein EJ, Coenye T, Shirtliff ME. Propionibacterium acnes: from commensal to opportunistic biofilm-associated implant pathogen. Clin Microbiol Rev. 2014; 27: 419-40.

Alves FR, Siqueira JF Jr, Carmo FL, Santos AL, Peixoto RS, Rôças IN, Rosado AS. Bacterial community profiling of cryogenically ground samples from the apical and coronal root segments of teeth with apical periodontitis. J Endod. 2009; 35: 486-92.

Amaral RR, Braga T, Siqueira JF Jr, Rôças IN, da Costa Rachid CTC, Oliveira AGG, de Souza Côrtes MI, Love RM. Root Canal Microbiome Associated With Asymptomatic Apical Periodontitis as Determined by High-Throughput Sequencing. J Endod. 2022; 48: 487-495.

Anderson M, Willis T, Canonical Analysis of Principal Coordinates: A Useful Method of Constrained Ordination for Ecology. Ecology. 2003; 84: 511-25.

Aronesty E. Comparison of Sequencing Utility Programs. TOBIOIJ. 2013; 7: 1-8.

Barbosa AS, Isaac L. Complement Immune Evasion by Spirochetes. Curr. Top. Microbiol. Immunol. 2018; 415: 215–38.

Belstrøm D, Holmstrup P, Nielsen CH, Kirkby N, Twetman S, Heitmann BL, Klepac Ceraj V, Paster BJ, Fiehn NE. Bacterial profiles of saliva in relation to diet, lifestyle factors, and socioeconomic status. J Oral Microbiol. 2014; 6: 1-9.

Bray R, Curtis J. An Ordination of the Upland Forest Communities of Southern Wisconsin. Ecological Monographs. 1957; 27: 325–49.

Chao A. Nonparametric Estimation of the Number of Classes in a Population. Scand. J. Stat. 1984; 11: 265–70.

Chavez de Paz LE. Redefining the persistent infection in root canals: possible role of biofilm communities. J Endod. 2007; 33: 652-62.

Chen C, Hemme C, Beleno J, Shi ZJ, Ning D, Qin Y, Tu Q, Jorgensen M, He Z, Wu L, Zhou J. Oral microbiota of periodontal health and disease and their changes after nonsurgical periodontal therapy. ISME J. 2018; 12: 1210-1224.

Clarke KR. Non-Parametric Multivariate Analyses of Changes in Community Structure. Austral Ecol. 1993; 18: 117–43.

Costalonga M, Herzberg M. The Oral Microbiome and the Immunobiology of Periodontal Disease and Caries. Immunol Lett. 2014; 162: 22-38.

Cutress TW, Ainamo J, Sardo-Infirri J. The community periodontal index of treatment needs (CPITN) procedure for population groups and individuals. Int Dent J. 1987; 37: 222-33.

Dashper S, Seers CA, Tan KH, Reynolds EC. Virulence Factors of the Oral Spirochete Treponema Denticola. J. Dent. Res. 2011; 90: 691–703.

de Brito Luciana Carla Neves, Doolittle-Hall Janet, Lee Chun-Teh, et al. The apical root canal system microbial communities determined by next-generation sequencing. Sci Rep. 2020; 10: 10932.

Dramis A, Aldlyami E, Grimer RJ, Dunlop DJ, O'Connell N, Elliott T. What is the significance of a positive Propionibacterium acnes culture around a joint replacement? Int Orthop. 2009; 33: 829-33.

Durack J, Lynch S. The Gut Microbiome: Relationships with Disease and Opportunities for Therapy. J. Exp. Med. 2019; 216: 20–40.

Edgar RC, Haas BJ, Clemente JC, Quince C, Knight R. UCHIME improves sensitivity and speed of chimera detection. Bioinformatics. 2011; 27: 2194-200.

Foo LH, Balan P, Pang LM, Laine ML, Seneviratne CJ. Role of the oral microbiome, metabolic pathways, and novel diagnostic tools in intra-oral halitosis: a comprehensive update. Crit Rev Microbiol. 2021; 47: 359-375.

Gohl DM, Vangay P, Garbe J, MacLean A, Hauge A, Becker A, Gould TJ, Clayton JB, Johnson TJ, Hunter R, Knights D, Beckman KB. Systematic improvement of amplicon marker gene methods for increased accuracy in microbiome studies. Nat Biotechnol. 2016; 34: 942-9.

Gomes BP, Lilley JD, Drucker DB. Associations of endodontic symptoms and signs with particular combinations of specific bacteria. Int Endod J. 1996; 29: 69-75.

Gomes BP, Pinheiro ET, Gadê-Neto CR, Sousa EL, Ferraz CC, Zaia AA, Teixeira FB, Souza-Filho FJ. Microbiological examination of infected dental root canals. Oral Microbiol Immunol. 2004; 19: 71-6.

Gomes BP, Herrera DR. Etiologic role of root canal infection in apical periodontitis and its relationship with clinical symptomatology. Braz Oral Res. 2018; 32: e69.

Gomes BPFA, Francisco PA, Godoi EP Jr, Endo MS, Barbosa-Ribeiro M, Delboni MG, Pecorari VGA. Identification of Culturable and Nonculturable Microorganisms, Lipopolysaccharides, and Lipoteichoic Acids From Root Canals of Teeth With Endodontic Failure. J Endod. 2021; 47: 1075-1086.

Griffee MB, Patterson SS, Miller CH, Kafrawy AH, Newton CW. The relationship of Bacteroides melaninogenicus to symptoms associated with pulpal necrosis. Oral Surg Oral Med Oral Pathol. 1980; 50: 457-61.

Guerrero-Preston R, Godoy-Vitorino F, Jedlicka A, Rodríguez-Hilario A, González H, Bondy J, Lawson F, Folawiyo O, Michailidi C, Dziedzic A, Thangavel R, Hadar T, Noordhuis MG, Westra W, Koch W, Sidransky D. 16S rRNA amplicon sequencing identifies microbiota associated with oral cancer, human papilloma virus infection and surgical treatment. Oncotarget. 2016; 7: 51320-51334.

Haapasalo M, Singh U, McBride BC, Uitto VJ.. "Sulfhydryl-Dependent Attachment of Treponema Denticola to Laminin and Other Proteins." INFIBR. 1991; 59, 4230–37.

Haapasalo M, Udnæs T, Endal U. "Persistent, Recurrent, and Acquired Infection of the Root Canal System Post-Treatment." Endod Topics. 2003; 6: 29–56.

Hajishengallis G, Lamont RJ. Dancing with the Stars: How Choreographed Bacterial Interactions Dictate Nososymbiocity and Give Rise to Keystone Pathogens, Accessory Pathogens, and Pathobionts. Trends Microbiol. 2016; 24: 477-489.

Hayashi F, Smith KD, Ozinsky A, Hawn TR, Yi EC, Goodlett DR, Eng JK, Akira S, Underhill DM, Aderem A. The innate immune response to bacterial flagellin is mediated by Toll-like receptor 5. Nature. 2001; 410: 1099-103.

Hedman WJ. An investigation into residual periapical infection after pulp canal therapy. Oral Surg Oral Med Oral Pathol. 1951; 4: 1173-9.

Hong BY, Lee TK, Lim SM, Chang SW, Park J, Han SH, Zhu Q, Safavi KE, Fouad AF, Kum KY. Microbial analysis in primary and persistent endodontic infections by using pyrosequencing. J Endod. 2013; 39: 1136-40.

Hsiao William Wl, Li Kevin L., Liu Zhenqiu, Jones Cheron, Fraser-Liggett Claire M., Fouad Ashraf F. Microbial transformation from normal oral microbiota to acute endodontic infections. BMC Genomics. 2012; 13: 345.

Huse SM, Welch DM, Morrison HG, Sogin ML. Ironing out the wrinkles in the rare biosphere through improved OTU clustering. Environ Microbiol. 2010; 12: 1889-98.

Huttenhower, Curtis, et al. Human Microbiome Project Consortium. Structure, function and diversity of the healthy human microbiome. Nature. 2012; 486: 207-14.

İriboz E., Arıcan Öztürk B., Kolukırık M., Karacan I., Sazak Öveçoğlu H. Detection of the unknown components of the oral microflora of teeth with periapical radiolucencies in a Turkish population using next-generation sequencing techniques. Int Endod J. 2018; 51: 1349–57.

Kakehashi S, Stanley HR, Fitzgerald RI. The effects of surgical exposures of dental pulps in germ-free and conventional laboratory rats. Oral surg oral med oral pathol. 1965; 20: 340-9.

Kesavalu L, Holt S, Ebersole J. Lack of Humoral Immune Protection against Treponema Denticola Virulence in a Murine Model. Infect. Immun. 1999; 67: 5736–46.

Kingsley VV, Hoeniger JF. Growth, Structure, and Classification of Selenomonas. *Bacteriol. Rev*. 1973: 37; 479–521.

Klindworth A, Pruesse E, Schweer T, Peplies J, Quast C, Horn M, Glöckner FO. Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. Nucleic Acids Res. 2013; 41: 1-11.

Korzen BH, Krakow AA, Green DB. Pulpal and periapical tissue responses in conventional and monoinfected gnotobiotic rats. Oral Surg Oral Med Oral Pathol. 1974; 37: 783-802.

Leite FR, Nascimento GG, Demarco FF, Gomes BP, Pucci CR, Martinho FC. Prevalence of treponema species detected in endodontic infections: systematic review and meta-regression analysis. J Endod. 2015; 41: 579-87.

Lenartova M, Tesinska B, Janatova T, Hrebicek O, Mysak J, Janata J, Najmanova L. The Oral Microbiome in Periodontal Health. Front Cell Infect Microbiol. 2021; 11: 1-16.

Lew HP, Quah SY, Lui JN, Bergenholtz G, Hoon Yu VS, Tan KS. Isolation of alkaline tolerant bacteria from primary infected root canals. J Endod. 2015; 41: 451-6.

Li L, Hsiao W, Nandakumar R, Barbuto S, Mongodin E, Paster B, Fraser-Liggett C, Fouad A. Analyzing Endodontic Infections by Deep Coverage Pyrosequencing. J Dent Res. 2010; 89: 980–4.

Lin LM, Di Fiore PM, Lin J, Rosenberg PA. Histological study of periradicular tissue responses to uninfected and infected devitalized pulps in dogs. J Endod. 2006; 32: 34-8.

Liu X, Zhu Y, Yang ST. Butyric Acid and Hydrogen Production by Clostridium Tyrobutyricum ATCC 25755 and Mutants. Enzyme Microb. Technol.. 2006; 38: 521–28.

Love RM, Jenkinson HF. Invasion of dentinal tubules by oral bacteria. Crit Rev Oral Biol Med. 2002; 13: 171-83.

Mason MR, Nagaraja HN, Camerlengo T, Joshi V, Kumar PS. Deep sequencing identifies ethnicity-specific bacterial signatures in the oral microbiome. PLoS One. 2013; 8: 1-2.

Melville TH, Birch RH. Root Canal and Periapical Floras of Infected Teeth. Oral Surgery, Oral Medicine, and Oral Pathology. 1967; 23: 93-98.

Methé, Barbara A., et al. A Framework for Human Microbiome Research. Nature. 2012; 486: 215-21.

Miller D, Frederick J, Sarkar J, Marconi R. The Treponema Denticola AtcR LytTR Domain-Containing Response Regulator Interacts with Three Architecturally Distinct Promoter Elements: Implications for Understanding the Molecular Signaling Mechanisms That Drive the Progression of Periodontal Disease. Mol. Oral Microbiol. 2014; 29: 219–32.

Miller WD. The Agency of Acids in the Production of Caries of the Human Teeth, with Comparative Analysis of Carious Dentine and Dentine Softened by Acids. The Dental Cosmos. 1883; 25: 337-344.

Mimee M, Citorik R, Lu T. Microbiome Therapeutics – Advances and Challenges. Adv. Drug Deliv. Rev. 2016: 105; 44–54.

Möller AJ, Fabricius L, Dahlén G, Ohman AE, Heyden G. Influence on periapical tissues of indigenous oral bacteria and necrotic pulp tissue in monkeys. Scand J Dent Res. 1981; 89: 475-84.

Mongaret C, Velard F, Reffuveille F. Cutibacterium acnes: the Urgent Need To Identify Diagnosis Markers. Infect Immun. 2021; 89: e00753-20.

Nardello Laura C. L., Amado Pamela P. P., Franco Diego C., et al. Next-Generation Sequencing to Assess Potentially Active Bacteria in Endodontic Infections. J Endod. 2020; 46: 1105–12.

Nair PN. Abusing technology? Culture-difficult microbes and microbial remnants. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 2007; 104: 569-70.

Ng HM, Slakeski N, Butler C, Veith P, Chen YY, Liu SW, Hoffman B, Dashper S, Reynolds E. The Role of Treponema Denticola Motility in Synergistic Biofilm Formation With Porphyromonas Gingivalis. Front. Cell. Infect. Microbiol. 2019; 9: 1-15.

Ordinola-Zapata R, Mansour D, Saavedra F, Staley C, Chen R, Fok A. In Vitro Efficacy of a Non-Instrumentation Technique to Remove Intracanal Multispecies Biofilm. Int. Endod. J. 2022; 55; 495–504.

Orstavik D, Kerekes K, Eriksen HM. The periapical index: a scoring system for radiographic assessment of apical periodontitis. Endod Dent Traumatol. 1986; 2: 20-34.

Ozok AR, Persoon IF, Huse SM, Keijser BJ, Wesselink PR, Crielaard W, Zaura E. Ecology of the microbiome of the infected root canal system: a comparison between apical and coronal root segments. Int Endod J. 2012; 45: 530-41.

Paster BJ, Boches SK, Galvin JL, Ericson RE, Lau CN, Levanos VA, Sahasrabudhe A, Dewhirst FE. Bacterial diversity in human subgingival plaque. J Bacteriol. 2001; 183: 3770-83.

Patel S, Brown J, Pimentel T, Kelly RD, Abella F, Durack C. Cone beam computed tomography in Endodontics - a review of the literature. Int Endod J. 2019; 52: 1138-1152.

Provenzano JC, Antunes HS, Alves FR, Rôças IN, Alves WS, Silva MR, Siqueira JF Jr. Host-Bacterial Interactions in Post-treatment Apical Periodontitis: A Metaproteome Analysis. J Endod. 2016; 42: 880-5.

Ricucci D, Siqueira JF Jr. Fate of the tissue in lateral canals and apical ramifications in response to pathologic conditions and treatment procedures. J Endod. 2010; 36: 1-15.

Robinson BG, Boling LR. "The Anachoretic Effect in Pulpitis I. Bacteriologic Studies." JADA. 1941; 28: 268–82.

Rocas IN, Siqueira JF Jr. Culture-independent detection of Eikenella corrodens and Veillonella parvula in primary endodontic infections. J Endod. 2006; 32: 509-12.

Rocas IN, Siqueira JF Jr. Species-directed 16S rRNA gene nested PCR detection of Olsenella species in association with endodontic diseases. Lett Appl Microbiol. 2005; 41: 12-6.

Rocas IN, Siqueira JF. Identification of bacteria enduring endodontic treatment procedures by a combined reverse transcriptase-polymerase chain reaction and reverse-capture checkerboard approach. J Endod 2010;36:45-52.

Rolph HJ, Lennon A, Riggio MP, Saunders WP, MacKenzie D, Coldero L, Bagg J. Molecular identification of microorganisms from endodontic infections. J Clin Microbiol. 2001; 39: 3282-9.

Roux V, Robert C, Raoult D. Non-Contiguous Finished Genome Sequence of Phocaeicola Abscessus Type Strain 7401987T. SIGS. 2013; 9: 351–58.

Santarelli A, Mascitti M, Galeazzi R, Marziali A, Busco F, Procaccini M. Oral ulcer by Sphingomonas paucimobilis: first report. Int J Oral Maxillofac Surg. 2016; 45: 1280-2.

Santos Adriana L., Jr José F. Siqueira, Rôças Isabela N., Jesus Ederson C., Rosado Alexandre S., Tiedje James M. Comparing the Bacterial Diversity of Acute and Chronic Dental Root Canal Infections. PLOS ONE 2011; 6: e28088.

Shannon CE, Weaver W. The Mathematical Theory of Communication. University of Illinois Press, 1949.

Schloss PD, Westcott SL, Ryabin T, Hall JR, Hartmann M, Hollister EB, Lesniewski RA, Oakley BB, Parks DH, Robinson CJ, Sahl JW, Stres B, Thallinger GG, Van Horn DJ, Weber CF. Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. Appl Environ Microbiol. 2009; 75: 7537-41.

Singh R, Stine OC, Smith DL, Spitznagel J, Labib M, Williams H. Microbial Diversity of Biofilms in Dental Unit Water Systems. Appl. Environ. Microbiol. 2003; 69: 3412–20.

Siqueira JF, Rôças IN, Souto R, de Uzeda M, Colombo AP. Microbiological evaluation of acute periradicular abscesses by DNA-DNA hybridization. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 2001; 92: 451-7.

Siqueira JF Jr, Rocas IN. Diversity of endodontic microbiota revisited. J Dent Res. 2009; 88: 969-81.

Siqueira JF Jr. Endodontic infections: concepts, paradigms, and perspectives. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 2002; 94: 281-93.

Siqueira JF Jr, Rôças IN, Silva MG. Prevalence and clonal analysis of Porphyromonas gingivalis in primary endodontic infections. J Endod. 2008; 34:1332-1336.

Siqueira José F., Rôças Isabela N., Ricucci Domenico. Biofilms in endodontic infection. Endodontic Topics. 2010; 22: 33–49.

Siqueira José F., Antunes Henrique S., Rôças Isabela N., Rachid Caio T. C. C., Alves Flávio R. F. Microbiome in the Apical Root Canal System of Teeth with Post-Treatment Apical Periodontitis. PLoS One 2016; 11:e0162887.

Sizova M, Muller P, Stancyk D, Panikow N, Mandalakis M, Hazen A, Hohmann T, Doerfert S, Fowle W, Earl A, Selson K, Epstein S. Oribacterium Parvum Sp. Nov. and Oribacterium Asaccharolyticum Sp. Nov., Obligately Anaerobic Bacteria from the Human Oral Cavity, and Emended Description of the Genus Oribacterium. Int. J. Syst. Evol. 2014: 64; 2642–49.

Socransky SS, Haffajee AD, Cugini MA, Smith C, Kent RL Jr. Microbial complexes in subgingival plaque. J Clin Periodontol. 1998; 25: 134-44.

Sundqvist G, Figdor D, Persson S, Sjögren U. Microbiologic analysis of teeth with failed endodontic treatment and the outcome of conservative re-treatment. Oral Surg Oral Med Oral Pathol Oral Radiol Endod. 1998; 85: 86-93.

Wu S, Yu F, Ma L, Zhao Y, Zheng X, Li X, Li Z, He X, Zhou J. Do Maternal Microbes Shape Newborn Oral Microbes? Indian J Microbiol. 2021; 61: 16-23.

Xu X, He J, Xue J, Wang Y, Li K, Zhang K, Guo Q, Liu X, Zhou Y, Cheng L, Li M, Li Y, Li Y, Shi W, Zhou X. Oral cavity contains distinct niches with dynamic microbial communities. Environ Microbiol. 2015; 17: 699-710.

Yang J, Yan H. TLR5: beyond the recognition of flagellin. Cell Mol Immunol. 2017; 14: 1017-1019.

Zandi Homan, Kristoffersen Anne K., Ørstavik Dag, Rôças Isabela N., Siqueira José F., Enersen Morten. Microbial Analysis of Endodontic Infections in Root-filled Teeth with Apical Periodontitis before and after Irrigation Using Pyrosequencing. J Endod. 2018; 44: 372–8.

APPENDIX

Table 1. Demographic and clinical characteristics of the studied population.

Variable	Categories	Counts	%
Age	≥65 years	28	43.0
	<65	37	56.9
Gender	Female	30	46.2
	Male	35	53.8
Sinus Tract	Present	14	21.5
	Absent	51	78.5
Percussion	Non-sensitive	38	58.5
	Sensitive	27	41.5
Probing Depth	≥4mm	19	29.2
	<4mm	46	70.8
Caries	Present	32	49.2
	Absent	33	50.8

Table 2. Summary (mean ± standard deviation) of sequencing data obtained among all samples. Unnormalized data.

N	Coverage	No. reads	OTUs	Shannon	Chao 1
65	0.997 ± 0.003	36,560 ± 28,388	143.6 ± 94.4	2.14 ± 0.82	353.14 ± 258.5

Table 3. Periapical Index Score (PAI) and associated reads, OTUs, Coverage, Alpha Diversity, Shannon and Chao1 Index. Unnormalized data.

Group	**Reads**	**OTUs observed**	**Coverage**	**Shannon**	**Chao**
PAI ≤ 2	44510±30315	146.1±88.4	0.997±0.00	1.929±0.64	357.9±265.0
PAI = 5	32531±27386	134.5±92.3	0.997±0.00	2.203±0.64	339.4±288.5
p-value (one-way ANOVA)	*0.290*	*0.747*	*0.526*	*0.285*	*0.867*

Table 4. Analysis of Similarity (ANOSIM) was performed in all samples (N=71) and excluding the four samples that presented the non-oral genera *Sphingomonas* (N=67). Overall results were not affected by the presence of this possible contaminant. The Radiographic Periapical Index (PAI) was the only variable with a significant *P-value*. PAI score ≤ 2 is denoted as 0, whereas PAI score > 5 is denoted as 1. Samples with *Sphingomonas* were not present in the radiographic analysis. Unnormalized data.

Comparison	**R-value (N=71)**	**P-value**	**R-value (N=67)**	**P-value**
Gender	-0.026	0.934	-0.011	0.665
Older adult yes-no	0.043	0.072	0.017	0.191
Percussion yes-no	-0.049	0.970	-0.047	0.899
Periodontal probe	-0.063	0.827	-0.025	0.619
Restoration yes-no	0.003	0.351	0.029	0.064
Sinus tract	-0.070	0.774	-0.078	0.827
Rad. PAI 0-1	0.104	**0.039**	-------	-------

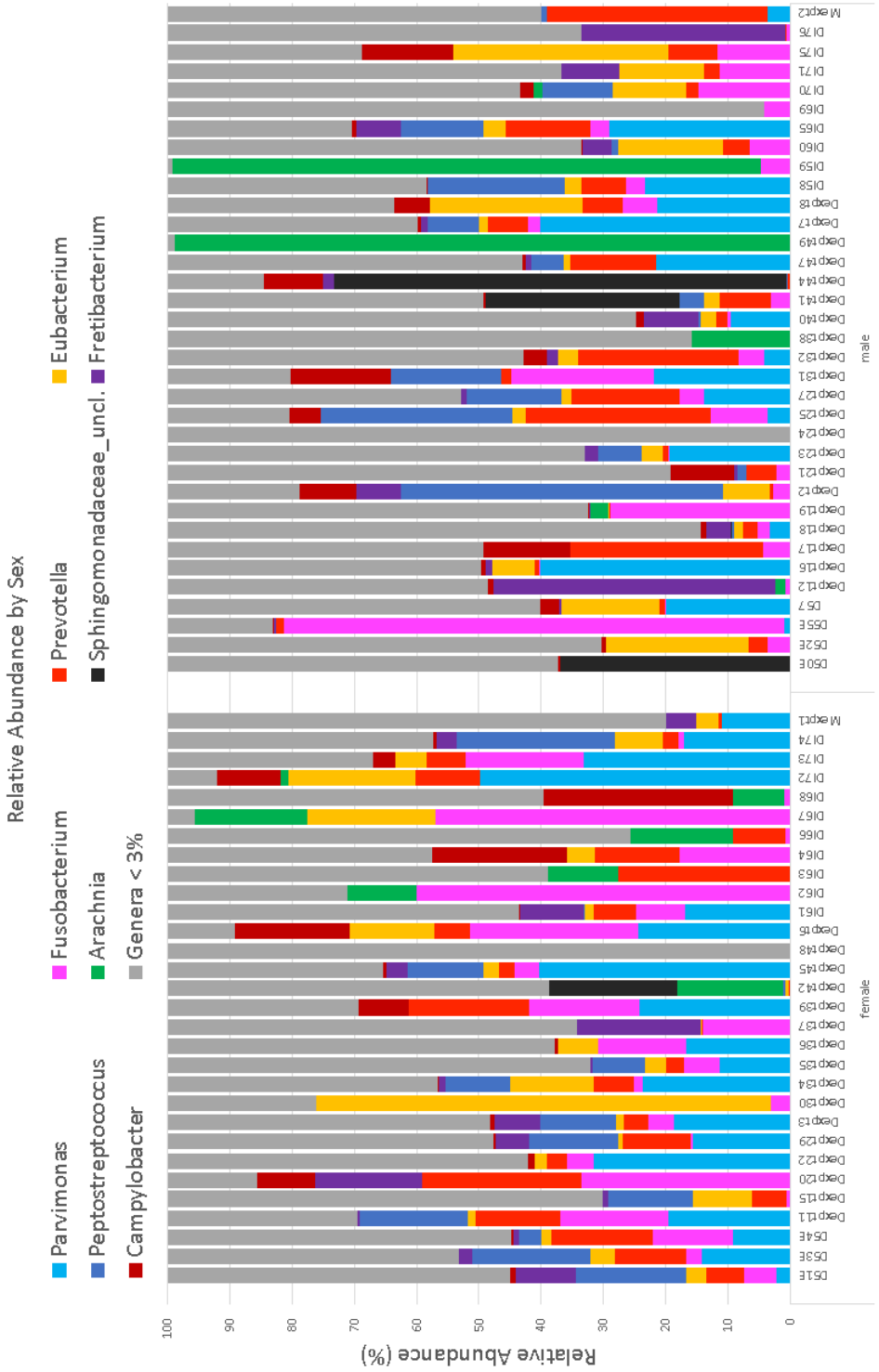

Figure 1. Prokaryotic composition of non-surgical samples. Relative abundances of predominant genera. Genera reflecting a mean < 3% of sequence reads among all samples were consolidated.

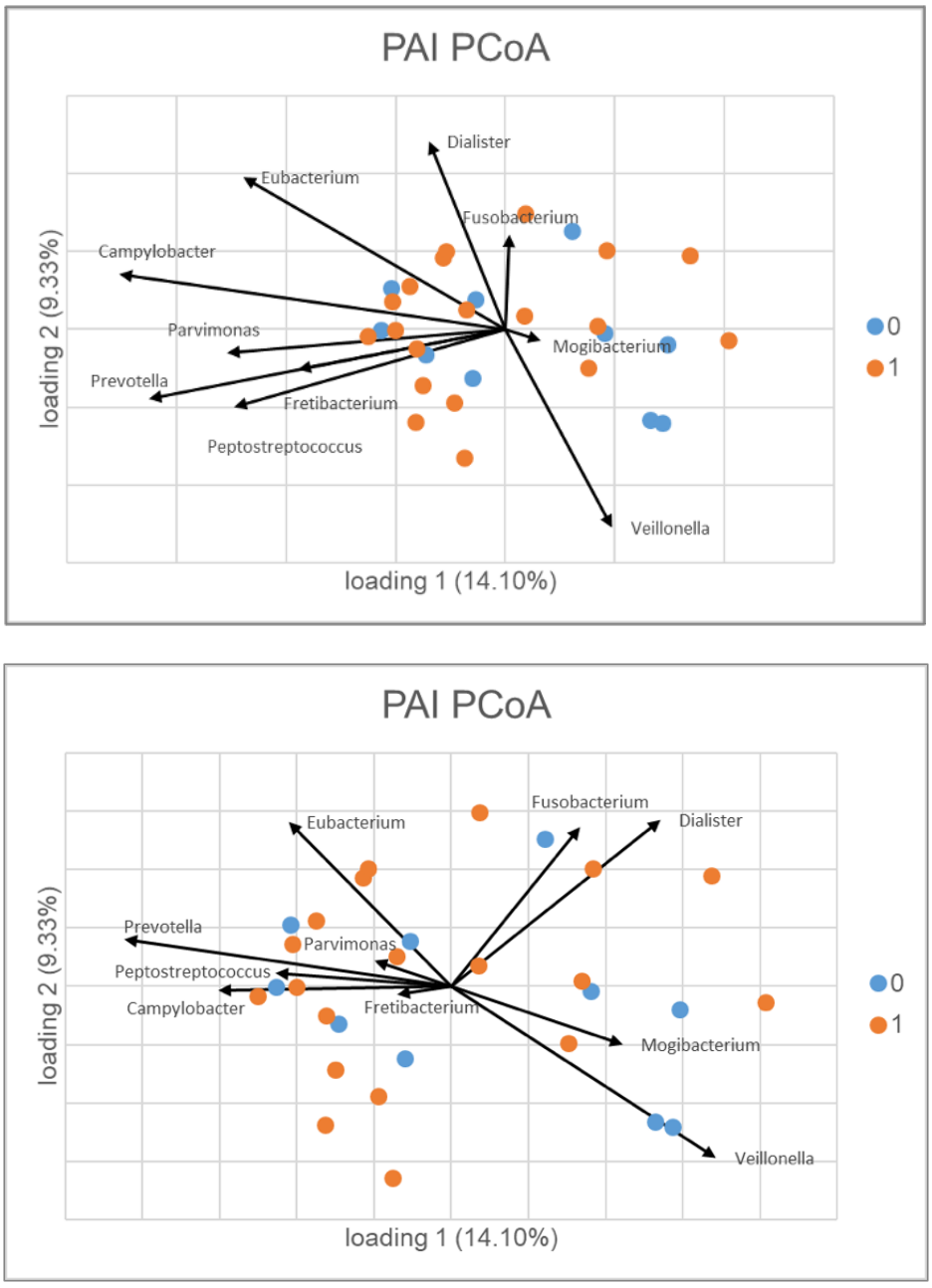

Figure 2. (Top) Principal Coordinate Analysis (PCoA) relative to Periapical Index Score (PAI). Lower figure excludes two outliers 0= PAI 2, 1=PAI 5. Unnormalized data.

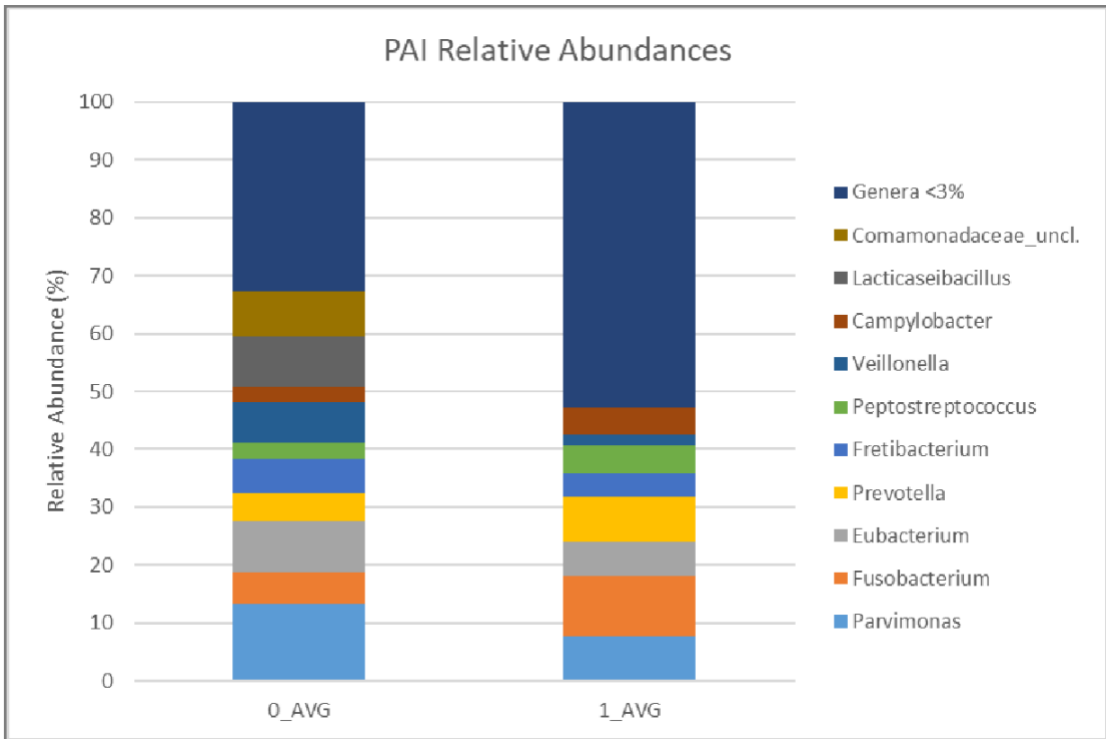

Figure 3. Periapical Index (PAI) score versus relative abundance. PAI score ≤ 2 is denoted as 0 (n = 12), whereas PAI score > 5 is denoted as 1 (n = 19).

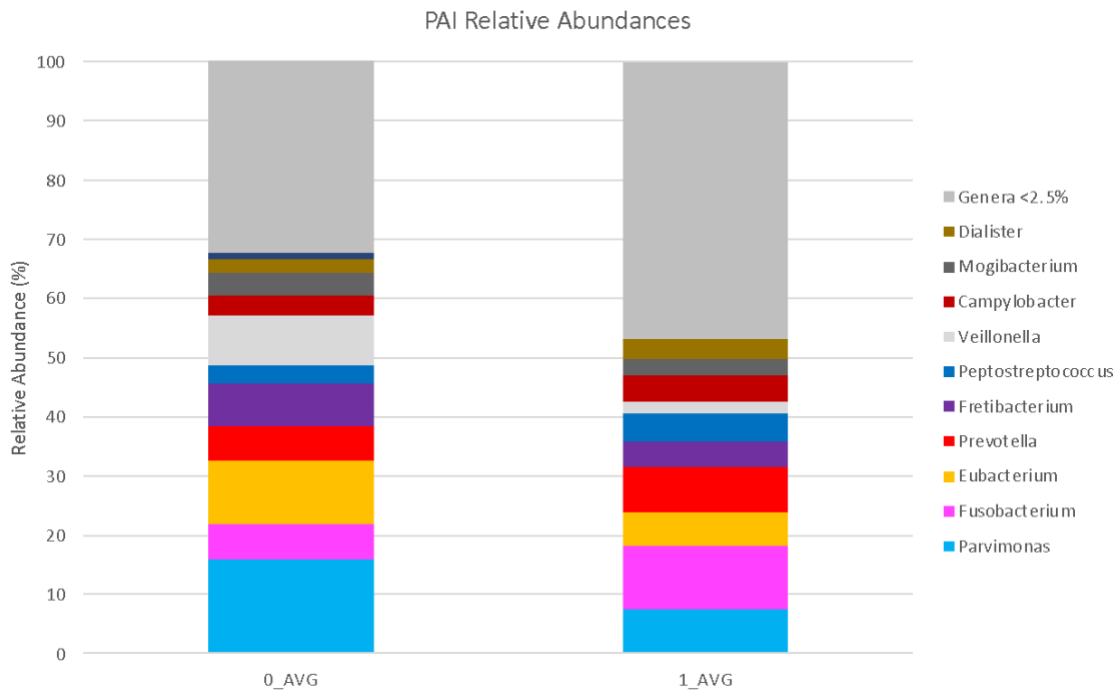

Figure 4. Periapical Index (PAI) score versus relative abundance. PAI score ≤ 2 is denoted as 0, whereas PAI score > 5 is denoted as 1. Samples #24 and #69 were considered outliers and were removed from this analysis due to over-abundance of *Lacticaseibacilus* and *Comamonadaceae*, respectively.

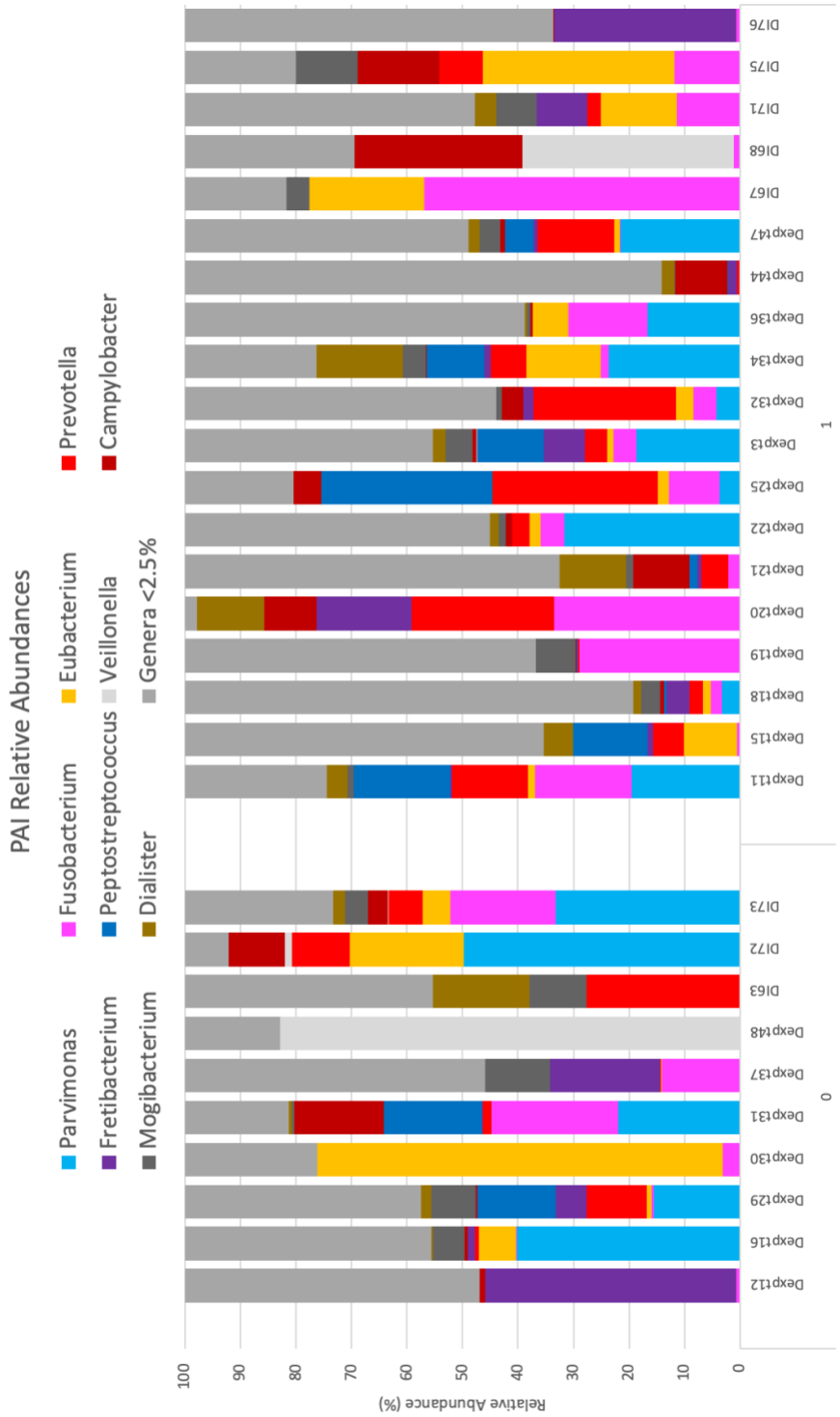

Figure 5. Prokaryotic composition of non-surgical samples related to Periapical Index score. Relative abundances of predominant genera. Genera reflecting a mean < 2.5% of sequence reads among all samples was consolidated. 0= PAI 2, 1=PAI 5.

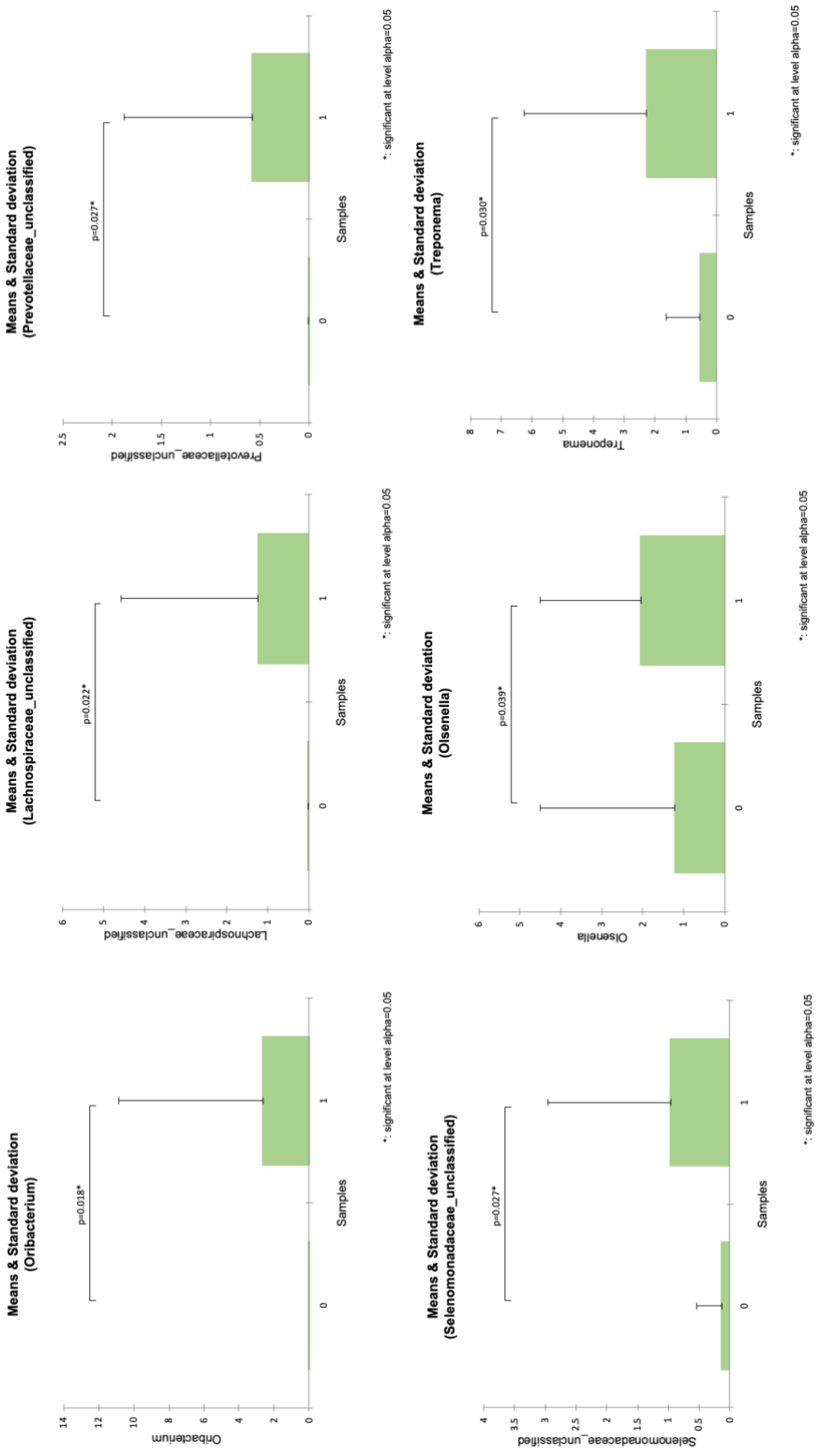

Figure 6. Relative abundance of significant species found in teeth with PAI 5. Kruskal Wallis test $P < 0.05$.

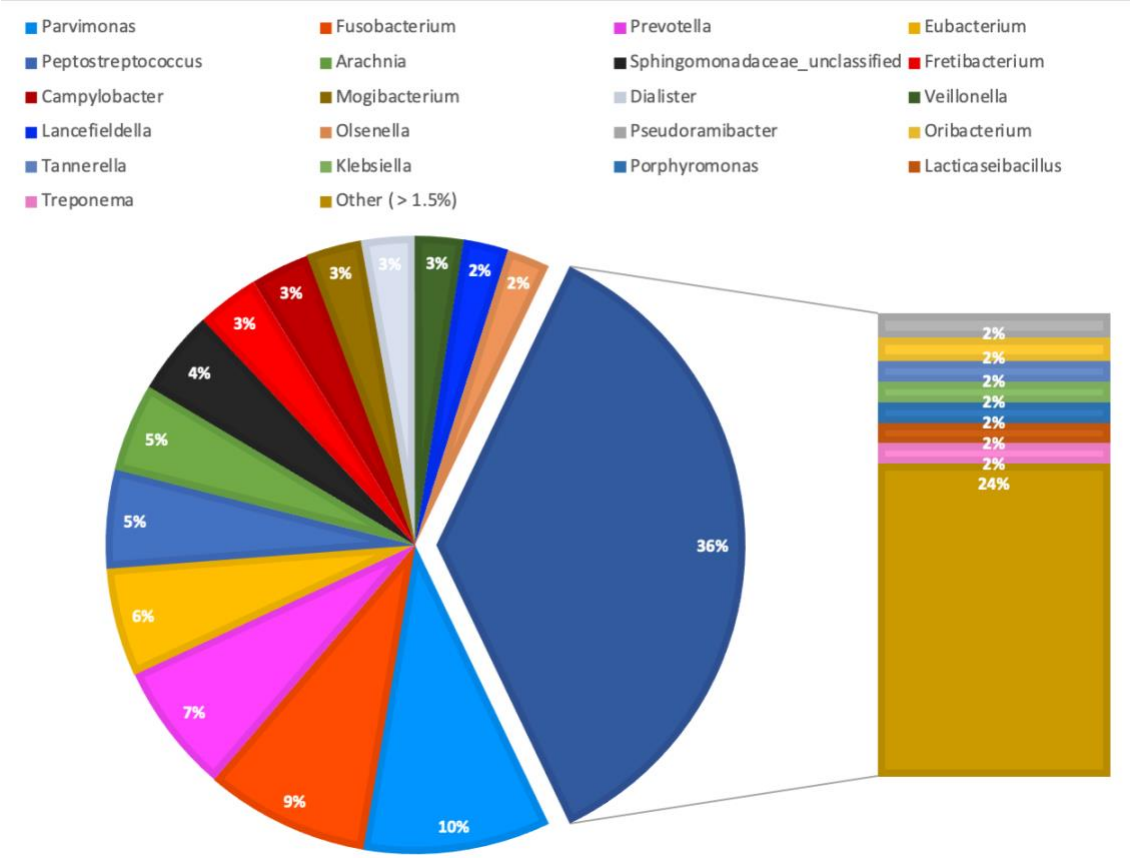

Figure 7. Prokaryotic composition of non-surgical samples. Relative abundances of predominant genera. Genera reflecting a mean < 1.5% of sequence reads among all samples were consolidated.