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- **Clinical Resident**, Endodontic program, School of Dental Medicine, University at Buffalo, USA. 2018-2020.
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SCIENTIFIC PUBLICATIONS

- **Alquria TA**, Acharya A, Tordik P, Griffin I, Martinho FC. Impact of root canal disinfection on the bacteriome present in primary endodontic infection: A next generation sequencing study. **Int Endod J.** 2024 May 3. doi: 10.1111/iej.14074. Epub ahead of print. PMID: 38700876.
- **Alquria TA**, Kabir B, Acharya A, Tordik P, Griffin I, Martinho FC. Clinical Investigation of Bacteriome in Primary Endodontic Infections with Apical Periodontitis Using High-throughput Sequencing Analysis. **Submitted.**
- **Alquria TA**, Alfirdous RA, Gupta S, Santamaria MP, Santamaria IF, Gomes APM, Tiradentes N, Silva EG, Martinho FC. Comparison of conventional and contemporary root canal disinfection protocols against bacteria, lipoteichoic acid (LTA), and lipopolysaccharide (LPS). **Sci Rep.** 2023 Jan 21;13(1):1206.
- AlSahafi R, Mitwalli H, Alhusein A, Balhaddad AA, **Alquria TA**, Melo MAS, Lynch CD, Oates TW, Zhang K, Xu HHK, Weir MD. Novel rechargeable nano-calcium phosphate and nano-calcium fluoride resin cements. **J Dent.** 2022 Nov;126:104312.
- Alfirdous RA, **Alquria TA**, Jacinto RC, Martinho FC. A modified dentine infection model with fluorescent lipopolysaccharide and lipopolysaccharides sampling technique to compare XP-Endo finisher and passive ultrasonic irrigation. **Int Endod J.** 2022 Oct;55(10):1081-1090.
- Velardi JP, **Alquria TA**, Alfirdous RA, Griffin IL, Tordik PA, Martinho FC. Efficacy of GentleWave System and Passive Ultrasonic Irrigation with Minimally Invasive and Conventional Instrumentation Technique against *Enterococcus faecalis* Lipoteichoic Acid in Infected Root Canals. **J Endod.** 2022 Jun;48(6):768-774.
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- Alfadda S, **Alquria T**, Karaismailoglu E, Aksel H, Azim AA. Antibacterial Effect and Bioactivity of Innovative and Currently Used Intracanal Medicaments in Regenerative Endodontics. **J Endod.** 2021 Aug;47(8):1294-1300.

- Azim AA, **Alquria T**, Wang HH, Piasecki L. Management of Root Fenestration Using Buccal Decortication and Guided Tissue Regeneration: A Case Report and 3-dimensional Analysis. **J Endod.** **2021** Jan;47(1):125-132.
- **Alquria T**, Al Gady M, Khabeer A, Ali S. Types of polymerisation units and their intensity output in private dental clinics of twin cities in eastern province, KSA; a pilot study. **J Taibah Univ Med Sci.** **2018** Dec 14;14(1):47-51.

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- **Project:** Impact of Root Canal Disinfection on Endodontic Bacteriome. **Oral presentation at the 2024 IADR/AADOCR/CADR General Session & Exhibition, New Orleans, Louisiana, USA, March 13-16, 2024.**
- **Project:** Impact of Root Canal Disinfection on Endodontic Bacteriome. **Poster Presentation Competition in UMSOD Research Day, Maryland, Baltimore, USA, March 6, 2024.**
- **Project:** Piezo-Electric use in Root-End Surgery: A Safe and Conservative Surgical Approach. **Table clinic presented at American Association of Endodontists (AAE19) Montreal, Canada, April 10-13, 2019.**
- **Project:** Comparison of oral hygiene practices and oral health problems among smoker and non-smoker male adolescents in the Eastern Province of Saudi Arabia. **Poster presented at (IADR) Boston, USA, Mar 11-14, 2015.**

SCIENTIFIC PRESENTATIONS

- **Root Canal Anatomy and Morphology**, Endodontics Department, University at Buffalo, School of Dental Medicine.
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- **Suturing in Endodontic Surgery**, Endodontics Department, University at Buffalo, School of Dental Medicine.

- **Root Canal Obturation**, Endodontics Department, University at Buffalo, School of Dental Medicine.
- **The outcome of Non-surgical Endodontic Re-treatment**, Endodontics Department, University at Buffalo, School of Dental Medicine.
- **Pain Medication and Antibiotics in Endodontics**, Endodontics Department, University at Buffalo, School of Dental Medicine.
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AWARDS & RECOGNITION

- **A Fellowship of Royal College of Dentists** of Canada in Endodontics, Canada. 2022.
- **“Teaching Assistant of the Year” Award** (2015-2016), College of Dentistry, University of Dammam, Saudi Arabia. 2017.
- **Saudi Arabian Government Scholarship**, Ministry of Higher Education, Saudi Arabia. 2015.
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- **The Ministry of Higher Education Appreciation Certificate** for Outstanding Contribution in the Sixth Annual Student Scientific Conference, Saudi Arabia. 2015.
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- **Member in Organization Committee** of the Annual Dental Symposium, organizing and coordinating between different organization committees. College of Dentistry, University of Dammam, Saudi Arabia. 2013-2018.
- **Co-founder and member of Bareeq Volunteer Club,** the first independent students volunteering club at the university. Carried out several healthcare and community outreach programs, which included education, screening, and treatment at the College of Dentistry, University of Dammam, Saudi Arabia. 2012-2018.

ABSTRACT

Title of Dissertation: Clinical Investigation of the Impact of Endodontic Disinfection on the Bacteriome of Root Canal Infection Using Next-Generation Sequencing on the Illumina MiSeq Platform.

Theeb Alquria, BDS, FRCDC, Doctor of Philosophy, 2024.

Dissertation Directed by: Frederico C. Martinho, DDS, MSc, PhD, Clinical Professor, Director, Predoctoral Endodontics, Department of Advanced Oral Sciences and Therapeutics, University of Maryland School of Dentistry, Baltimore, MD, USA.

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The primary cause of root canal infection is bacteria and their by-products, making disinfection of the root canal system a key goal in endodontic therapy. However, the complex anatomy of root canal systems, particularly the isthmus and its ramifications, poses challenges for effective disinfection. Currently, no disinfection protocol can eliminate all bacterial contents from root canal infections, driving the ongoing search for an optimal disinfection approach. Recently, next-generation sequencing (NGS), particularly the Illumina MiSeq platform, has been widely explored in endodontic infections due to its low sequencing error rates, cost-effectiveness, and high-quality reads. Leveraging advanced sequencing techniques to reveal the bacteriome of root canal infections and assess the impact of current disinfection methods could enable the development of more targeted and effective disinfection protocols. This dissertation presents an interventional clinical study aiming to investigate the diversity and composition

of the bacteriome in primary endodontic infection (PEI) with apical periodontitis (AP) and evaluate the impact of root canal disinfection on the endodontic bacteriome using NGS on the Illumina MiSeq Platform. First, we characterized the bacteriome in PEI with AP, identified core and rare bacteriome species, and analyzed community diversity metrics using the Illumina MiSeq platform. Our results showed that Bacteroidetes, Firmicutes, Synergistetes, Fusobacteria, and Actinobacteria were the most abundant bacterial phyla. We identified 113 genera and 215 species. Analysis revealed differences in abundant taxa among distinct age, gender, symptomatology, and lesion size groups. These findings suggest that the bacteriome in PEI with AP is complex and has high microbial heterogeneity among patients.

Moreover, age, gender, symptomatology, and lesion size might play a role in the abundant taxa present in PEI with AP. Second, we determined quantitatively and qualitatively the impact of chemomechanical preparation (CMP) using 2.5% sodium hypochlorite (NaOCl) on the bacteriome found in PEI with AP using the Illumina MiSeq platform. Despite a significant decrease in bacterial abundance, our findings demonstrated a distinct community composition and increased alpha diversity after CMP using . We observed differential enrichment of specific taxa, including *Stenotrophomonas_unclassified*, *Enterococcus_unclassified*, and *Actinomyces_unclassified*, suggesting lower effectiveness of CMP using 2.5% NaOCl against these taxa. Findings from this dissertation highlight the complexity and heterogeneity of the bacteriome in PEI with AP, emphasizing the influence of patient-related factors on microbial diversity. The research highlighted the limited effectiveness of current endodontic disinfection protocols, specifically the use of 2.5% NaOCl, in reducing bacterial abundance while revealing limitations against certain taxa.

These insights provide a foundation for developing more targeted and effective disinfection strategies, potentially leading to improved outcomes in endodontic therapy.

Clinical Investigation of the Impact of Endodontic Disinfection on the Bacteriome of
Root Canal Infection Using Next-Generation Sequencing on the Illumina MiSeq Platform

by
Theeb Abdullah Alquria

Dissertation submitted to the Faculty of the Graduate School of the
University of Maryland, Baltimore in partial fulfillment
of the requirements for the degree of
Doctor of Philosophy
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DEDICATION

“When others shy away from a challenge, dare to take it on. Triumph will make you a hero, and even if you fall short, the honor of your brave attempt will always be yours”

—Hadi Bin Masoud Alquria (Grandfather)

I dedicate this dissertation to God. All that I have, all that I am, and all that I do is because of and for You.

To my dear Father and Mother, for your continuous love and limitless support. You have endured so much for my sake and have shared with me moments of joy and hardship. Your prayers have always surrounded me until I became what I am now. Your encouragement and belief in me have been the foundation of my success.

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to me, as well as a friend, colleague, and neighbor. I have learned a lot from him throughout my PhD. program and during my studies at the dental school. Dr. Balhaddad supported me in every way, standing by me at every step, and never hesitated to offer advice or assistance. I credit Dr. Balhaddad for every success in my career. We have shared many wonderful times together, and I look forward to continuing this valued friendship in the future.

I have special gratitude for my clinical research team, Dr. Ina L. Griffin, Dr. Swati Gupta, and Dr. Binait Kabir, for their outstanding help and support throughout my research.

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LIST OF ABBREVIATIONS

ALDE	ANOVA-like differential expression tool for HTS sequencing data
AP	Apical periodontitis
ASVs	Amplicon sequence variants
Bp	Base pairs
Ca(OH) ₂	Calcium hydroxide
CBCT	Cone beam computed tomography
CFU	Colony-forming unit
CMP	Chemomechanical preparation
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
EPT	Electric pulp test
EPS	Extracellular polymeric substance
H ₂ O ₂	Hydrogen peroxide
HOMD	Human Oral Microbiome Database
HTS	High-throughput sequencing
HVRs	Hypervariable regions
IGS	Institute for Genome Sciences
IRB	Institutional Review Board
LDA	Linear discriminant analysis
LEFSe	Linear effect size
NaOCl	Sodium hypochlorite

NGS	Next-generation sequencing
ONT	Oxford nanopore technologies
OTUs	Operational taxonomic units
PacBio	Pacific Biosciences
PCR	Polymerase chain reaction
PEI	Primary endodontic infection
PoCA	Principal coordinate analysis
POP	Pain on palpation
PPs	Paper points
qPCR	Quantitative polymerase chain reaction
RCS	Root canal sampling
RDP	Ribosomal Database Project
RNA	Ribonucleic acid
rRNA	Ribosomal ribonucleic acid
RT-qPCR	Real-time quantitative PCR
SECOM	Sparse estimation of correlations among microbiomes
SRL	Size of radiolucent area
STROBE	Strengthening the reporting of observational studies in epidemiology
S	Sample
T-RFLP	Terminal-restriction fragment length polymorphism
TTP	Tenderness to percussion
UMSOD	University of Maryland School of Dentistry
WL	Working length

CHAPTER ONE

INTRODUCTION

1.1 Background

1.1.1 Anatomy of the dental pulp

The dental pulp is a mass of connective tissue that is highly vascularized and innervated. It is surrounded and protected by dentin, enamel, and cementum within the pulp chamber. The pulp and dentin are closely interrelated and dependent on each other's development and survival. The association between the pulp and dentin is often termed the pulp-dentine complex (1). The vitality of this complex is essential for root development, tissue regeneration, and repair (2–4). Moreover, the complex provides defensive responses against external stimuli. The defensive responses include inflammatory and immune reactions, decreased dentin permeability, and tertiary dentin formation (5).

1.1.2 Microbial role in pulpal disease

Irritation of the dental pulp tissue can lead to pulpal disease. Various factors can cause pulpal irritation, including thermal, mechanical, microbial, radiation, or electrical stimuli (5). However, in 1965, Kakehashi et al. emphasized microbes as the primary cause of pulpal disease (6). The study involved 15 conventional and 21 germ-free rats as subjects. Dental pulps were exposed to the oral cavity in all subjects. The result showed that pulpal necrosis and periapical inflammation manifested only in the presence of microbes (conventional rats).

On the other hand, exposed pulps in germ-free rats exhibited signs of tissue repair. Subsequent studies conducted on monkeys showed similar findings (7,8). Currently, it is acknowledged that while noninfectious agents (e.g., thermal and mechanical factors) may initiate pulpal and apical diseases, the microbial component is the main factor for the development and progression of the disease (5,9,10).

1.1.3 Microbial pathways to the pulp

Dental caries is the common microbial pathway to the dental pulp (11) (Figure 1.1). However, microorganisms can access the pulpal space through alternative pathways, such as exposed dentinal tubules due to periodontal disease, operative procedures, and trauma (fracture). Cervical root resorption is a potential entry point into the dentinal tubules (12). Anachoresis, defined as pulpal infection resulting from bacteria in the bloodstream, is also considered a pathway for microorganisms to reach dental pulp (13). In teeth with existing root canal treatment, reinfection may occur due to the presence of microorganisms that survived the disinfection process of the initial root canal treatment or managed to re-enter the root canal through coronal leakage (14).

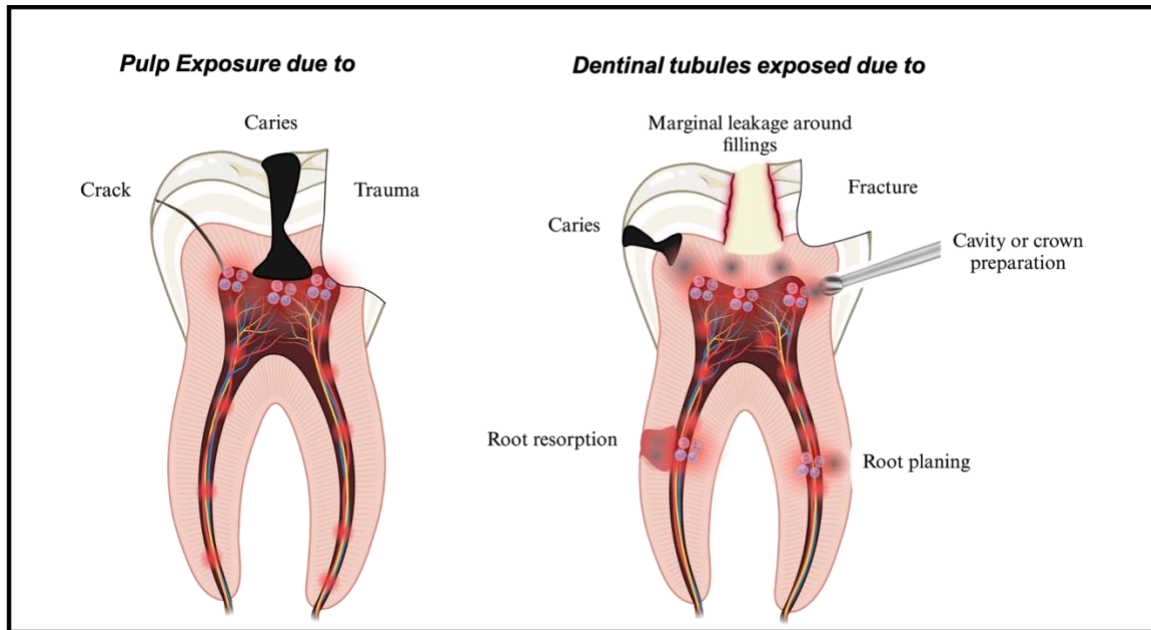


Figure 1.1: Illustration of the various microbial pathways to the dental pulp (left) and to the dentinal tubules (right).

1.1.4 Microbial interactions within the infected dental pulp

The protective layers of the dental pulp (enamel and dentin) can be breached by caries, tooth wear, fractures, or dental procedures. When these protective layers are compromised, the dental pulp becomes exposed to noxious agents. These noxious agents can cause pulp inflammation (i.e., pulpitis) and, if untreated, complete necrosis of the dental pulp (15). In necrotic pulp, defensive mechanisms are compromised, allowing microbes to inhabit the pulpal space and proliferate extensively (9).

Various factors influencing microbial activity in a specific environment include local pH, oxygen abundance, redox potential, availability of selective nutrients, and the condition of local host defenses (16). In primary root canal infection, the microbial composition changes over time (17). At the initial stage of the infection, aerobes and facultative anaerobes dominate the canal, where there is a high source of nutrition from the

oral cavity. As the infection progresses, the abundance of nutrition decreases, and obligate anaerobes tend to dominate the canal. Conversely, the challenging conditions of secondary infections support species with enhanced survival capabilities. *Enterococci* and *Streptococci* are commonly detected at a high prevalence in secondary root canal infections (18,19).

Planktonic bacteria are essential for initiating root canal biofilm (20). A biofilm is a stationary multicellular bacterial community where bacteria firmly attach to a surface and are surrounded by extracellular polymeric substances (EPSs) (21). Nair was the first to report the presence of bacteria in the form of biofilm in infected root canals in 1987 (22). Furthermore, biofilms have been noted in cases of secondary infections (23).

1.1.5 Apical periodontitis

Apical periodontitis (AP) emerges as a host defense response to prolonged root canal infection with complete pulp necrosis. AP prevents the spread of the bacteria and its byproducts from the root canal to the periapical bone and the surrounding tissues. Clinical manifestations of AP may include distraction of the surrounding periapical tissue, including periodontal ligament cementum and alveolar bone. Pain on percussion or biting, tenderness on palpation, and swelling can also be associated with the AP. Despite the resorption of the periapical bone and damage to the periodontal tissue, AP is a component of the defensive process. Following successful root canal treatment and effective bacterial elimination, the periapical tissue can heal gradually, and bone regeneration will occur as part of the healing process (12).

1.1.6 Root canal treatment

1.1.6.1 The goals of root canal treatment

The main goal of endodontic treatment (root canal treatment) is to eliminate irritants from the root canal system, fill or seal the cleaned and shaped canal, and prevent any subsequent recontamination of the sealed root canal. Primary endodontic infection (PEI) is caused by microorganisms that initially invade and colonize the necrotic pulp. Treating PEI involves removing the necrotic pulp tissue and bacteria and its by-products from the root canal to stimulate tissue healing and bone regeneration of existing AP and prevent the development of new periapical inflammation (24).

Nonsurgical root canal treatment is recommended under the following circumstances:

- Pulpitis (vital pulp): where the dental pulp undergoes reversible or irreversible inflammation, the treatment is performed to preserve periapical health and prevent the onset of AP.
- Pulp necrosis (non-vital pulp) is often associated with AP; treatment eliminates the source of infection and allows the periapical health process to initiate.

Nonsurgical root canal treatment procedures include multiple steps. The first step involves mechanical instrumentation to scrape the infected dentin wall and prepare the canal for the obturation material. The second step includes chemical irrigation with antibacterial properties to disinfect the root canal. These two steps are referred to as chemomechanical preparation (CMP). Following the CMP, the root canal needs to be obturated to seal the canal apically, and coronal restoration needs to be applied to seal the canal coronally. If a root canal treatment is performed in more than one visit, an intracanal medicament should be applied in the canal space between the visits. Therefore, single-visit

root canal treatment differs from multiple visits, as it is conducted in one appointment without intracanal medication.

1.1.7 Causes for root canal treatment failure

1.1.7.1 Intra-radicular infections

The main factors contributing to the failure of root canal treatment are inadequate disinfection or an inadequate seal of the root canal; the latter can lead to bacteria and their byproducts reaccessing the root canal system (14). Bacteria can be introduced to the previously treated root canal due to inadequate obturation, poor coronal seal, poor isolation, and contamination during the treatment (25). The survival of the existing bacteria can be attributed to various factors. The root canal anatomy is complex; as a result, a portion of the root canal wall is hard to reach via chemomechanical disinfection procedures. The bacteria can survive disinfection procedures by using these hard-to-reach areas as a shelter. The efficacy of the disinfection procedure can also be affected by the occurrence of procedural errors during the treatment. Common procedural errors in root canal treatment include instrument separation and ledge formation (14). Additionally, bacteria can be located deep in the dentinal tubules or accessory canals and isthmus, thus evading disinfection procedures (26). Lastly, some bacteria showed high resistance to the root canal treatment disinfection. *Streptococci* and *Enterococcus faecalis* are facultative species commonly detected and have specific features that enable them to survive disinfection and adverse environmental conditions (14,27).

Considering these factors and understanding the limitations of radiographs, root canal treatment can fail despite how good it appears on the radiograph (14,28).

1.1.7.2 Extra-radicular infections

AP is a defensive mechanism to confine and prevent bacterial infection from spreading to the periradicular areas. In specific circumstances, the bacteria can breach the root canal apex and bind to the external surface of the root (27). Extra-radicular infections can be formed once the bacteria reach the external root surface. They can also arise from the extrusion of infected dentine chips during instrumentation. The incidence of extra-radicular infection is relatively low, ranging from 6–10%, with a limited number of species capable of residing beyond the root canal. *Actinomyces* and *Propionibacterium spp.* are among the most frequently isolated species in these cases (26).

1.1.7.3 Radicular cysts

A radicular cyst is a type of cyst that forms at the apex (tip) of the root. It occurs as a result of AP and is lined by epithelium (26). The question of whether radicular cysts respond to root canal treatment has been widely studied for a long time. It depends on the type of cyst; pocket cysts connected to the root apex can be resolved following nonsurgical root canal treatment. On the other hand, true cysts, completely isolated in the periapical area, are self-sustaining and unlikely to heal following root canal treatment (27).

1.1.7.4 Foreign body reactions

Extrusion of the endodontic material during root canal treatment can cause irritation and inflammation in periapical tissues. Endodontic materials include gutta-percha and root canal sealer (26).

1.1.7.5 Scar tissue

Scar tissue (also referred to as fibrous tissue) formation can be a part of the healing process in apical lesions. Radiographically, the presence of scar tissue may sometimes be

misinterpreted as an indication of treatment failure. The incidence of scar tissue is relatively low, accounting for less than 7% of cases, and it is often associated with post-surgical endodontic interventions (29).

1.2 Microbiological analyses of microorganisms in infected root canals

Microbiological detection methods can be broadly divided into open-ended and closed-ended approaches (Figure 1.2). The open-ended approach allows for identifying a wide range of species in the sample (though, in practice, it primarily identifies the prevailing ones based on the method's depth of coverage), providing insights into diversity (including relative abundance and richness). On the other hand, the closed-ended approach involves identifying specific target species using a method that indicates whether the species are present or absent and incorporating quantification. Endodontic microbiology studies are divided into five generations based on chronology and technology (30,31).

1.2.1 Culturing techniques studies

The first generation of studies employed culture-based methods, emphasizing the importance of anaerobic culture techniques over aerobic methods. This trend became prominent in endodontic research from the late 1960s to the mid-1970s (32–34). These studies help understand the role of bacteria in the development of AP. They revealed numerous types of bacteria, particularly anaerobic bacteria (such as *Fusobacterium nucleatum*, *Prevotella* spp., *Porphyromonas* spp., *Parvimonas micra*, and *Pseudoramibacter alactolyticus*), associated with primary root canal infection (32,34–43). Furthermore, facultative bacteria such as *Enterococcus faecalis* and *streptococci* were identified as being associated with secondary root canal infection (44–47). In current

studies that utilize culture techniques for detecting and identifying endodontic bacteria, it is recommended to strictly adhere to anaerobic methodologies. (48).

1.2.2 Molecular techniques studies

Molecular microbiology techniques first appeared in endodontic research in the late 1990s and continued through the subsequent four generations of studies. The initial implementation of molecular methods in endodontics involved closed-ended approaches, representing the second generation of studies. Among the methods using closed-ended approaches are polymerase chain reaction (PCR) and the traditional checkerboard hybridization assay, which employs entire genomic coverage probes. Using these techniques allows for heightened sensitivity compared to culture methods. They have been applied to identify bacterial species that are challenging to cultivate (48). Some of these species are recognized as periodontal pathogens, but their presence in endodontic infections has not been reported. These species include *Tannerella forsythia* (49) and *Treponema denticola* (50). Additionally, anaerobic bacteria with previous difficulties in cultivation, such as *Dialister* species, *Filifactor alocis*, and treponemes were initially recognized within endodontic infections and were prevalent at significant rates (51–58). Furthermore, the second-generation studies revealed that numerous species that can be cultivated exhibited higher prevalence compared to studies utilizing culture methods (59–62), thereby reinforcing their association with AP. These studies have broadened the list of potential endodontic pathogens.

Third-generation studies utilized advanced DNA-based molecular techniques. These included open-ended methods such as broad-range PCR followed by cloning and Sanger sequencing terminal-restriction fragment-length polymorphism (T-RFLP) or

denaturing gradient gel electrophoresis (DGGE). These methodologies are intended to detect the presence of almost all bacterial species in a given sample, including those that can be cultivated and those that are yet to be cultivated within microbial communities. Despite these techniques requiring significant effort, time, and cost, they allow for identifying only a few samples per study, with recognition limited to dominant community members. Nevertheless, applying these techniques in analyzing root canal infection enabled researchers to create a comprehensive list of cultivable and yet-to-be-cultivated species that may contribute to infections in the oral cavity (63–70). Moreover, these methods facilitated the profiling of the composition of bacterial communities in endodontic infection, laying the groundwork for conceptualizing the community as the primary unit influencing pathogenicity in AP (71).

The findings from the third generation of studies provided the basis for the fourth generation. This subsequent generation used closed-ended molecular techniques, including PCR, reverse-capture checkerboard hybridization, and microarrays, which were applied to many endodontic samples. These methods were utilized to explore the frequencies and associations among bacteria, both those that can be cultivated and those that are yet to be cultivated, in cases of AP. Findings from the fourth generation introduced as-yet-uncultivated bacteria, including members of the phyla Synergistetes and Bacteroidetes, into the list of potential endodontic pathogens (72–75).

The fifth generation of studies utilizes high-throughput sequencing (HTS), also referred to as next-generation sequencing (NGS) technologies, to conduct a thorough and open-ended analysis of endodontic infections. Exploration employing these approaches has revealed an unexpectedly more diverse bacterial population in root canal infections. This includes low-abundance taxa that escaped detection in prior study generations (76–84). Studies from the fifth generation provide valuable insights into profiling and comparing bacterial communities linked to diverse clinical conditions (85–87).

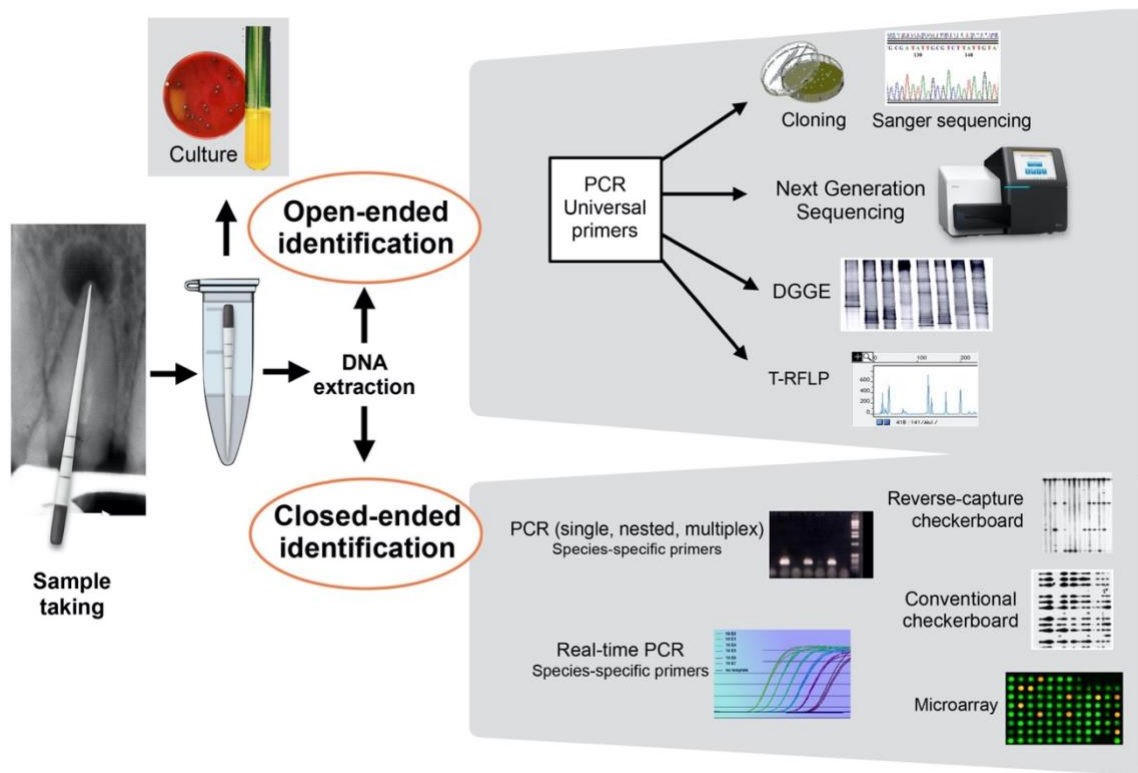


Figure 1.2: Methods used in the study of the endodontic microbiome. Open-ended identification methods can identify all bacteria in the sample, whilst closed-ended methods target selected microbial species or groups (48).

1.3 NGS technologies

1.3.1 Overview

NGS technology, or high-throughput or massively parallel sequencing, is a broad term covering a variety of advanced techniques capable of sequencing DNA or RNA at a massive rate and great depth (88).

The establishment of NGS can be linked to Frederick Sanger's pioneering work in developing a method for DNA sequencing utilizing a chain-termination approach (89). Following extensive improvements over the years, the Human Genome Project achieved its successful culmination in 2001, predominantly relying on Sanger sequencing and automated capillary electrophoresis as indispensable tools (90).

The Human Genome Project played a crucial role in advancing cutting-edge sequencing technologies, creating more powerful instruments to enhance speed and resolution. This initiative significantly expedited the overall progress and evolution of NGS (91).

The NGS technologies differ from the Sanger method in three fundamental aspects. First, bacterial cloning of DNA fragments is not required; instead, NGS libraries are prepared in a cell-free system. Second, they generate higher sequencing reactions in parallel, ranging from hundreds to millions. Third, the sequencing output is directly detected without electrophoresis (92).

Human microbiome analysis involves studying microbial communities on and within the human body. Human microbiome studies aim to comprehend microbes' role in health and disease (93). The emergence of NGS initiated a revolution in metagenomic sequencing and analysis. “Metagenomic” refers to the genomic analysis of microbial

communities directly extracted from environmental samples, providing insights into microorganisms' diversity, functions, and interactions within a given ecosystem. The enhanced sequencing efficiency (high-throughput), cost reduction, and technological advancements have substantially transformed the metagenomics landscape (94).

1.3.2 NGS instruments

In 2005, Life Sciences introduced the pioneering NGS instrument known as Roche 454 Pyrosequencing. Subsequently, different companies have offered various sequencing platforms with distinct technologies and specifications. Some of the main platforms included the following:

- Oxford Nanopore Technologies (ONT) platforms (e.g., MinION, GridION, PromethION)
- Illumina platforms (e.g., HiSeq and MiSeq series, NovaSeq, NextSeq)
- Pacific Biosciences (PacBio) platforms (e.g., Sequel II)
- Ion Torrent platforms
- Roche 454 Pyrosequencing (discontinued)

The various NGS technologies follow the common principle of massive parallel sequencing. Yet, distinctions arise in specific aspects including sequencing chemistry, read length, running time, throughput per run, and reads per run (95). Illumina technology, as detailed in Table 1.1, employs paired-ended overlapping reads, contributing to an extension of the total fragment length and an enhancement of sequence quality (95,96). Additionally, the Illumina MiSeq platform demonstrates a notably low error rate in comparison to benchtop sequencers (97).

Table 1.1: Illumina benchtop sequencer comparison table (95,96).

	iSeq 100	MiniSeq	MISeq Series	NextSeq 550 Series	NextSeq 1000 & 2000
Run Time	9.5–19 hrs	4–24 hours	4–55 hours	12–30 hours	11-48 hours
Maximum Output	1.2 Gb	7.5 Gb	15 Gb	120 Gb	360 Gb
Maximum Reads Per Run	4 million	25 million	25 million	400 million	1.2 billion
Maximum Read Length	2 × 150 bp	2 × 150 bp	2 × 300 bp	2 × 150 bp	2 × 300 bp

1.4 Human microbiome investigation using NGS

1.4.1 Oral microbiome

With the emergence of NGS technologies, numerous studies have been conducted to explore the oral microbiome. Keijser et al. employed 454 GS FLX pyrosequencing to examine 71 saliva and 98 supragingival samples (98). They identified 28,978 unique variable (V6) tag sequences from 22 taxonomic phyla. However, out of the 22 taxonomic phyla, 99.6% of these sequences belong to Actinobacteria, Bacteroidetes, Firmicutes, Fusobacteria, Proteobacteria, Spirochetes, or candidate division TM7. The results indicated greater diversity in plaque bacteria compared to saliva. The number of genera detected in plaque bacteria was 267 versus 185 detected from saliva. Similarly, when examining operational taxonomic units (OTUs) at a 3% difference, the plaque bacteria exhibited higher diversity, with 10,000 OTUs compared to 5,600 OTUs detected in saliva. However, despite this apparent increase in diversity, the rarefaction curves estimating bacterial richness indicated that richness was still incomplete in plaque and saliva samples. In 2009, Lazarevic utilized the Illumina Genome Analyzer system GAII to investigate the extent of sequencing depth (96). The analysis of three individuals' saliva and oropharyngeal swabs resulted in 1,373,824 sequencing reads, with 330,815 representing distinct taxa. Their result showed greater coverage depth than the previous oral microbiota studies.

Additional investigations employed NGS to explore the oral microbiome in samples taken from periodontitis and dental caries (99), experimental gingivitis (100), periodontal samples (101), and both healthy and failing implants (102). Findings from these studies provide a deep understanding of oral microbiota diversity.

1.4.2 Endodontic microbiome

Li et al. used NGS for the first time in 2010 to analyze root canal infection (103). They used Sanger sequencing and GS FLX pyrosequencing to analyze samples collected from seven teeth using paper points. The quality assessment approved a total of 200,129 sequencing reads. The pyrosequencing analysis resulted in higher taxonomic detection at various levels than Sanger sequencing. The sequencing analysis from pyrosequencing yielded 13 phyla, 22 classes, 43 orders, and 97 families, compared to eight phyla, 10 classes, 11 orders, 20 families, and 25 genera from Sanger sequencing. Bacteroidetes was the most abundant phyla, constituting approximately 59.44% of the microbial population in the sample, followed by Firmicutes, representing 19.92%. Other abundant phyla comprising less than 5% were Actinobacteria, Fusobacteria, Proteobacteria, and Spirochetes. In addition to the most abundant phyla, six other phyla were identified for the first time in endodontic infections: Acidobacteria, Chloroflexi, Tenericutes, Cyanobacteria, Deinococcus-Thermus, and OD1. Most of the identified genera were found in relatively low abundance. The technique employed in this study effectively identified low-abundance bacteria and unveiled the bacterial diversity within the endodontic microbiota that was not previously accessible. In 2011, Siqueira et al. focused on analyzing the microbiota of apical root canals, employing multiplex tag-encoded FLX titanium amplicon pyrosequencing for their investigation (80). Cryogenically grinding 10 apical root specimens for sequencing

identified 84 genera affiliated with ten phyla. An average of 37 taxa were detected from the apical root segment. These findings were significantly higher than what Siqueira et al. reported in 2007 (41), where the mean of detected taxa from the apical root segment was three species. Saber et al. reported a similar finding in 2012 (104). In another study, Santos et al. (2011) investigated chronic and acute root canal infections through pyrosequencing, detecting 13 phyla and 67 genera. Findings from this study showed that acute infections exhibit greater bacterial diversity than chronic infections. Additionally, the composition of bacterial communities was found to vary between individuals (87). A subsequent study analyzed extracted teeth and compared the coronal and apical microbiota, describing much more intricate apical diversity than previously emphasized (78). From 2011 to 2015, a series of studies were published examining factors of root canal infections, indicating that the diversity is significantly more extensive than previously expected (76,82,83,85,105). Recently, the Illumina MiSeq platform has been used to investigate the bacteriome in teeth with PEIs (77,112–129), secondary/persistent infections (116,124,127–129), and teeth with endodontic-periodontal lesions (130,131). The findings from Illumina MiSeq studies are discussed in detail in Chapters Two and Three of this thesis.

1.5 Central hypothesis

The bacteriome of root canal infections varies among patients based on their clinical, radiographic, and demographic factors. Additionally, the bacteriome of root canal infections before disinfection differs from the bacteriome after disinfection.

1.6 Specific aims

Aim I: To characterize the bacteriome in PEI with AP, identify core and rare bacteriome species and community diversity metrics, and analyze the relationships among the bacteriome composition, diversity and features, and patient variables using the Illumina MiSeq platform.

Aim II: To investigate the bacteriome present in teeth with PEI and AP and to determine quantitatively and qualitatively the impact of CMP using 2.5% sodium hypochlorite (NaOCl) on the bacteriome found in PEI with AP using the Illumina MiSeq platform.

1.7 Significance

Illumina MiSeq is a high-throughput sequencing platform that offers substantially greater sequencing coverage with lower sequencing error rates and lower cost compared to the other sequencing platforms. To the best of our knowledge, we will be the first to describe the bacteriome of PEI with AP before and after disinfection using the Illumina MiSeq platform. Using HTS before and after the disinfection will allow us to identify the most persistent bacteria after root canal therapy. We will be the first to investigate the association between the bacteriome in PEI with AP and patient demographic factors as well as clinical and radiographic findings using Illumina MiSeq as an HTS platform.

CHAPTER TWO

Clinical Investigation of Bacteriome in Primary Endodontic Infections with Apical Periodontitis Using High-throughput Sequencing Analysis

Chapter abstract

This study characterized the bacteriome in primary endodontic infection (PEI) with apical periodontitis (AP), identified core and rare bacteriome species and community diversity metrics, and analyzed the relationship between the bacteriome composition, diversity and features, and patient variables. Twenty-seven patients with PEI and AP were sampled. The DNA was extracted and quantified using qPCR. Raw V3-V4 amplicon sequencing data were processed with the DADA2 pipeline to generate amplicon sequence variants (ASVs), and taxonomic assignment of the ASVs up to the species level was done against the HOMD. Core bacteriome and differential abundance analyses were performed using ANCOM. Alpha diversity was determined using Chao1, Shannon, and Simpson indexes. LeFse analysis was used to identify abundant taxa. SECOM analysis estimated linear and nonlinear relationships among bacteria. 24/27 root canal samples were analyzed, and 3 RCS were filtered out with a low read count. The bacterial phyla with top mean relative abundance were Bacteroidetes, Firmicutes, Synergistetes, Fusobacteria, and Actinobacteria. A total of 113 genera and 215 species were identified. The samples were gathered into three clusters. LeFse analysis identified differences in abundant taxa between distinct age, gender, symptomatology, and lesion size groups. SECOM distance analysis indicated *Slackia exigua* as the node with the highest degree. In conclusion, the bacteriome in PEI with AP is complex and had high microbial heterogeneity among the patients in this

study. Moreover, age, gender, symptomatology, and lesion size might play a role in the abundant taxa present in PEI with AP.

2.1 Introduction

Apical periodontitis (AP) is one of the most common oral diseases, affecting 52% of the adult population worldwide (106). AP involves pathologic changes in the periradicular tissues, including the alveolar bone, periodontal ligament, and cementum (24). The primary cause of AP is the presence of bacteria in the root canal system (6). Therefore, the knowledge of the root canal bacterial community in AP is essential for a better understanding of the pathogenesis of the disease and the establishment of effective and targeted future therapeutic protocols.

Endodontic bacterial community studies have evolved over the last two decades (79,107,108). Culture-dependent methods failed to reveal uncultivated bacterial species (107). Early close-ended molecular techniques could detect cultivated and uncultivated bacteria species but were limited to targeted bacterial species and failed to disclose unknown species not yet identified (107). High-throughput sequencing (HTS), an open-ended molecular technique known as next-generation sequencing (NGS), has been explored for endodontic infections lately (79,107,108). The HTS is a DNA-based analysis that can yield many DNA sequences in a single run (109). Moreover, HTS has an extensive sampling depth and coverage, allowing for the detection of the most dominant bacterial community members and low-abundance taxa (110).

Several HTS technologies have become available over the last decade and are widely used in microbiome studies (111). The two most common NGS platforms explored

in endodontics has been 454 pyrosequencing and Illumina-based technologies (79). Early NGS studies investigated bacterial communities in primary endodontic infections (PEIs) using 454 pyrosequencing technology (80,85,86). However, like any other technology, the 454-pyrosequencing platform has some disadvantages (97). After 454-pyrosequencing technology was discontinued, the Illumina MiSeq platform is currently the commonly most used technology in oral research (79,107,108). It is favored for its exceptional combination of low sequencing error rates, high-quality reads, and cost-effectiveness (48).

Recently, the Illumina MiSeq platform has been used to investigate the bacteriome in teeth PEIs (77,112–129), secondary/ persistent infections (116,124,127–129), and teeth with endodontic-periodontal lesions (130,131) (Table 2.1). Although these studies have contributed to a better understanding of the bacteriome involved in PEIs, more clinical studies are needed for a better understanding of the pathogenesis of AP and to better define the bacteriome of PEI for future development of the targeted therapeutic approach. Therefore, this clinical study characterized the bacteriome in PEI with AP, identified core and rare bacteriome species and community diversity metrics, and analyzed the relationship between the bacteriome composition, diversity and features, and patient variables.

Table 2.1 Illumina Miseq platform studies in primary endodontic infection (PEI) with apical periodontitis (AP).

Reference #	Author	Year	Type of study	Platform	Region	OTU	ASV	LEfSe	Sampling Technique	Other site sampled	Type of infection (# of samples)
15	Rôças et al.	2016	Ex vivo	Illumina Miseq	V4	Yes	No	No	Caries excavator	No	Primary endodontic infection (n=10)
16	Slaton K et al.	2017	In vivo	Illumina Miseq	V4	Yes	No	No	Syringe	No	Primary endodontic infection (n=9)
17	Persoon et al.	2017	Ex vivo	Illumina Miseq	V3-V4	Yes	No	No	Cryopulverization	No	Primary endodontic infection (n=26)
18	Irıboz et al.	2018	In vivo	Illumina Miseq	V3-V4	Yes	No	No	Paper-point	No	Primary endodontic infection (n=20)
19	Boullaguet et al.	2018	Ex vivo	Illumina Miseq	V3-V4	Yes	No	No	File	No	Primary endodontic infection (n=21) and secondary/ persistent infection (n=22)
20	Tawfik et al.	2018	In vivo	Illumina Miseq	V3-V4	Yes	No	No	File/ Paper-point	No	Primary endodontic infection (n=19)
21	Qian et al.	2019	Ex vivo	Illumina Miseq	V3-V4	Yes	No	No	Cryopulverization	No	Primary endodontic infection (n=23) and secondary/ persistent infection (n=8)
22	de Brito et al.	2020	In vivo	Illumina Miseq	V3-V4	Yes	No	No	File	No	Primary endodontic infection (n=15)
23	Nardello et al.	2020	In vivo	Illumina Miseq	V4-v5	Yes	No	No	Paper-point	No	Primary endodontic infection (n=5)
24	Moraes et al.	2020	In vivo	Illumina Miseq	V1-V3	Yes	No	Yes	Paper-point	Yes*	Primary endodontic infection (n=12)
25	Zahrán et al.	2021	In vivo	Illumina Miseq	V3-V5	Yes	No	No	Paper-point	No	Irreversible pulpitis (n=30)
26	Amaral et al.	2022	In vivo	Illumina Miseq	V3-V4	Yes	No	No	Paper-point	No	Primary endodontic infection (n=25)
27	Zahrán et al.	2022	In vivo	Illumina Miseq	V3-V4	Yes	No	No	Paper-point	No	Primary endodontic infection (n=75)
28	de Castro Kruly et al.	2022	In vivo	Illumina Miseq	V3-V4	Yes	No	No	Paper-point	No	Primary endodontic infection (n=10) and secondary/ persistent infection (n=9)
29	Manoharan	2020	In vivo	Illumina Miseq	V3-V4	No	Yes	No	Paper-point	No	Primary endodontic infection (n=52)
30	Fouad et al.	2022	In vivo	Illumina Miseq	V4	Yes	No	Yes	File and paper-point	No	Permanent tooth with a necrotic pulp (n=97)
31	Ordinola-Zapata	2023	In vivo	Illumina Miseq	V3-V4	Yes	No	No	File and paper-point	No	Primary endodontic infection (n=31) and secondary/persistent infection (n=27)
32	Kesim et al.	2022	In vivo	Illumina Miseq	V3-V4	No	Yes	Yes	Paper-point	No	Primary endodontic infection (n=10) and secondary/ persistent infection (n=10)
33	Georgiou et al.	2023	Ex vivo	Illumina Miseq	V4	Yes	No	Yes	Cryopulverization	Yes+	Primary endodontic infection (n=7) and secondary/ persistent infection (n=20)
34	Gomes et al.	2022	In vivo	Illumina Miseq	V3-V4	Yes	No	No	Paper-point	Yes†	Endodontic-periodontal lesion (n=10)
35	Gomes et al.	2023	In vivo	Illumina Miseq	V3-V4	Yes	No	Yes	Paper-point	Yes	Endodontic-periodontal lesion (n=15)

* Saliva/ supragingival biofilm; + Blood; and †Periodontal pocket.

2.2 Materials and Methods

2.2.1 Patients and case selection

Twenty-seven patients with PEI with AP who presented to the postgraduate endodontic clinic at the University of Maryland School of Dentistry (UMSOD) for root canal treatment meeting the inclusion and exclusion criteria were enrolled in this study. The research protocol was approved by the Institutional Review Board (IRB) of the University of Maryland Baltimore (HP-00082693). All patients included consented for the treatment by signing consent forms. A medical history was recorded for each individual, followed by clinical and radiographic examination. The pulpal diagnosis was determined using a cold test (Endo Ice, 1,1,1,2- tetrafluoromethane; Coltène/Whaledent AG, Altstätten, Switzerland) and an electric pulp test (EPT, Kerr Corporation, Brea, CA). The periapical diagnosis was evaluated using percussion and palpation tests and periapical radiographs. Clinical and radiographic features such as tenderness to percussion (TTP) and pain on palpation (POP), and size of radiolucent area (SRL) were collected. The following patient data were tabulated for further analysis: age [< 60 and ≥ 60 years old], gender [Male and female], behavioral factor [smoker and non-smoker], symptomatology [symptomatic or asymptomatic], and lesion size [< 5 or ≥ 5 mm].

The inclusion criteria were: (a) adult subjects, age >18 years old; (b) healthy individuals, with no significant medical history; (c) primary endodontic infection; (d) molars with pulp necrosis with no response to cold and EPT test; (e) mature apices; and (f) presence of AP detected radiographically. The exclusion criteria were (a) teeth with extensive caries and coronal destruction that prevented a proper rubber dam isolation, (b) crown or root fracture, (c) exposed pulp chamber to the oral cavity, (d) acute apical abscess,

(e) probing > 4 mm, (f) open apexes, (g) critical anatomy, and (h) calcified canals. Patients under antibiotic therapy in the last three months were excluded. A tooth that yielded no bacterial DNA recovery after DNA extraction was also excluded.

2.2.2 Root canal sampling (RCS)

Disinfection for the operative field was performed as previously described (132). Briefly, the tooth was isolated with a rubber dam. The crown and surrounding structures were cleansed with 30% hydrogen peroxide (H₂O₂) for 30 seconds, followed by 5.25% NaOCl for 30 seconds, and then inactivated with 5% sodium thiosulfate. Disinfection of the external surfaces of the crown was checked by taking a swab sample from the crown surface and streaking it onto blood agar plates, which were incubated at 37°C in an aerobic and anaerobic atmosphere. A two-stage access cavity was prepared using a sterile high-speed carbide bur under manual irrigation with sterile saline (132). The first stage involved the removal of major contaminants, including carious lesions and restorations. In the second stage, the access cavity was disinfected before entering the pulp chamber according to the abovementioned disinfection protocol. The disinfection of the internal surface of the access cavity was evaluated as previously described. In the second stage, a new sterile bur was used under irrigation with a sterile saline solution to access the canals. The efficacy of the decontamination protocol was confirmed by PCR analysis using universal bacterial primers. The root canal samples were taken from the palatal canals from maxillary molars and distal canals from mandibular molars (133). One tooth was sampled per patient. Before sampling, the mesial and distal buccal canals in the maxillary molars and mesial buccal and mesial lingual canals in the mandibular molars were sealed with Top dam light-curing resin gingival dam (FGM, California). The root canal samples were collected using sterile

paper points (Dentsply-Maillefer, Ballaigues, Switzerland). Three sterile paper points were consecutively placed into the full length of the canal, remaining in position for 60 sec. All paper points were stored in 500 µl DNA/RNA Shield (ZYMO RESEARCH, CA, USA) and at -80°C for future bacterial analysis.

2.2.3 DNA Extraction, sequencing and analysis

2.2.3.1 DNA Extraction, qPCR, Sequencing and Analysis

The Institute for Genome Sciences (IGS) at the University of Maryland School of Medicine conducted the DNA extraction and NGS sequencing. Briefly, the DNA from the root canal samples was extracted using the MagAttract Power Microbiome DNA/RNA Kit (Qiagen, Hilden, Germany) and bead-beating on the TissueLyser II according to the manufacturer's instructions and automated on a Hamilton STAR robotic platform (Hamilton Company, Reno, NV, USA). The same procedures were followed for negative controls (water). After DNA isolation, a 2-step PCR targeting the V3-V4 region of the 16S rRNA gene was used for DNA library preparation as previously described (134) using a unique dual indexing strategy (Illumina). Next-generation sequencing was performed on the Illumina MiSeq platform using paired-end 300 bp (600 cycles). The sequence data were processed according to Holm et al. (134). DADA2 was used to generate amplicon sequence variants (ASVs) and SILVA v132 16S rRNA gene database to train the RDP Naïve Bayesian classifier for taxonomic classification of ASVs (134,135). Sequence data were processed using the DADA2 pipeline, and the amplicon sequence variants (ASV) taxonomy assignment was done with the Human Oral Microbiome Database (HOMD). Downstream analyses were performed with R packages 'decontam,' 'phyloseq,' 'microbiome,' and 'microbiomeMarker.' Contaminant taxa were removed. Alpha and beta

diversity metrics were computed. Associations of alpha diversity were estimated for five selected categorical patient variables (age [< 60 and ≥ 60 years old], gender [Male and female], behavioral factor [smoker and non-smoker], symptomatology [symptomatic or asymptomatic], and lesion size [< 5 or ≥ 5 mm]) and tested using t-tests and ANOVA after normality testing. PERMANOVA was applied to test for associations with beta diversity community structure. A 'core bacteriome' was defined as ASVs with a detection threshold of 0.0001 and prevalence in a minimum of 30% of samples. Marker species of each clinical category were determined using Linear discriminant analysis (LDA) and effect size (LEFSe) analysis.

Quantitative PCR method (qPCR)

Triplicate reactions were prepared using 1.5 ul template genomic DNA in a 10 ul reaction scale with 1.8 uM of forward & reverse primer, 225 nM probe, and 1x Quanta Toughmix rox mastermix. Reactions were cycled 10 min at 95°C for Taq activation, 15 s at 95°C for denaturation and 1 min at 60°C for annealing and extension x 40 cycles. Cycle threshold values (i.e., Ct value) for each 16 S qPCR reaction were obtained using an instrument automated Ct threshold and automatic baseline in the CFX384 instrument software (Biorad, Hercules, CA). Absolute abundance was determined using a gene-specific standard curve covering a minimum of 6 orders of magnitude. Standards and positive and no-template were run in triplicate on a per-plate basis to ensure plate to plate reproducibility.

2.2.3.2 Amplicon sequence variants (ASVs) analysis - Raw V3-V4 amplicon sequencing data were processed with the DADA2 pipeline to generate amplicon sequence variants (ASVs), and taxonomic assignment of the ASVs up to the species level was done against

the Human Oral Microbiome Database (HOMD) with the RDP classifier to generate 7 level taxonomy. The further analysis did not include samples with <1000 reads/sample. The ASV count table was further processed in R (version 4.3.0) using the packages ‘phyloseq,’ ‘vegan,’ ‘microbiomeMarker,’ and ‘microbiome,’ designed for microbiome data analysis. First, the ASV table was imported into the R environment as a phyloseq object. Contaminant ASVs were identified using the package ‘decontam’ using the qPCR data as a quantitative metric and negative control, with the combined method (threshold=0.5) (136) as it provides improved contaminant detection (137). Contaminant ASVs were filtered, and the clinical samples' ASV table was rarefied to even depth for downstream analysis. Relative abundance plots at Phylum, Genus, and Species taxonomic levels were plotted after aggregating taxa of the same type to summarize the taxonomic composition of the root canal bacteriome in PEI with AP. Core and rare species were identified. Rare species are important determinants of microbial and functional biodiversity. Rare species were defined as those with prevalence below the 25th percentile of relative abundance, as reported earlier (138). Core species analysis was performed using a detection threshold=0.00001 and prevalence threshold=0.3 with the package ‘microbiome’ using the species level abundance table with compositional transformation. The core species were plotted as a heatmap, where expression values were denoted as low ="yellow, " high="red," and value="grey.”

2.2.3.3 Alpha diversity analysis - The influence of five selected categorical patient variables listed above were analyzed to assess their effects on root canal bacteriome parameters: age [< 60 and ≥ 60 years old], gender [Male and female], behavioral factor [smoker and non-smoker], symptomatology [symptomatic or asymptomatic] and lesion

size [< 5 or ≥ 5 mm]. The qPCR counts, and alpha community diversity measures (Chao1, Shannon, and Simpson metrics) were computed and compared between categories for the five clinical variables using Wilcoxon's test (for two groups) with a p-value of 0.05. The Chao1 index quantifies species richness, the Shannon diversity index quantifies species richness and evenness, and the Simpson index quantifies species dominance. A higher Chao1 value represents greater species richness, while a higher Shannon index represents higher diversity.

2.2.3.4 Beta diversity and LEfSe (Linear discriminant analysis Effect Size) -

Phylogenetic (weighted Unifrac) and non-phylogenetic (Bray-Curtis) beta diversity indices were computed. Principal Coordinate Analysis (PoCA) plots were generated, and PERMANOVA ADONIS (with 999 permutations) was applied to test the effect of clinical variables on beta diversity metrics. To characterize the inter-subject variability and 'community types' based on bacteriome community composition, hierarchical cluster analysis was performed using the Bray-Curtis distances with the Ward method, optimal cluster number was selected using gap statistics, and driver taxa for each cluster were determined using the LEfSe (Linear discriminant analysis Effect Size) using the 'MicrobiomeMarker' pipeline (139). Chi-Square tests were applied to assess the association of microbial community clusters with age [< 60 and ≥ 60 years old], gender [Male and female], behavioral factor [smoker and non-smoker], symptomatology [symptomatic or asymptomatic], and lesion size [< 5 or ≥ 5 mm]. Marker taxa for each variable were assessed using LEfSe (using the 'microbiomeMarker' pipeline, one of the most widely applied tools for microbiome biomarker discovery (140). Briefly, LEfSe identifies taxonomic features that best explain differences between two or more groups by

quantifying LDA (linear discriminant analysis) effect sizes. The threshold values used for LEfSe analysis were a Wilcoxon's cut-off value of 0.05 and an LDA cut-off value of 4. LEfSe analysis was applied to all taxa ranks.

2.2.3.5 Sparse estimation of correlations among microbiomes (SECOM distance) -

SECOM analysis was used for estimating linear and nonlinear relationships among bacteria while maintaining sparsity (141). The bacterial species level correlation was generated using MicrobiomeAnalyst (permutations=100, and Secom distance, $p=0.05$).

2.3 Results

A total of twenty-seven patients enrolled in this study. All sterility controls were negative (27/27). The total read count for 28 samples (including 27 clinical samples and 1 negative control) was 3634488. Three samples with low read count (less than 1000 reads/sample), which did not yield any non-chimeric reads, were filtered, leaving 24 samples for further analysis. The demographic, clinical, and radiographic features of the 24 patients included are summarized in Table 2.2. The qPCR counts for the 16S rRNA gene in the 24 samples ranged from 154.68 to 24447.55 (mean 6895 ± 8494.411). After trimming, quality filtering, and chimera removal of reads from the remaining 24 clinical samples and 1 negative control sample, 1462156 non-chimeric reads with an average of 52219.86 reads/sample (3.47% singletons) were analyzed further to formulate an ASV count table with 1667 ASVs. Seventy-one contaminant ASVs were identified using the DECONTAM pipeline and filtered, followed by removing the control sample and rarefaction to even depth (1324 ASVs).

2.3.1 Bacterial community - Nine bacteria phyla were detected across all samples (Figure 2.1). The bacterial phyla with top mean relative abundance across the samples were Bacteroidetes (35.70%), Firmicutes (27.69%), Synergistetes (11.77%), Fusobacteria (8.85%) and Actinobacteria (7.34%). Proteobacteria showed a mean relative abundance of 7.08%. Rarer phyla included Spirochaetes (1.55%), Chloroflexi (0.02%), and Saccharibacteria (TM7) (0.01%). Twenty-four ASVs were unmapped to any phylum. In terms of detection frequency, Bacteroidetes, Firmicutes, and Actinobacteria were detected in all 24 samples, Fusobacteria and Proteobacteria in 20/24 samples, Synergistetes in 16/24 samples, Spirochetes in 12/ 24 samples, and rare phyla Saccharibacteria (TM7) in 4/24 samples and Chloroflexi in 2/24 samples (Figure 2.1). A total of 113 genera were identified. 1199 ASVs were mapped to the genus level. The top 50 genera by mean relative abundance are shown in Figure 2.2. The 10 most abundant genera included Prevotella, Porphyromonas and Fusobacterium, Bacteroidaceae [G-1], Parvimonas, Fretibacterium, Pyramidobacter, Dialister, Olsenella, and Peptostreptococcus, which had a mean relative abundance >2%. In terms of prevalence (proportion of samples detected), Prevotella, Porphyromonas, Fusobacterium, Bacteroidaceae [G-1], Parvimonas, Fretibacterium, and Pyramidobacter showed a prevalence of 70% or higher.

Table 2.2 Demographic, clinical, and radiographic features distribution of the twenty-four patients analyzed

Variables			Median and Range values
Age	≤ 60 years (n=19)	> 60 years (n=5)	47.5 years (25 – 76 years)
Gender	Male (n=10)	Female (n=14)	-
Tooth	Maxilla (n=16)	Mandible (n=8)	-
Smoking	Smoker (n=7)	Non-smoker (n=17)	-
Symptomatology *	Yes (n=5)	No (n=19)	-
Lesion size	< 5 mm (n=18)	≥ 5 mm (n=6)	4 mm (2 – 7 mm)

* TTP = Tenderness to percussion (n=3); POP = Pain on palpation (n=0).

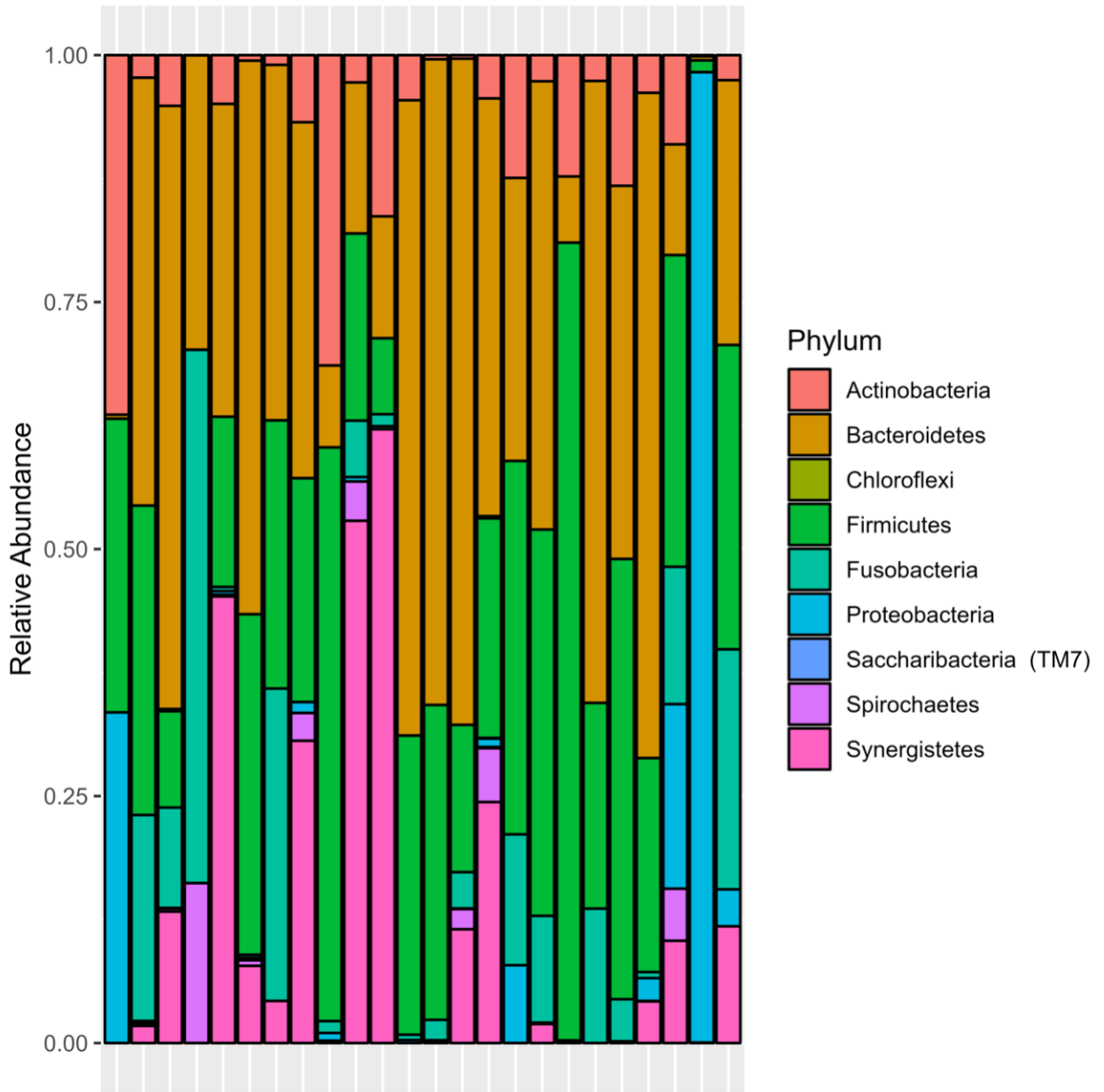


Figure 2.1 - Relative abundance bar plots of 9 bacterial phyla observed in the 24 samples.

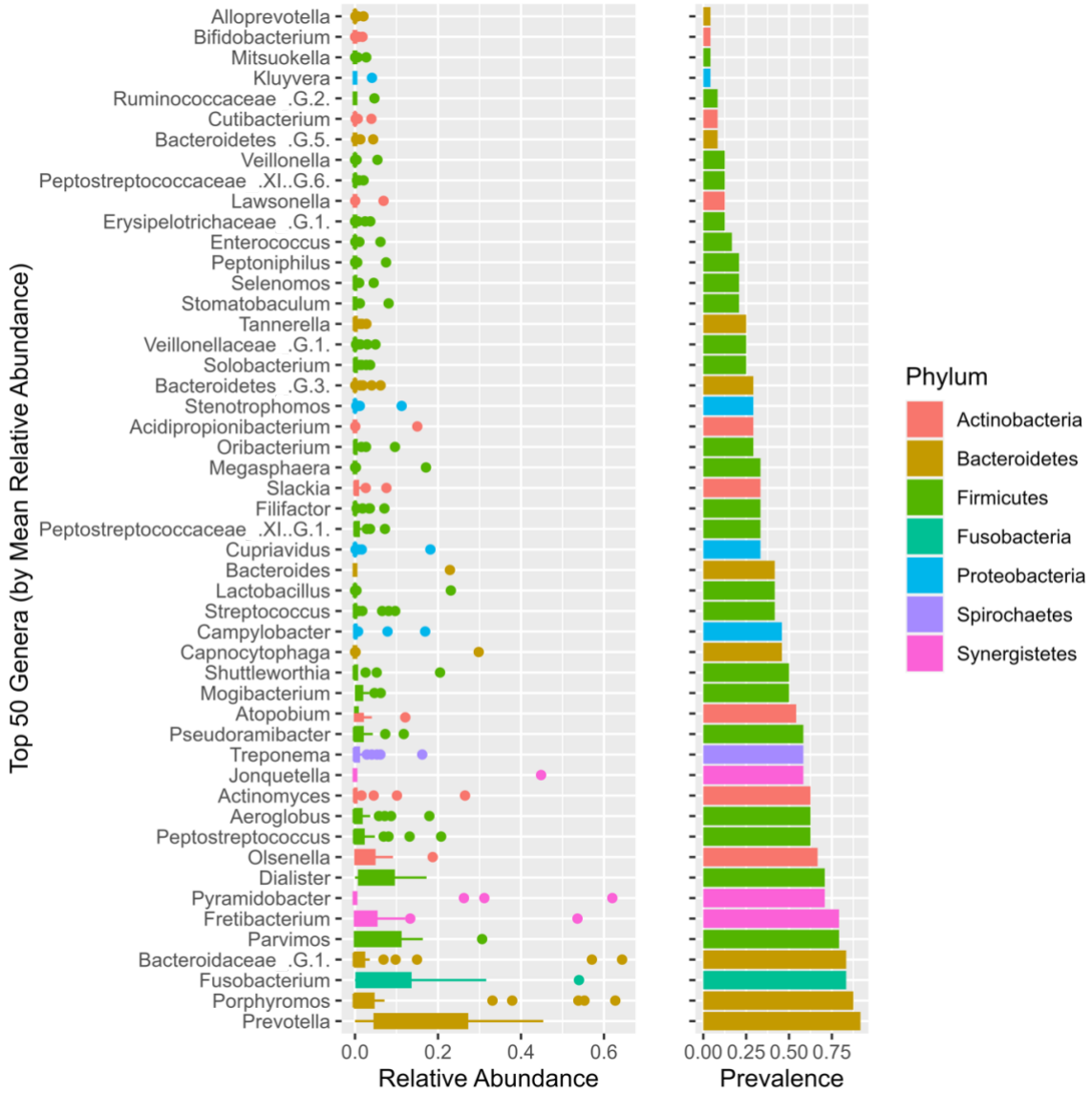


Figure 2.2 - The top 50 genera (by mean relative abundance) are depicted as relative abundance boxplots and prevalence (proportion of samples in which present). Box plots depict the median, 25th, and 75th percentiles as a box and 1.5 times the interquartile range as whiskers, and outliers are seen as dots. The colors of the bars correspond to their respective Phyla.

2.3.2 Core bacteriome - A total of 963 ASVs were annotated to the species level, and 215 species were identified. Twenty-four species were determined as core species at the prevalence threshold of 0.3 and are depicted in Figure 2.3a. Among these, *Dialister invisus*, *Slackia exigua*, *Prevotella oris* and *Parvimonas micra* were prevalent in >70% of all samples. A phylogenetic tree of the core species is shown in Figure 2.3b, showing that 10/24 core species belonged to the phylum Firmicutes and 7/24 to Bacteroidetes. Fifty-four rare species are depicted in Figure 2.4 as a phylogenetic tree.

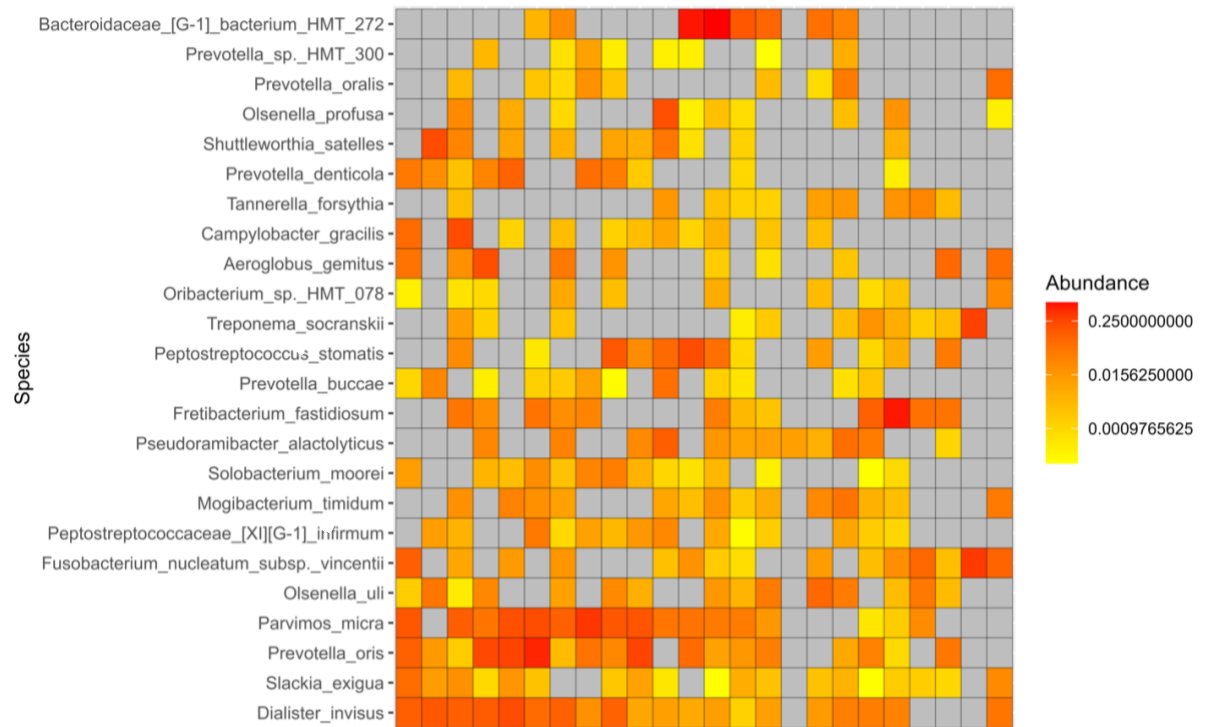


Figure 2.3a - Core bacteriome: A heatmap of 24 species determined as core species using the species-level abundance table. The core species are arranged in increasing order of prevalence.

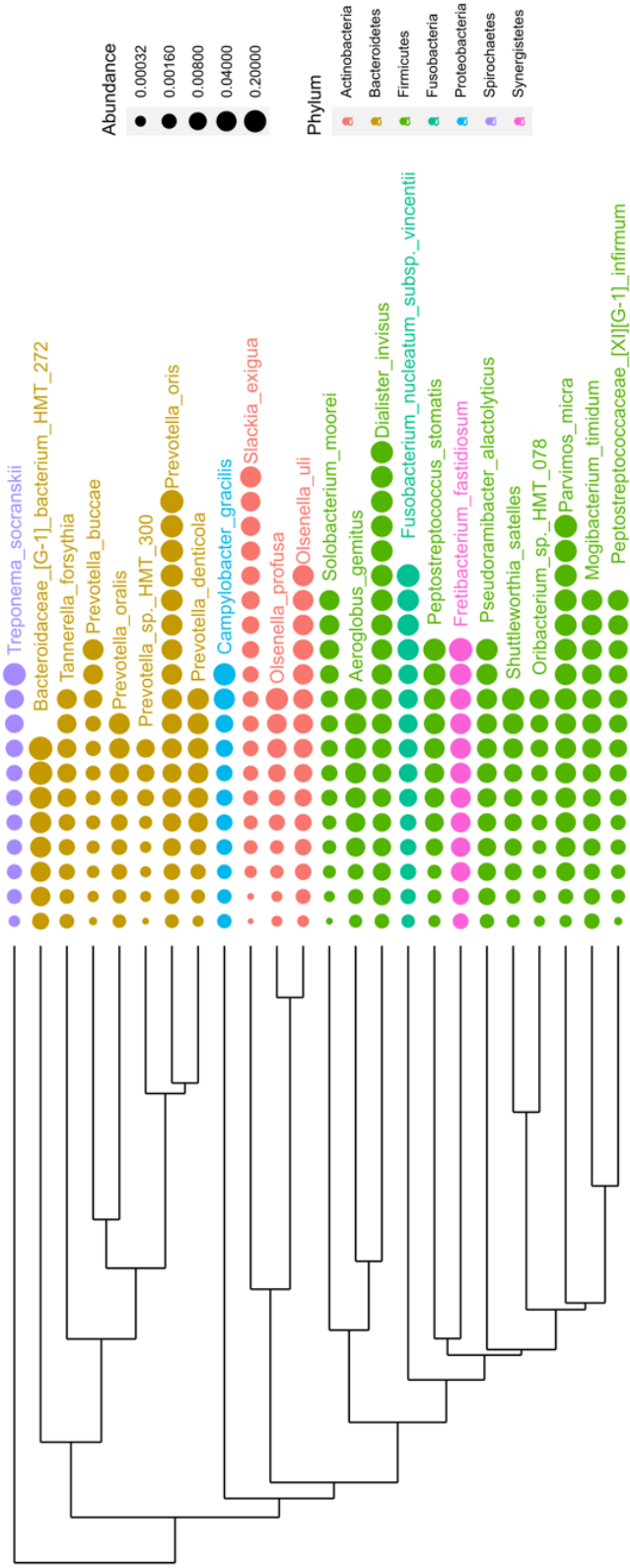


Figure 2.3b- Core bacteriome: A phylogenetic tree of the core species with tips and tip labels colored by Phylum. The size of the tips represents the abundance of samples

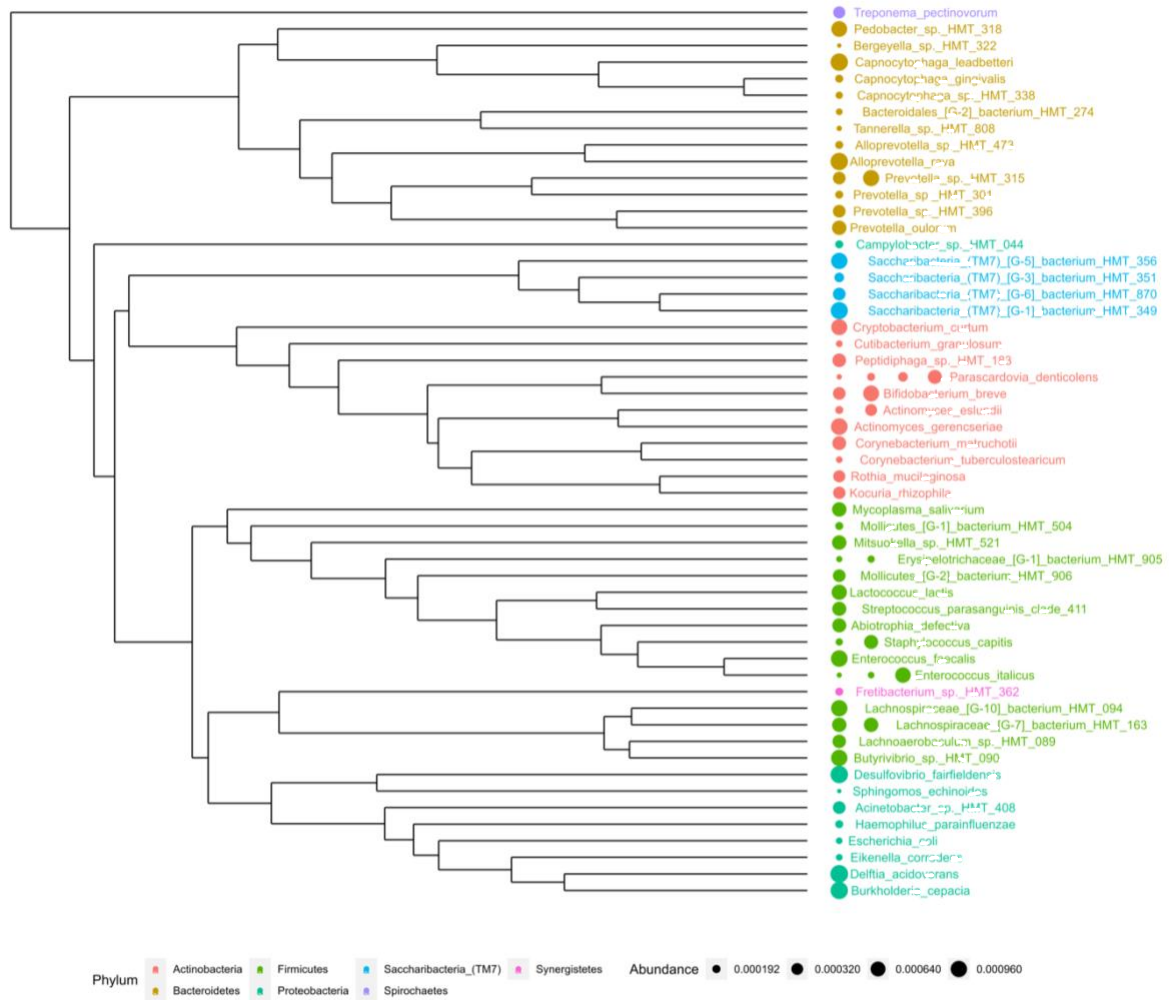


Figure 2.4 - A phylogenetic tree of the rare species with tips and tip labels colored by Phylum. The size of the tips represents the abundance of samples.

2.3.3 Alpha and beta diversity analysis- Shapiro-Wilk normality testing indicated a normal distribution of Chao1 ($W = 0.96$, $p\text{-value} = 0.43$) and Shannon ($W = 0.95$, $p\text{-value} = 0.32$) metrics, whereas Simpson ($W = 0.84$, $p\text{-value} = 0.002$) index showed non-normal distribution. Alpha diversity estimates showed no significant differences between samples grouped by selected variables (Figure 2.5). Beta diversity measures showed no significant differences in the Weighted UniFrac (phylogenetic) diversity of samples grouped by any of the 5 patient variables tested.

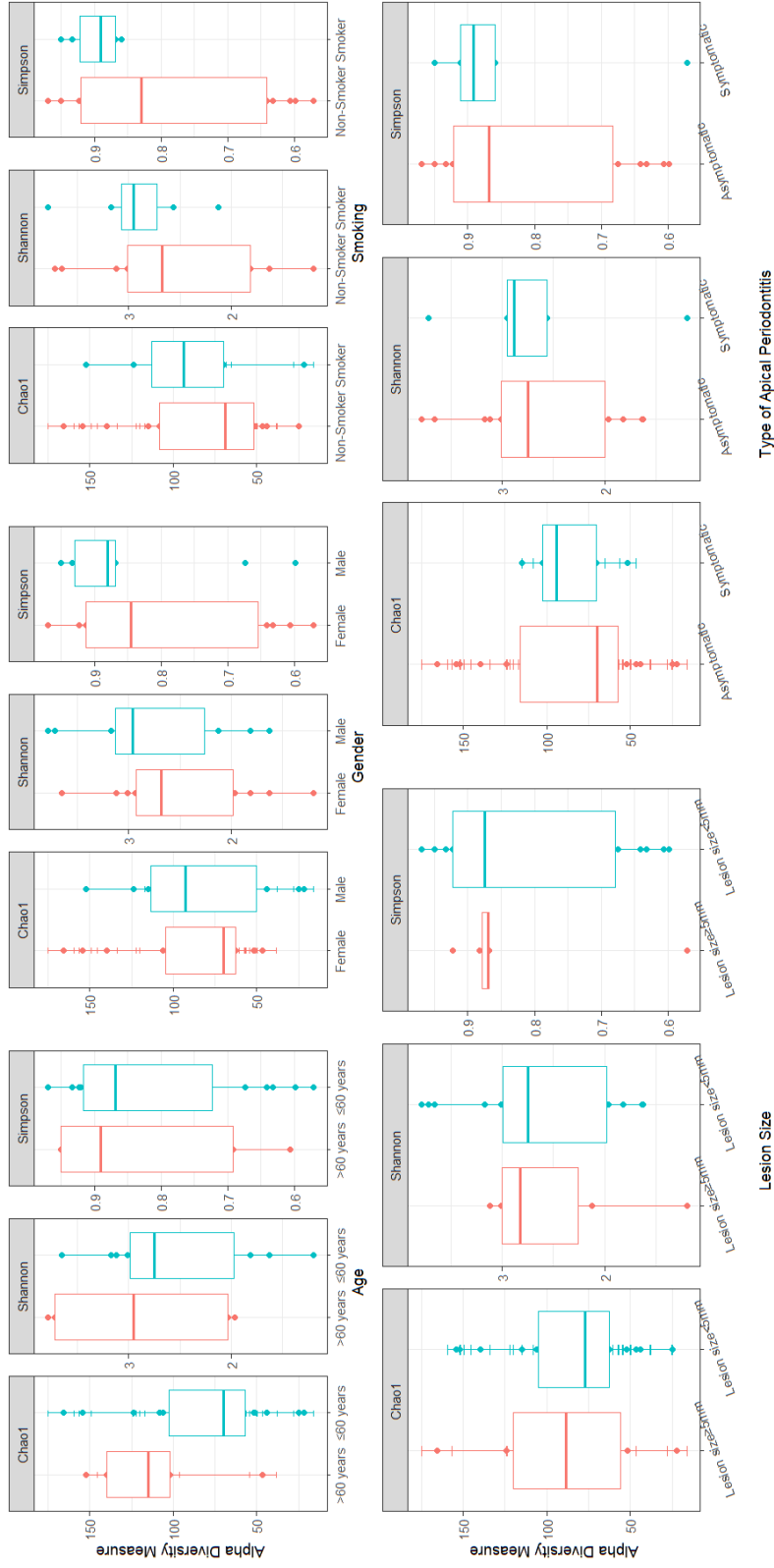


Figure 2.5 – Boxplots of alpha diversity metrics; Chao1 index, Shannon index, and Simpson’s index are shown. Samples were grouped by categories of the 5 selected patient variables.

2.3.4 Cluster analysis – Community typing with hierarchical clustering on Bray-Curtis distances clustered the samples into 3 clusters (Figure 2.6a). The clusters did not show a significant association with the patient’s variable categories. LEfSe analysis for driver taxa showed Cluster 1 was marked by enrichment with *Porphyromonas endodontalis*, Cluster 2 was driven by enrichment in unidentified species of Fusobacterium, along with *Dialister invisus*, and Cluster 3 drivers were Bacteroidetes species *Bacteroidaceae [G-1] bacterium HMT 272* and *Prevotella HMT 596* along with Synergistetes *Pyramidobacter piscolens* (Figure 2.6b).

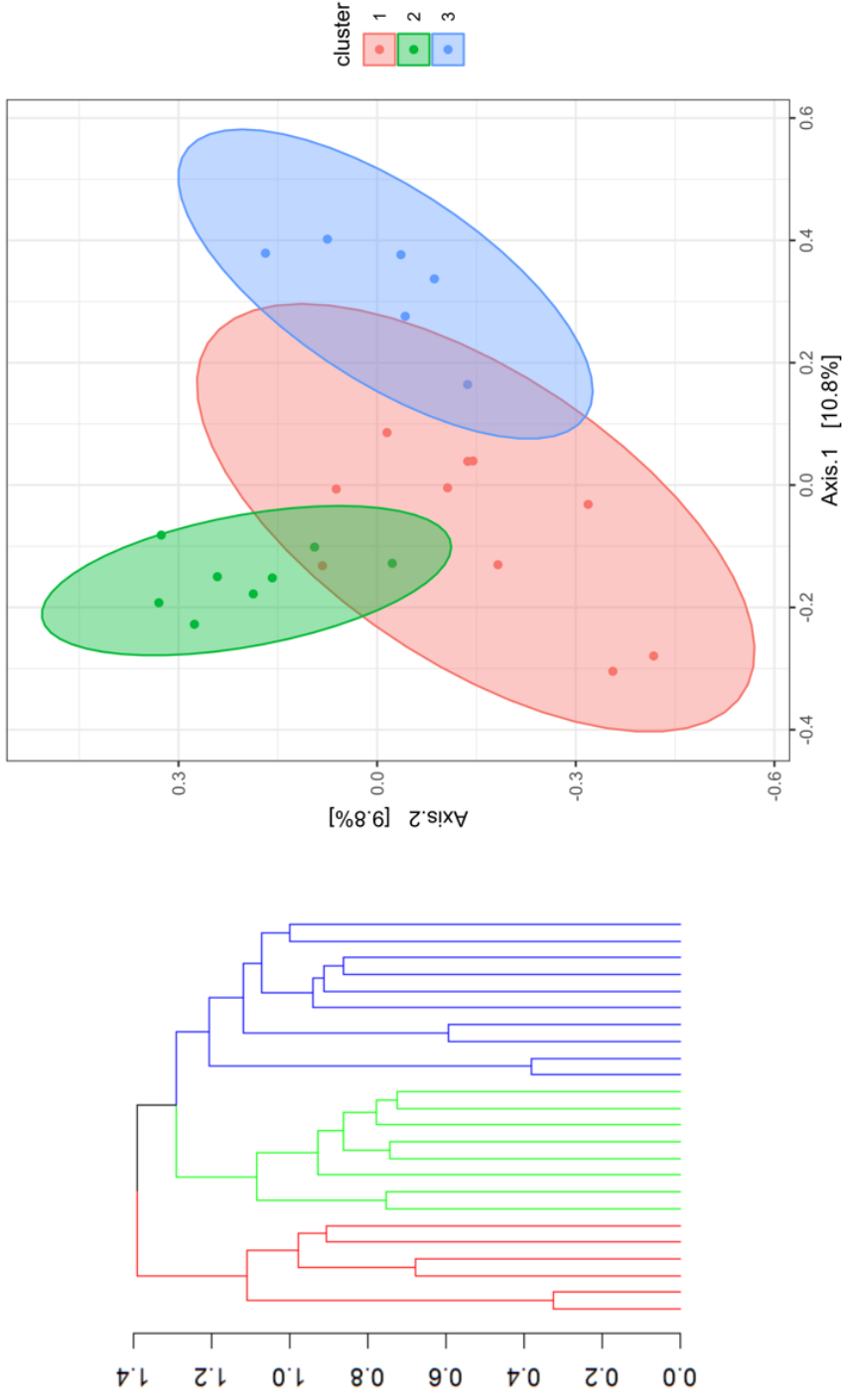


Figure 2.6a – *Cluster analyses*: bacteriome community typing by hierarchical clustering with Bray-Curtis dissimilarity matrix (Ward method). Gap statistic was used to select the optimal cluster number, and 3 clusters were identified. The left panel shows a dendrogram of samples with branch colors corresponding to cluster membership. The right panel shows an ordination plot using the Bray-Curtis dissimilarity with samples colored by cluster membership. Ellipses represent 95% confidence intervals.

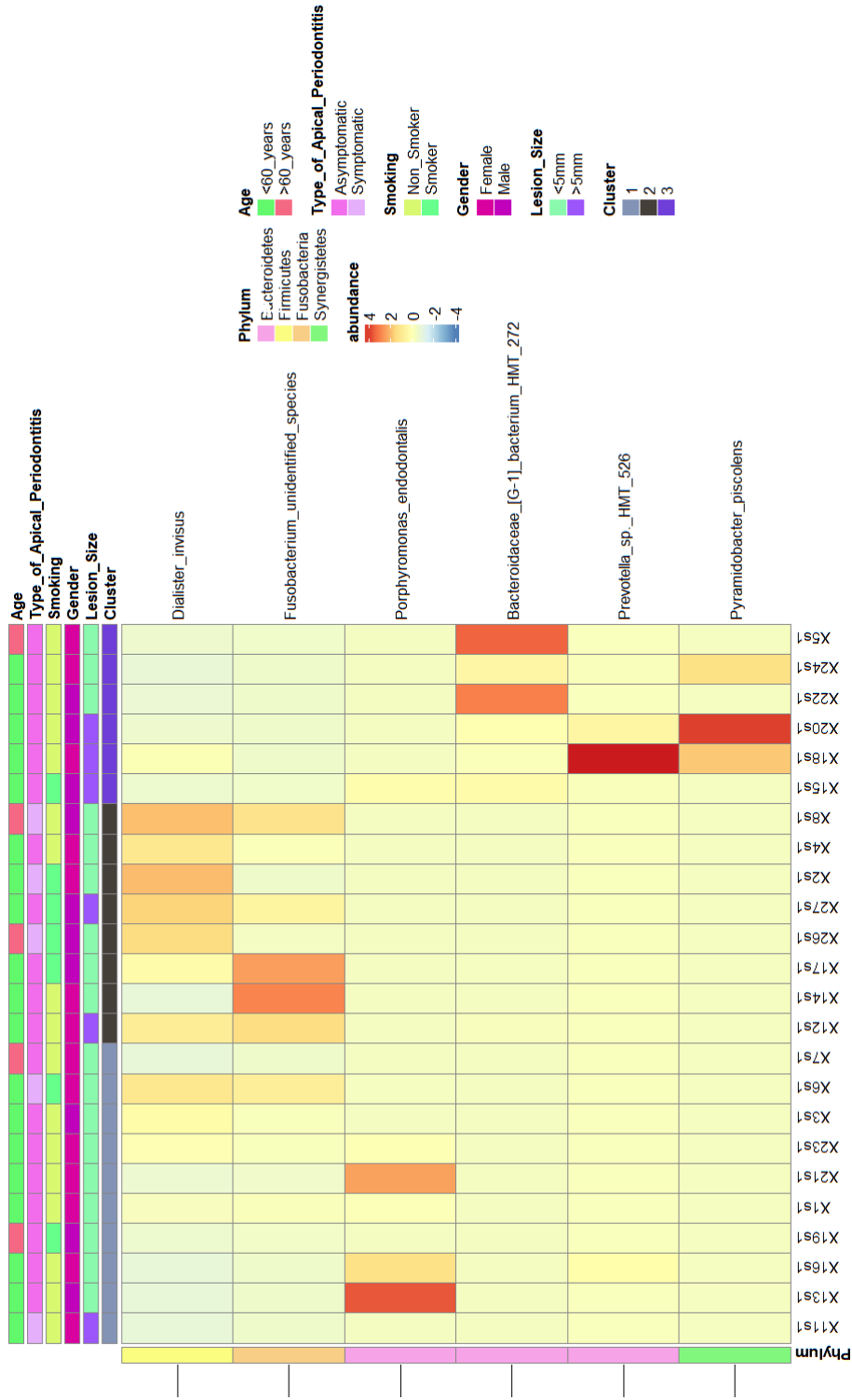


Figure 2.6b – *Cluster analyses*: Heatmap depicts the abundances of driver species of the three community-type clusters. Samples are clustered by cluster type, and clinical variable categories are annotated.

2.3.5. LEfSe (LDA, linear discriminant analysis effect size) - The results of the LEfSe analysis are depicted as effect size bar plots for significant markers with LDA effect sizes > 2 in Figure 2.7. *Fretibacterium* was the topmost discriminator taxa of Age > 60 years (Figure 2.7). Species discriminating in males were *Mogibacterium timidum* and *Prevotella oralis* (Figure 2.7). *Megasphaera sp. HMT 123* and *Treponema socranskii* were discriminant species for smoking (Figure 2.7). The two discriminators in symptomatic patients were *Peptostreptococcaceae [XI][G-2] bacterium HMT 091* and *Prevotella sp. HMT 315* (Figure 2.7). Lesion size ≥ 5 mm was marked by a number of *Prevotella sp. HMT 820*, *Prevotella multisaccharivorax*, *Prevotella sp. HMT 376*, *Prevotella sp. HMT 443* and *Prevotella dentalis*, while lesion size < 5 mm was marked by *Peptostreptococcus stomatis* (Figure 2.7).

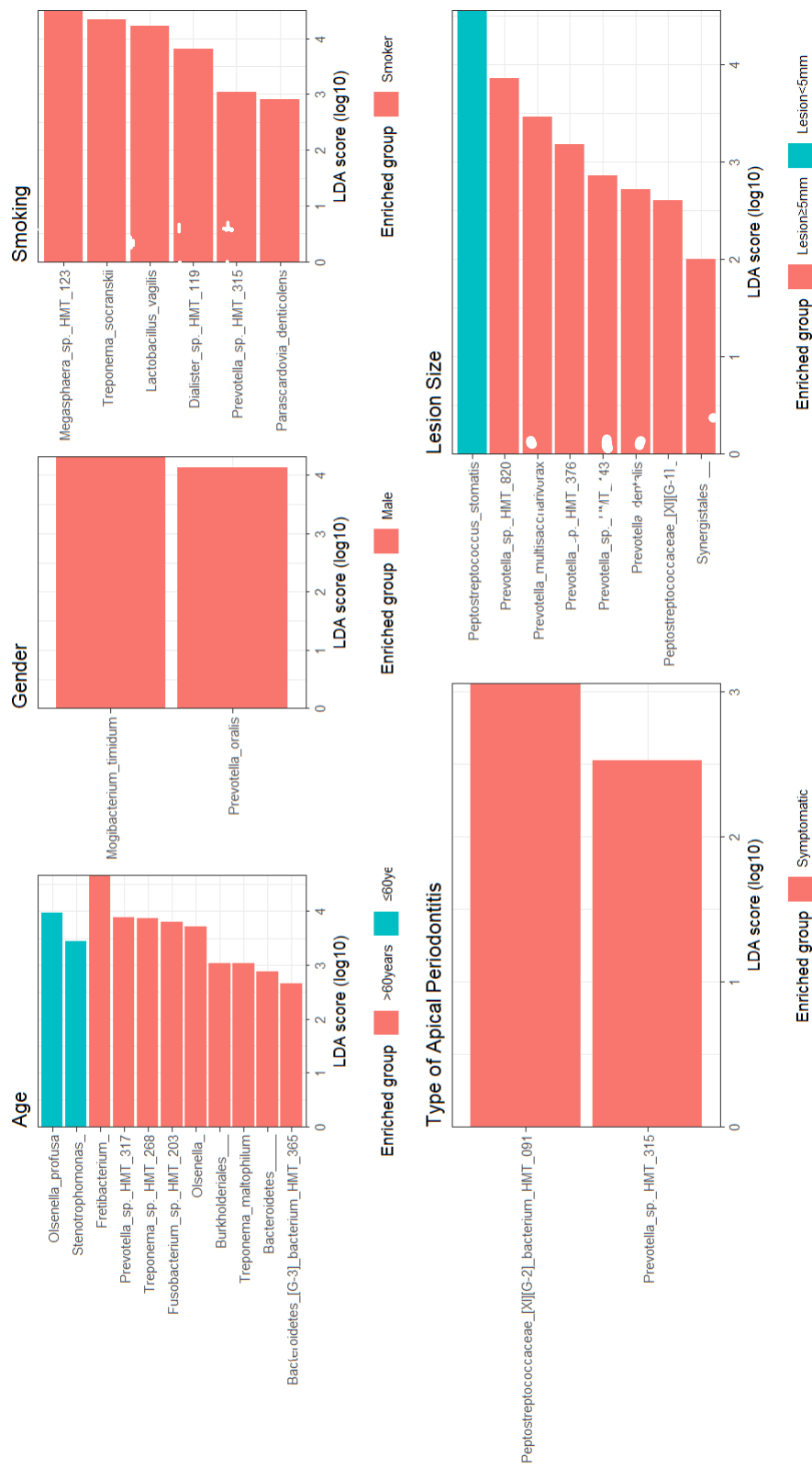


Figure 2.7 – Linear discriminant analysis Effect Size: effect size bar plots of marker taxa for the 5 selected patient variables determined by LEfSe analysis.

2.3.6 *SECOM distance analysis* - SECOM distance analysis indicated *Slackia exigua* as the node with the highest degree (Figure 2.8).

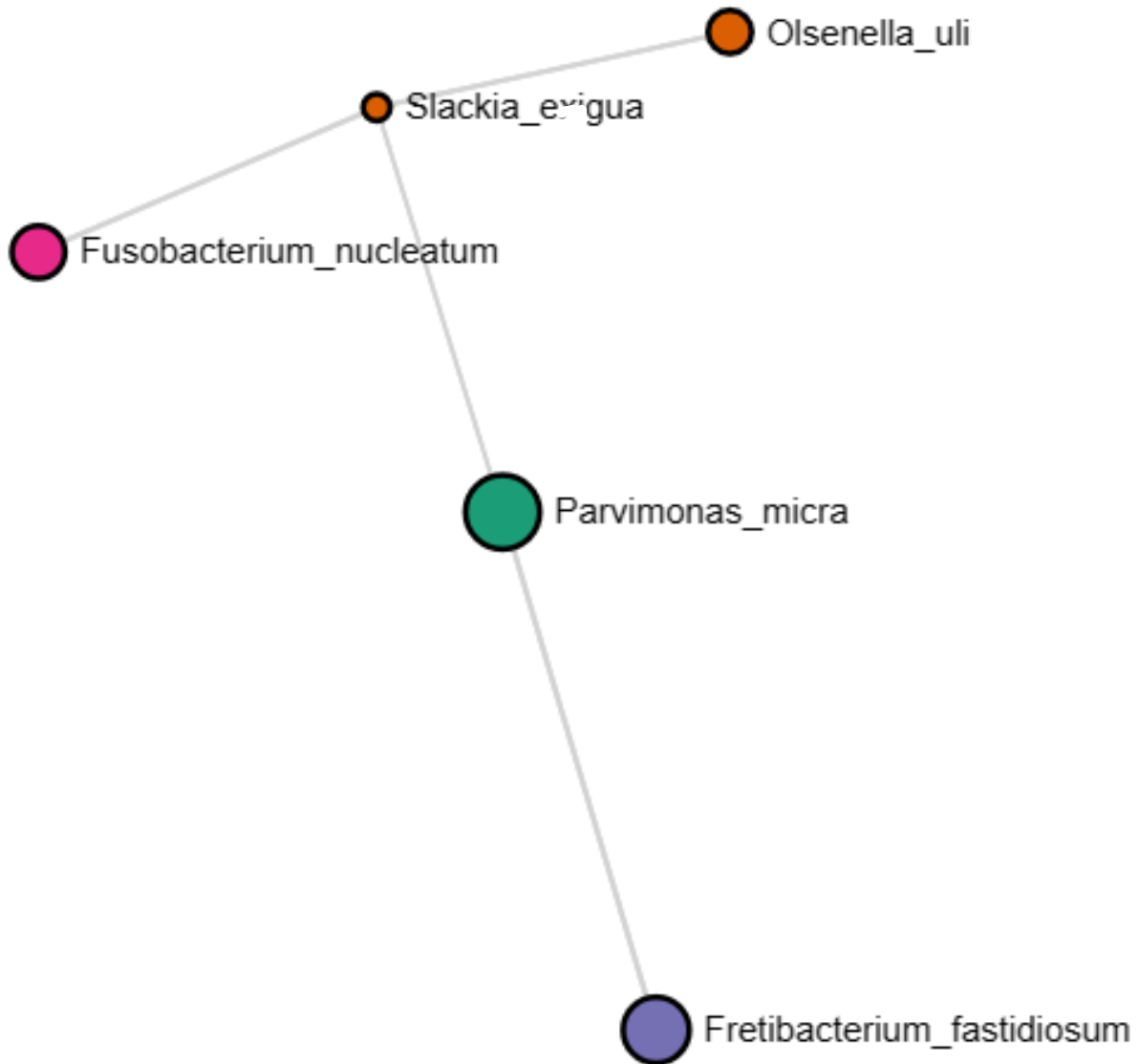


Figure 2.8 - Sparse estimation of correlations among microbiomes (SECOM distance): *Slackia exigua* as the node with the highest degree.

2.4 Discussion

Unlike previous NGS studies using 454 pyrosequencing technology (80,85,86), we used the Illumina MiSeq platform to investigate bacteriome in PEI with AP. The Illumina MiSeq method has improved the performance and quality of NGS studies related to elucidating the microbiomes in a diagnostic and ecological context, helping to reveal the bacterial composition (97,108,110). While the Roche 454 GS-FLX could sequence read lengths of 700 bases (bp) and generate ~1,000,000 shotgun and 700,000 amplicon reads per run, the Illumina MiSeq reads 2 x 250 bp and can generate 6.8 million (LRGC routinely getting N15 M) per run, which allows a deeper analysis of the bacterial community (97). Thus, differences in the bacterial community are expected when comparing these two NGS platforms. Additionally, among a wide range of NGS technologies, Illumina MiSeq has the lowest rate of sequencing errors and cost per sample (48).

Here, we target the 16S rRNA gene because it is highly conserved and ubiquitous among bacteria (142). It is the gold standard for bacterial taxonomy (142), even though for particular groups, it may not reach identification to the species level (143). The 16S rRNA gene contains nine hypervariable regions (HVRs, V1-V9), which differ between genera and bacterial species. Due to the high diversity of bacteria encountered in PEI, we sequenced the V3 - V4 regions, which yielded high-quality sequencing data (144) . Many of the Illumina MiSeq studies in PEI sequenced V3-V4 region (114–119,122–125,127,128,130,131); however, few of them have used amplification and sequencing based on different hypervariable regions such as V1-V3 (120), V3-V5 , V4 (112,113,126,129), and V4-V5 (77). Interestingly, differences among studies sequencing different hypervariable regions have been reported (145–147).

We used the amplicon sequence variants (ASVs) analysis instead of operational taxonomic units (OTUs) (77,112–124,126,127,129–131). Few in vivo studies are currently exploring ASV analysis in PEI (125,128). The ASV method has been used lately as a standard in bioinformatics processes and interpretation (148). OTU clustering has been debated because it causes the misassignment of query sequences to taxa (149), which could overestimate the infection's microbial abundance and bacterial diversity (150,151). Particularly, in ASV-based analysis, sequence reads are subject to a quality control analysis to remove low-quality samples, and the potential errors are denoised by computational methods (152). An advantage of ASVs over OTUs analysis is the sequence errors that are controlled up to one nucleotide difference, which confers higher sensitivity of ASVs in detecting biological variation (153). Therefore, caution must be taken when comparing bacterial communities between ASVs and OTUs studies.

The core bacteriome investigated in this study describes a group of microbial features highly representative of a particular environmental niche and are defined based on their detection and prevalence (154). We identified 24 species comprising the core intracanal bacteriome, largely supported by previous reports (79,108).). *Dialister invisus*, *Slackia exigua*, *Prevotella oris*, *Parvimonas micra*, and *F. nucleatum subsp. vincentii* were among the most prevalent and abundant core species. Several *Dialister* species have been consistently associated with endodontic infections, and *Dialister invisus* has been reported as abundant (125,155). The SECOM distance analysis indicated *Slackia exigua* as the node with the highest degree. *Slackia exigua* and *Mogibacterium timidum* are common species in root canal infection (125,156). *Slackia exigua* reduced the proliferation of stem cells in the apical papilla (157). Multiple *Prevotella* species were denoted as core species.

Consistent with our findings, *Prevotella oris* was reported as one of the most prevalent Prevotella species in PEIs (158). A consortium of *Atopobium rimae* and *Pseudoramibacter alactolyticus*, *Dialister invisus*, and *Fusobacterium nucleatum* have been associated with chronic lesions (159).

While common species are characteristic of a niche, rare species are increasingly recognized as key determinants of ecological and functional diversity (138). We characterized the rare species as those below the 25th abundance percentile. These included *Enterococcus faecalis*, commonly associated with endodontic failure and persistent periapical lesions. Several Proteobacterial species were noted among rare species. Proteobacteria are documented as low-abundance members of the intracanal microbial community in AP (77). They are shown to increase in relative abundance after disinfection (126) and secondary infection (108).

Microbial community typing enables the capture of inter-individual variation in microbiomes (160). We applied hierarchical clustering analysis and determined 3 clusters or ‘types.’ Cluster 1 was marked by enrichment with Porphyromonadaceae, including *Porphyromonas endodontalis* at the species level, which has been associated with symptomatic teeth (119). Cluster 2 was driven by enrichment in Fusobacteria at the phylum level and unidentified species of Fusobacterium, along with Veillonellaceae, including *Dialister invisus*. Cluster 3 showed a more diverse pattern of drivers with Bacteroidaceae, including *Bacteroidaceae [G-1] bacterium HMT 272* and Synergistetes, including *Pyramidobacter piscolens*. It was also demarcated by the enrichment of *Prevotella HMT 596*, which was previously documented as a core species in intracanal microbiomes (119). *Bacteroidaceae [G-1] bacterium HMT 272* abundance has decreased after instrumentation

(161). *Pyramidobacter piscolens* is one of the main Synergistetes species associated with endodontic infections (161). We found no significant differences in clinical variables between the cluster community types. Overall, community typing reflected the wide inter-individual variability in the intracanal bacteriome composition, and clinical traits were not discriminated against by exploratory microbiome-based unsupervised clustering. However, the statistical data from this exploratory analysis can inform power calculations (162) for future case-control studies to test the differences in alpha and beta diversity between clinical variables in PEI.

Linear discriminant analysis (LDA) is a supervised learning approach for discovering features that best classify and predict metadata class labels. LefSe is an algorithm for high-dimensional biomarker discovery and explanation that identifies genomic features such as genes, pathways, or taxa to characterize the differences between two or more selected conditions (140). Here, we used the LefSe analysis to identify marker-abundant taxa for selected patient variables, including age, gender, behavioral factors (smoker and non-smoker), symptomatology, and lesion size. Several of the current LefSe findings are supported by previous oral microbiome reports (161,163–166). Age has been associated with an increase in oral *Treponema* (163). Similarly, smoking is also documented to increase *Treponema* and *Dialister* species in the subgingival niche and saliva (164,165). Several *Prevotella* species were noted as discriminant taxa of larger periapical lesion size and are typically reported as one of the most frequent species in AP (166). *Peptostreptococcaceae* [G-2] bacterium HMT 091 was a marker of symptomatic AP, which has been earlier found to be linked to persistence after chemomechanical debridement (161). Overall, the current LDA analyses suggest that the core bacteriome in

endodontic infections should be defined based on selected patient variables and social behaviors. However, larger-sampled and case-control design studies are warranted to validate these biomarker species. The relevance of microbiome-based community types to longitudinal outcomes, such as the persistence of endodontic infection or treatment failure, also merits further investigation.

DNA sequencing-based techniques like targeted amplicon sequencing offer greater accuracy in capturing microbial complexity in low-biomass samples. However, contaminant DNA introduced from environmental or laboratory sources presents a significant challenge in low-biomass samples owing to their inherently low signal. To overcome such limitations, we applied a combined method of contaminant ASV identification using the negative control and the detection frequency in the DECONTAM algorithm, which outperforms the individual process. One of the limitations of this study is the paper-point sampling technique. Despite having obtained enough DNA biomass for the Illumina MiSeq technique for most samples analyzed, a particular concern exists in acquiring enough biomass samples from root canals using paper points exclusively. Alternatively, future NGS clinical studies, especially metagenomic or metabolomic ones, should consider pre-filing the canal walls with a small hand file before the paper-point sample.

Overall, this study described the diversity and inter-individual variations of the bacteriome involved in PEI with AP. Using previously described thresholds and methods, we described a core bacteriome consisting of 24 species with *Dialister invisus*, *Slackia exigua*, *Prevotella oris*, *Parvimonas micra*, and *F. nucleatum subsp. vincentii* as the most prevalent taxa and a rare or low-abundance bacteriome with multiple species noted as an

endodontic pathogens, highlighting their relevance to the diseased state. We also described ‘community types’ based on beta-diversity-based clustering and showed three distinct clusters driven by enrichment in *Porphyromonas endodontalis*, *Fusobacterium* and *Dialister invisus*, *Bacteroidaceae [G-1] bacterium HMT 272*, *Prevotella HMT 596*, and *Pyramidobacter piscolens*, respectively. Moreover, the LDA analysis identified discriminant bacterial species in distinct ages, genders, smoking, symptomatology, and lesion size groups, including multiple *Prevotella* species that discriminated against larger lesions. These findings provide valuable insights into the endodontic microbial community in PEI with AP.

2.5 Conclusion

The bacteriome in PEI with AP is complex and had high microbial heterogeneity among the patients. Moreover, age, gender, symptomatology, and lesion size might play a role in the abundant taxa present in PEI with AP.

CHAPTER THREE

Impact of root canal disinfection on the bacteriome present in primary endodontic infection: A next generation sequencing study¹

Chapter Abstract

Aim: To investigate the bacteriome present in teeth with primary endodontic infection (PEI) and apical periodontitis (AP) and to determine quantitatively and qualitatively the impact of chemomechanical preparation (CMP) using 2.5% sodium hypochlorite NaOCl on the bacteriome found in PEI with AP using the Illumina MiSeq platform.

Methodology: Thirty-six paired samples from 18 patients were successfully sequenced and analysed. Samples were collected at two sampling times: before (s1) and after (s2) CMP using 2.5% NaOCl. The DNA was extracted from s1 and s2 samples and quantified using quantitative PCR (qPCR). All 36 samples were sequenced using the Illumina MiSeq platform. Raw V3–V4 amplicon sequencing data were processed with the DADA2 pipeline to generate amplicon sequence variants (ASVs). Alpha diversity metrics representing abundance (Chao1) and diversity and evenness (Shannon, Simpson) were computed. The paired-sample Wilcoxon's test was used to compare alpha diversity metrics and qPCR counts between s1 and s2. The PERMANOVA method (with 999 permutations) was applied to compare community composition between sample types (s1 versus s2) and between patient IDs. ALDEx2 (ANOVA-like differential expression tool for high-

¹ Alquria, T.A., Acharya, A., Tordik, P., Griffin, I. & Martinho, F.C. (2024) Impact of root canal disinfection on the bacteriome present in primary endodontic infection: A next generation sequencing study. *International Endodontic Journal*, 57, 1124–1135.

throughput sequencing data) to investigate differentially abundant taxa between s1 and s2. A paired-sample Wilcoxon's test was used to compare alpha diversity metrics and qPCR counts between s1 and s2.

Results: The qPCR counts were significantly higher in s1 compared to s2 ($p = .0007$). The Chao1 index indicated no difference in alpha diversity ($p < .7019$); whereas Shannon ($p = .0056$) and Simpson ($p = .02685$) indexes showed higher values in s2. The PERMANOVA test using Adonis2 showed a significant effect of sample time on community composition ($R^2 = .0630$, $p = .012$). Patient ID also showed a significant effect on community composition ($R^2 = .6961$, $p = .001$). At the genus level, Dialister, Mogibacterium, Prevotella, and Olsenella were differentially enriched at s1, while Actinomyces, Stenotrophomonas_unclassified, Enterococcus_unclassified, and Actinomyces_unclassified were differentially enriched in s2.

Conclusion: The bacteriome present in teeth with PEI with AP is complex and diverse. CMP using 2.5% NaOCl showed a high quantitatively and qualitatively disinfectant impact on the bacteriome present in PEI with AP.

3.1 Introduction

The primary cause of root canal infection is bacteria and their by-products (6,167). Therefore, one of the main goals of endodontic therapy is to disinfect the root canal system (168). However, root canal systems have a complex anatomy in terms of the isthmus and its ramifications, which makes disinfection challenging (167,169). Currently, no disinfection protocol can eliminate all bacterial contents from root canal infection, and the search for an optimal root canal disinfection protocol continues.

The endodontic therapy performed in a single visit relies mainly on chemomechanical preparation (CMP), which comprises the mechanical instrumentation and irrigation of the root canal system. Sodium hypochlorite (NaOCl) is the commonly used irrigant for root canal disinfection (170,171). Among its numerous properties, NaOCl displays potent antimicrobial activity (132,172–174), can dissolve biofilm components and pulp tissue (175–178), and can reduce bacterial virulence factors such as endotoxins and lipoteichoic acid (132,179,180). NaOCl concentrations ranging from 0.5% to 8% have been used for root canal disinfection, and there is no consensus on the ideal concentration (170).

Numerous clinical studies have investigated the antimicrobial activity of CMP using 2.5% NaOCl to treat PEI (48). Early clinical studies used traditional culture-dependent methods to determine the residual bacterial load as the colony-forming unit (CFU) count after CMP using 2.5% NaOCl (132,168,181–183). Despite the CFU-count method having been widely used to evaluate the effectiveness of CMP, the root canal environment in a PEI is complex and hard to replicate in vitro on an agar plate; providing an ideal air atmosphere condition to culture all the cultivable bacterial species simultaneously is a challenge. Therefore, early culture-dependent methods studies could

underestimate the total residual infection after CMP, particularly the unculturable bacterial species load. Additionally, numerous clinical studies have used closed-ended molecular methods based on conventional polymerase chain reaction (PCR) for single specific species (48,184,185) and multiplex PCR platforms such as checkerboard DNA–DNA hybridization and reverse-capture checkerboard hybridization (186–191) to determine the effectiveness of CMP using 2.5% NaOCl against PEI. However, these closed-ended PCR studies were limited to species-specific primers and therefore failed to characterize qualitatively the total residual bacterial community and unknown species of as-yet-uncultivated bacteria (48). Additionally, real-time quantitative PCR (RT- qPCR) had been used solely or associated with other molecular techniques to quantify the residual bacterial load in PEI after CMP using 2.5% NaOCl (123,192–194).

More recently, the next-generation sequence (NGS) has been commonly explored in endodontic infections (48,79,108,161,195). The most common NGS platforms explored in endodontics are 454-pyrosequencing and Illumina MiSeq (10,79). With 454-pyrosequencing platform discontinuation, the Illumina MiSeq is currently the most used NGS platform for oral research because of its singular combination of low sequencing error rates, cost-effectiveness, and high-quality reads (10,79). To the best of our knowledge, clinical Illumina MiSeq study by Zahran et al. (2022) is the only one to evaluate the impact of CMP using 2.5% NaOCl against the bacterial community profile in PEI, but they focused mainly on infection control measures. This clinical cross-sectional interventional study is intended to investigate the bacteriome present in teeth with primary endodontic infection (PEI) and apical periodontitis (AP) and to determine quantitatively and qualitatively the impact of CMP using 2.5% NaOCl on the bacteriome found in PEI

with AP using the Illumina MiSeq platform. The null hypothesis is that no difference exists in the bacteriome before and after CMP using 2.5% NaOCl.

3.2 Materials and Methods

3.2.1 Patients and case selection

The present cross-sectional interventional study was performed in compliance with STROBE (Strengthening the Reporting of Observational Studies in Epidemiology) guidelines. Patients with primary endodontic infection (PEI) with apical periodontitis (AP) who sought treatment at the postgraduate endodontic clinic at the University of Maryland School of Dentistry meeting the inclusion criteria were selected for participation. A total of 27 patients with PEI and AP were enrolled in this study. Approval for the research protocol was obtained from the Institutional Review Board (IRB) of the University of Maryland (HP- 00082693). Patients were recruited between 2019 and 2022. All patients signed consent forms to participate in this research. Following the medical history, all patients underwent clinical and radiographic examination. The pulpal diagnosis was established using cold (Endo Ice – 1,1,1,2-tetrafluoromethane; Coltène/Whaledent, AG, Altstätten, Switzerland) and electric pulp tests (EPT, Kerr Corporation, Brea, CA). The periapical diagnosis was evaluated using percussion and palpation tests and periapical radiographs. The inclusion criteria were (a) adult subjects, age >18 years old; (b) healthy individuals with no significant medical history; (c) PEI; (d) molars with pulp necrosis with no response to cold and EPT test; (e) mature apices; and (f) presence of AP detected radiographically. The exclusion criteria were (a) teeth with extensive caries and coronal destruction that prevented a proper rubber dam isolation; (b) crown or root fracture; (c)

pulp chamber exposed to the oral cavity; (d) acute apical abscess; (e) probing >4 mm; (f) open apices; (g) critical anatomy; and (h) calcified canals. Patients who had undergone antibiotic therapy in the last 3 months were excluded. Patients with samples yielding low sequencing reading counts (<1000 reads) after DNA extraction were excluded.

3.2.2. Root canal sampling (RCS) before (s1) and after (s2) treatment

Disinfection of the operative field was performed as described elsewhere (132). Briefly, the tooth was isolated with a rubber dam. The crown and surrounding structures were cleansed with 30% hydrogen peroxide (H₂O₂) for 30s, followed by 5.25% NaOCl for 30s, and then inactivated with 5% sodium thiosulfate. Disinfection of the external surfaces of the crown was checked by taking a swab sample from the crown surface and streaking it onto blood agar plates, which were incubated at 37°C in an aerobic and anaerobic atmosphere. A two-stage access cavity was prepared using a sterile highspeed carbide bur under manual irrigation with sterile saline (132). The first stage involved the removal of major contaminants, including carious lesions and restorations. In the second stage, the access cavity was disinfected before entering the pulp chamber according to the above-mentioned disinfection protocol. The disinfection of the internal surface of the access cavity was evaluated as previously described. In the second stage, a new sterile bur was used under irrigation with a sterile saline solution to access the canals. The efficacy of the decontamination protocol was confirmed by PCR analysis using universal bacterial primers (126). The root canal samples were taken from the palatal canals from maxillary molars and distal canals from mandibular molars (133). One tooth was sampled per patient. Before sampling, the mesial and distal buccal canals in the maxillary molars and mesial buccal and mesial lingual canals in the mandibular molars were sealed with a top dam light-

curing resin gingival dam (FGM, California, US). The root canal samples were collected using sterile paper points (PPs) (Dentsply-Maillefer, Ballaigues, Switzerland) before (s1) and after treatment (s2). Three sterile PPs were consecutively placed into the full length of the canal, which was predetermined radiographically, remaining in position for 60s. All PPs were stored in 500 μ L DNA/RNA Shield (ZYMO RESEARCH, CA, USA) at -80°C for future bacterial analysis.

After obtaining the baseline sample (s1), the working length (WL) was determined to be 1mm from the radio- graphic apex using an electronic apex locator (Root ZX II, J. Morita, USA), followed by confirmation of canal patency using a #10 K-file. Initially, the canal was irrigated with 5mL of 2.5% NaOCl, then hand instrumented up to a #15 K-file, using a crown-down rotary instrument to a size of 40/0.04. In cases in which resistance was encountered before reaching the WL, smaller rotary files were used until WL was attained. A #10 K-file was used throughout the instrumentation to maintain patency. The root canals were irrigated with 5 mL of 2.5% NaOCl. After CMP, the NaOCl was neutralized with 5 mL of sterile 0.5% sodium thiosulfate for 1 min, then removed with 5 mL of sterile saline. Subsequently, the canal was irrigated with 5mL of 17% EDTA for 3min and rinsed with 5mL of sterile saline. The second bacteria sample (s2) was obtained as previously described in the s1 sample collection. All PPs were preserved in 500 μ L DNA/RNA Shield (ZYMO RESEARCH, CA, USA) and stored at -80°C for future bacterial analysis. All root canals were disinfected as described above and obturated with gutta-percha and AH Plus sealer using warm vertical condensation and backfill techniques. Access cavities in all instances were restored with 2 mm of Cavit TM (3M Dental Products, St Paul, MN, USA) and FiltekTM Z250 (3M Dental Products).

3.2.3 DNA extraction and sequencing:

The DNA extraction and NGS sequencing were conducted by the Institute for Genome Sciences (IGS) at the University of Maryland School of Medicine. In summary, DNA was extracted from the root canal samples using the MagAttract Power Microbiome DNA/RNA Kit (Qiagen, Hilden, Germany), with bead-beating on the TissueLyser II following the manufacturer's instructions and automated on a Hamilton STAR robotic platform (Hamilton Company, Reno, NV, USA). The same procedures were applied to negative controls (water). After DNA isolation, a two-step PCR targeting the V3–V4 region of the 16S rRNA gene was utilized for DNA library preparation, employing a unique dual indexing strategy (Illumina), as previously described (134). Next-generation sequencing took place on the Illumina MiSeq platform utilizing paired-end 300 bp (600 cycles). The sequence data were processed following Holm et al.'s methodology (134). DADA2 was employed to generate amplicon sequence variants (ASVs), and the SILVA v132 16S rRNA gene database was utilized to train the RDP Naïve Bayesian classifier for taxonomic classification of ASVs (134,135).

3.2.4 Statistical analysis

V3–V4 amplicon sequencing data were processed with the DADA2 pipeline to generate ASVs with a taxonomic assignment against the Human Oral Microbiome Database (HOMD) with the RDP classifier. Downstream analysis was performed in R software (version 4.3.0) using the phyloseq, vegan, decontam, microbiome Marker, and microbiome packages. Samples that had >1000 sequences after the filtration steps were included. Only samples in which both s1 and s2 paired samples for a patient were available were included to ensure paired sample comparison. Contaminant ASVs were identified at

a user-selected threshold with the Decontam method the combined approach and were filtered (137). Unique and shared ASVs between s1 and s2 samples were determined and depicted in a Venn diagram. The ASV table was rarefied to even depth. Relative abundance bar plots were drawn at the phylum level with

samples grouped by patient ID to visualize the shift in bacteriome composition. Alpha diversity metrics representing abundance (Chao1) and diversity and evenness (Shannon, Simpson) were computed. A paired-sample Wilcoxon's test was used to compare alpha diversity metrics and qPCR counts between s1 and s2. Weighted unifracs beta diversity was determined, and ordination plots were drawn. The PERMANOVA method (with 999 permutations) was applied using Adonis2 from the vegan package to compare community composition between sample types (s1 versus s2) and between patient IDs (intra-patient versus interpatient comparison). Differences in abundant taxa at phylum, class, order, family, and species levels between s1 and s2 were determined using paired sample Aldex2 in the microbiome Marker package using the Wilcoxon's test and 128 Dirichlet Monte Carlo simulations. A heatmap of abundant taxa was drawn, and Aldex effect sizes were plotted between the endodontic bacteriome in s1 and s2. A p-value threshold of .05 (two-sided) was applied for all statistical tests.

3.3 Result

Figure 3.1 shows the STROBE flowchart. Thirty-six paired samples from 18 patients were successfully sequenced and analysed to compare s1 and s2. After contaminant ASV filtering, 1 872 767 sequence reads with an average of 52021.31 reads/sample (sparsity = 0.96) were assigned to 2523 ASVs and analysed further.

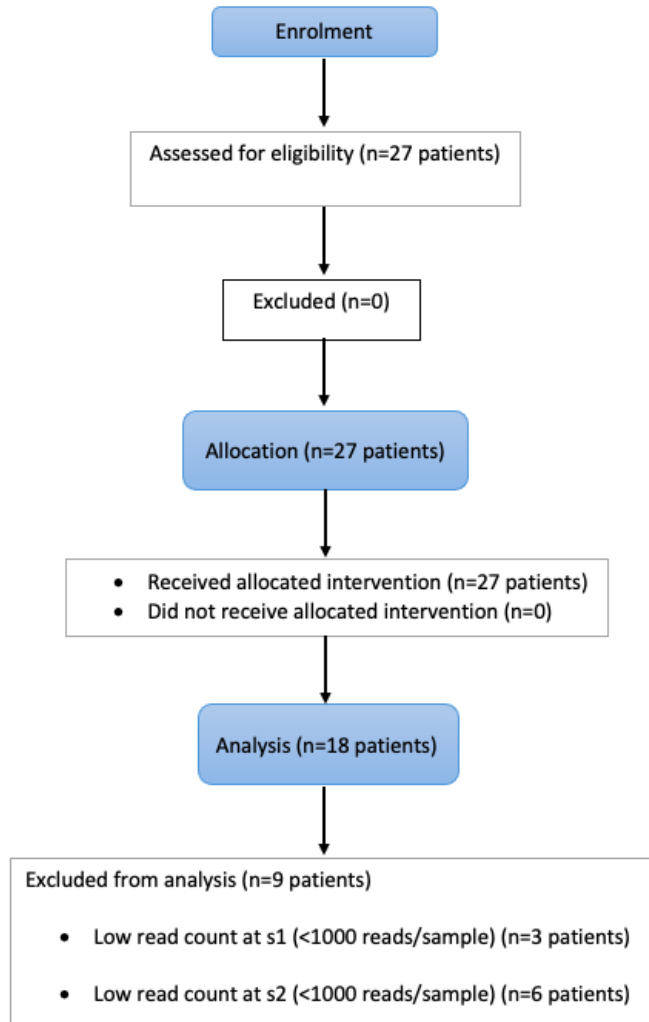


Figure 3.1: STROBE flow-chart

Figure 3.2 depicts a Venn diagram showing the unique and shared ASVs between s1 and s2; 996 ASVs were detected in s1, 1179 ASVs in s2, and 348 ASVs were shared.

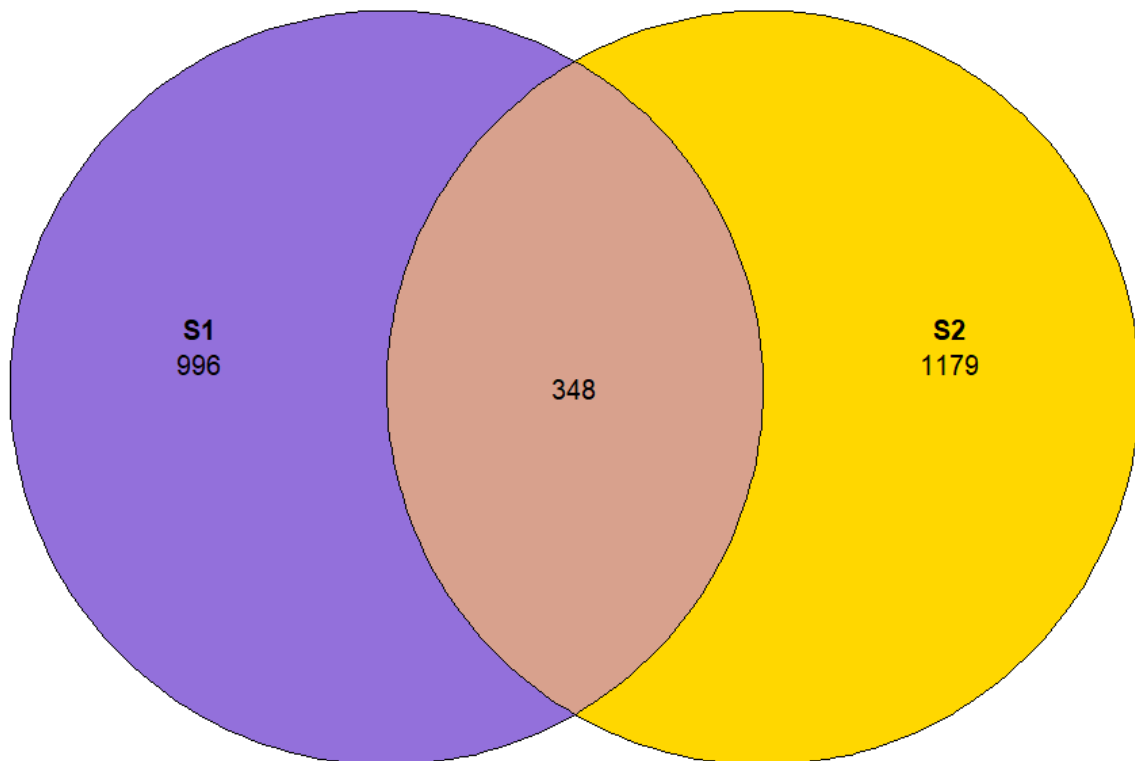


Figure3.2: Venn diagram showing shared and unique ASVs between s1 (pre-instrumentation) and s2 (post- instrumentation) samples.

The relative abundance of phyla is shown in Figure 3.3. The most abundant phyla at s1 were Bacteroidetes (38.37%), Firmicutes (19.58%), and Synergistetes (7.44%), while in s2, the most common were Firmicutes (30.77%), Bacteroidetes (24.11%), and Actinobacteria (8.04%).

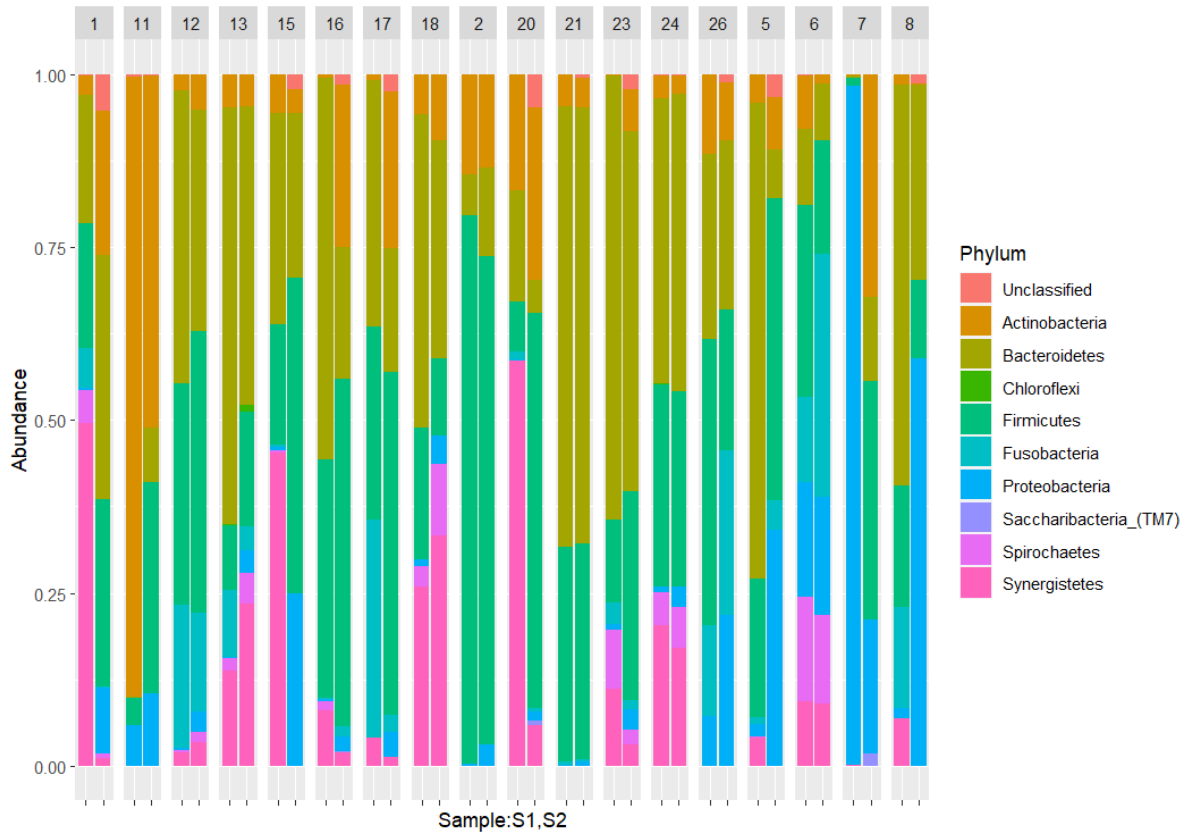


Figure 3.3- Relative abundance bar plots (100% stacked bar plots) of Phyla in s1 and s2 samples. Sample are grouped by patient ID.

The qPCR counts were significantly higher in s1 than s2 ($p = .0007$) (Figure 3.4). A Chao1 index indicated no difference in alpha diversity ($p < .7019$), whereas Shannon ($p = .0056$) and Simpson ($p = .02685$) indexes showed higher values in s2 (Figure 3.4).

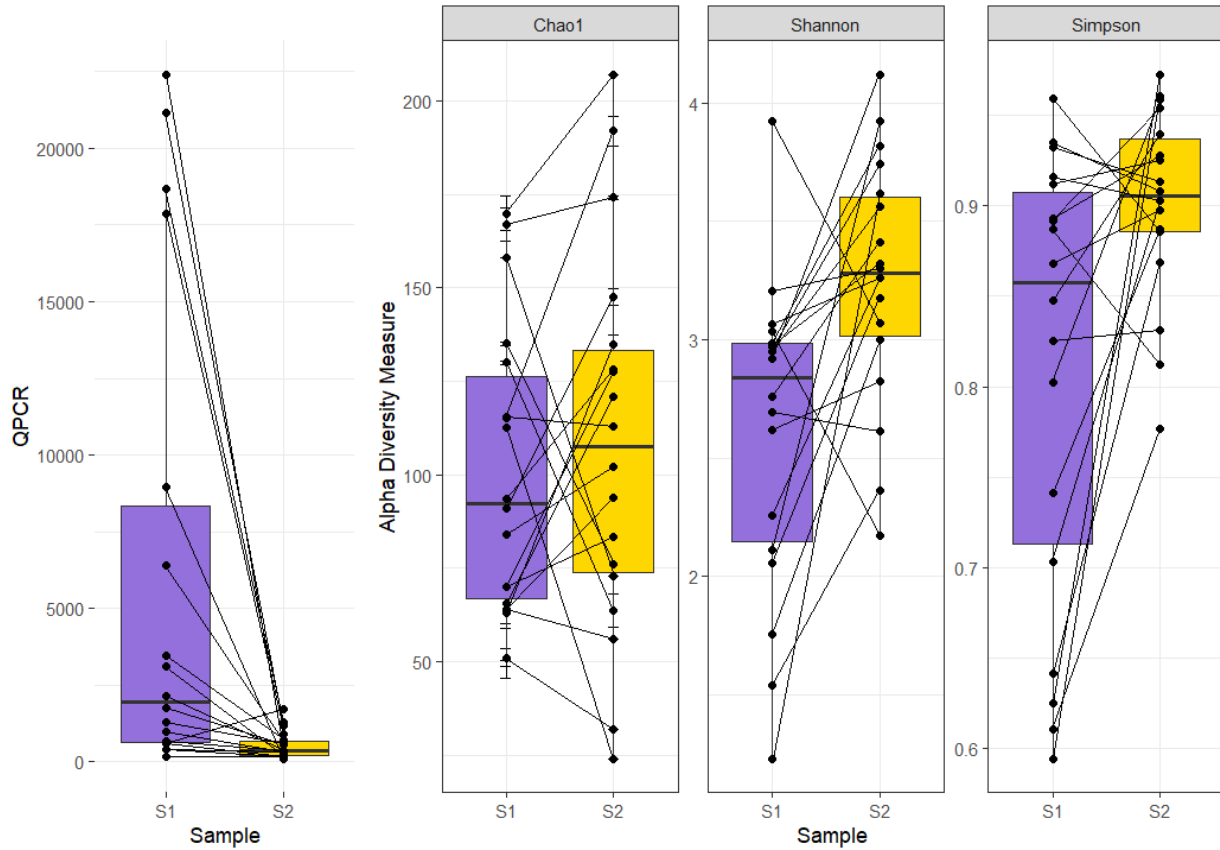


Figure 3.4- Boxplots showing the comparison of QPCR 16S rRNA gene copy counts and alpha diversity metrics. Black lines indicate paired samples from the same patient.

The ordination of the samples using the weighted Unifrac phylogenetic beta diversity metric is depicted in Figure 3.5. The PERMANOVA test using Adonis2 showed a significant effect of sample time on community composition ($R^2 = .0630$, $p = .012$) (Figure 3.5). Patient ID also showed a significant effect on community composition ($R^2 = .6961$, $p = .001$) (Figure 3.5).

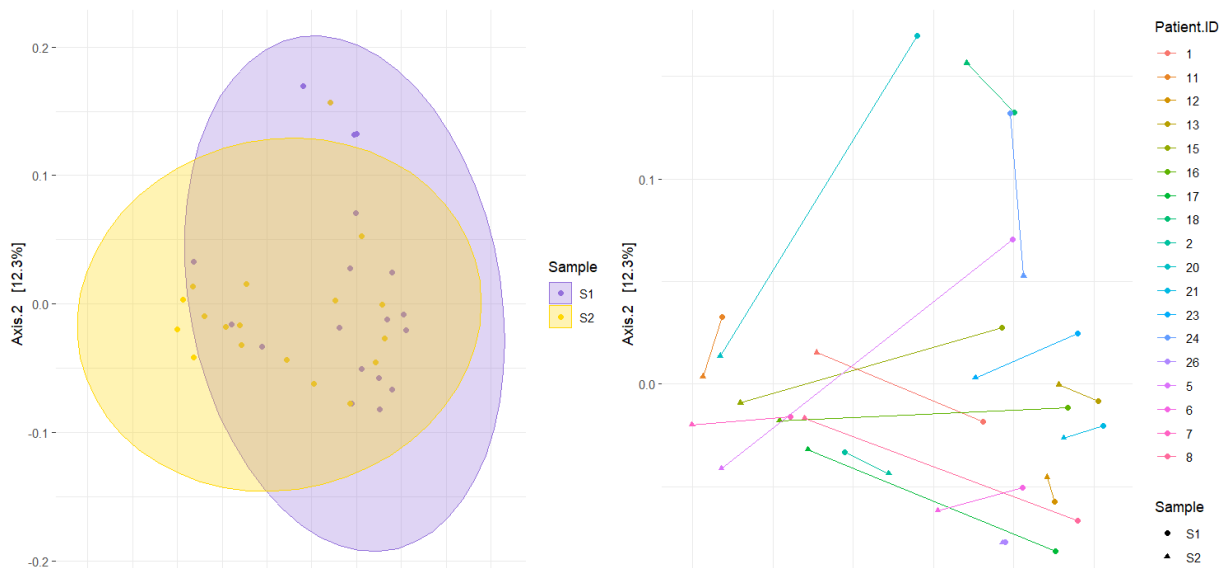


Figure 3.5- Ordination plot of Weighted Unifrac distances with samples coloured by Sample type (left) and Patient ID (right). Ellipses in the left plot represent 95% confidence intervals. Lines on the right plot join s1 and s2 samples from the same patient. PERMANOVA testing showed significant effects on both sample type ($R^2 = .0630$, $p = .012$) and Patient.ID ($R^2 = .6961$, $p = .001$) on community composition.

The CMP with 2.5% NaOCl showed a significant impact on the bacteriome composition. Aldex2 was applied to identify differentially abundant taxa at all seven taxonomic levels (Figure 3.6).

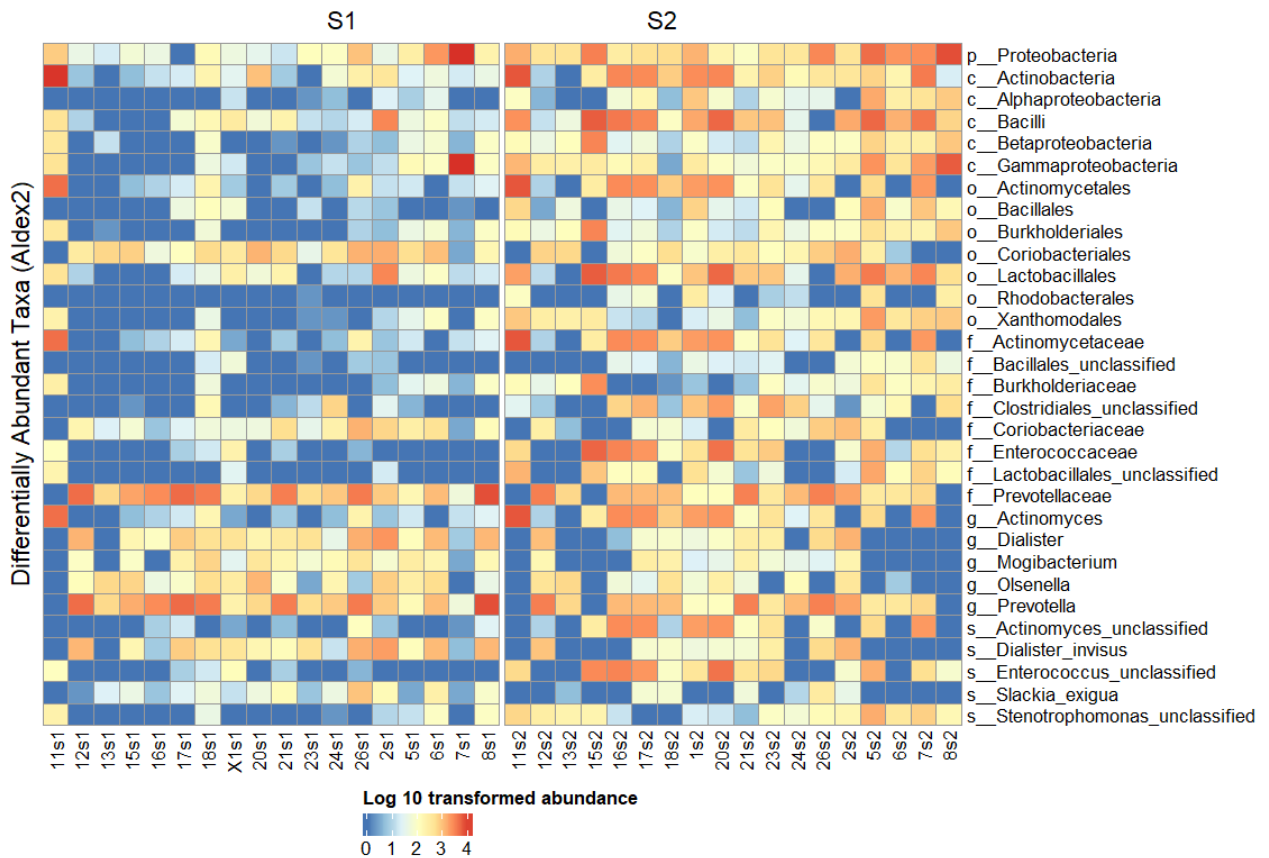


Figure 3.6- A heatmap of log 10 transformed abundances of 32 differentially expressed taxonomic features between s1 (before treatment) and s2 (after treatment) determined by the Aldex2 test. Rows represent taxonomic features. Columns represent samples and are grouped by sampling time (s1/s2).

At the phylum level, Proteobacteria were differentially enriched at s2 (Figure 3.7). At the genus level, Dialister, Mogibacterium, Prevotella, and Olsenella were differentially enriched at s1, while Actinomyces was enriched at s2 (Figure 3.7). At the species level, Stenotrophomonas_unclassified, Dialister_invisus, Enterococcus_unclassified, Slackia_exigua, and Actinomyces_unclassified species were differentially abundant between s1 and s2, with Dialister_invisus differentially enriched at s1 and Enterococcus_unclassified, Stenotrophomonas_unclassified, and Actinomyces_unclassified species differentially enriched at s2 (Figure 3.7). Table 3.1 shows the demographic data for the 18 patients analysed.

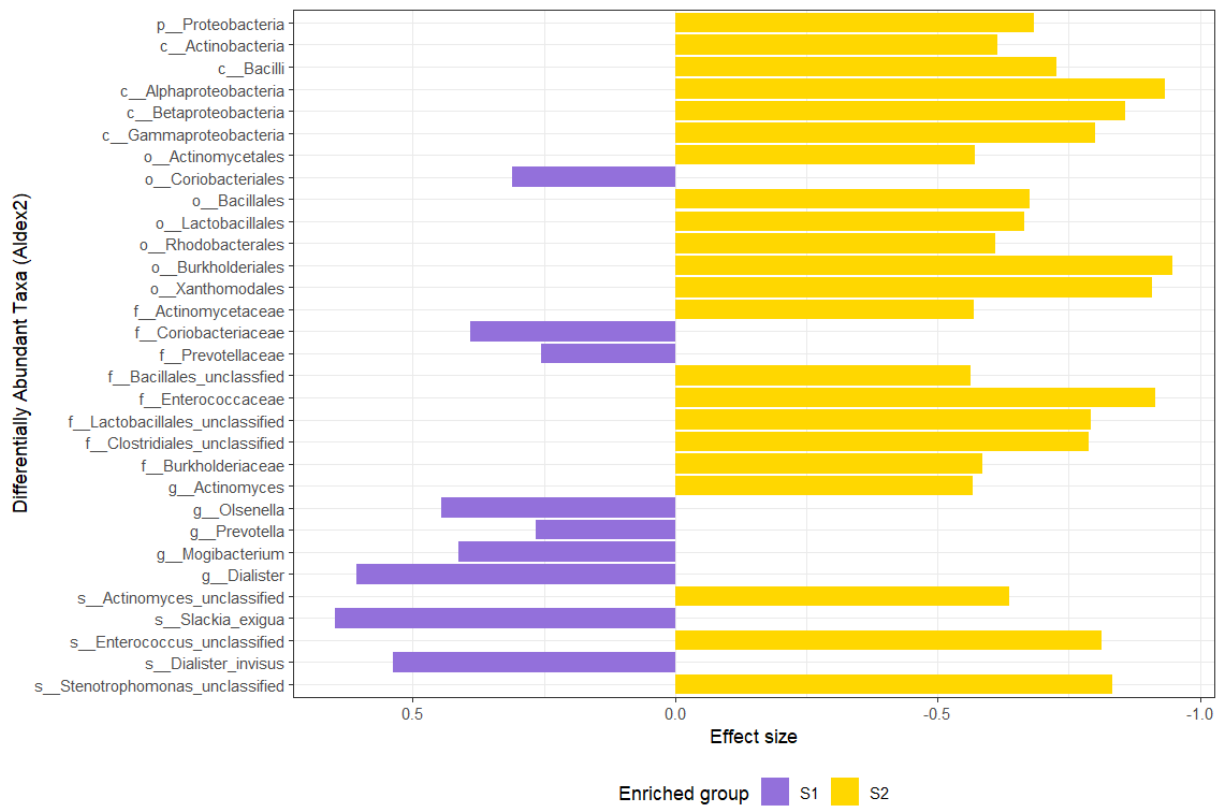


Figure 3.7- Plot of Aldex2 effect sizes for the differentially abundant features. Positive values represent features enriched in s1 and negative values represent features enriched in s2.

Table 3.1. Demographic, clinical, and radiographic features distribution of the eighteen patients analysed.

Variables			<i>Median and Range values</i>
Age	≤ 60 years (n=13)	> 60 years (n=5)	51.5 years (27 – 76 years)
Gender	Male (n=7)	Female (n=11)	-
Tooth	Maxilla (n=13)	Mandible (n=5)	-
Smoking	Smoker (n=5)	Non-smoker (n=13)	-
Symptomatology *	Yes (n=3)	No (n=15)	-
Lesion size	< 5 mm (n=14)	≥ 5 mm (n=4)	4 mm (3 – 7 mm)

* TTP = Tenderness to percussion (n=3); POP = Pain on palpation (n=0).

3.4 Discussion

This clinical cross-sectional interventional study successfully investigated the impact of CMP using 2.5% NaOCl in molars with PEI and AP. The qPCR showed a significant reduction in bacterial DNA count after treatment. Bacterial samples collected at s2 shared 348 ASVs with samples collected at s1, representing 13.8% of the total ASVs count. Shannon and Simpson diversity indexes revealed a higher diversity in the bacterial community in s2 than in s1. Beta diversity showed a significant dissimilarity in bacterial composition between s1 and s2 sampling times and revealed distinct clustering patterns among them. Patients' features showed a significant effect on community composition. *Slackia_exigua*, *Dialister_invisus*, *Stenotrophomonas_unclassified*, *Enterococcus_unclassified*, and *Actinomyces_unclassified* were differentially abundant species between s1 and s2, with *Enterococcus_unclassified*, *Stenotrophomonas_unclassified*, and *Actinomyces_unclassified* species enriched at s2.

One of the limitations of this study is the root canal sampling technique. Despite the PP sampling technique being the most common, it is limited to the main canal, thus

possibly underestimating the total bacterial composition in the endodontic infection, particularly in challenging areas to reach with the PP such as the isthmus, lateral canals, ramifications, dentinal tubules, and deeper layers of the dentine (48,196). Although cryogenically grinding (48,190,197) is an alternative sampling method that could overcome some PP sampling technique disadvantages, it cannot be applied to research of this nature, which includes patients with longitudinal analysis because of sample destruction. This study also lacks bacterium discrimination between live and dead because it is limited to DNA sequencing analysis. Despite the V3–V4 region from the 16s rRNA yields high-quality sequence data (144), it could have limited the identification of bacteria to the species level. We focused on the bacteriome analysis, which is the most analysed component of the human microbiome, and other microbiome aspects such as fungi, viruses, genes, and host immune response, and environmental analysis was not within the scope of this research.

In this study, we used the NGS platform Illumina MiSeq; it has several advantages over other sequencing platforms because it achieves a high quality of reads with relatively low costs (48,79,108). First, most clinical Illumina MiSeq studies have been focused on characterizing qualitatively the bacterial community profile present in endodontic infections (127,198–200). However, to date, few clinical Illumina MiSeq studies have explored CMP's effect on the bacteriome present in PEI (115,123,124,126). Recently, de Castro Kruly et al. (2022) and Íriboz et al. (2018) investigated changes in microbial diversity among patients with PEI before and after Ca (OH)₂ intracanal medication (115,124). Fouad et al. (2022) characterized changes in the bacteriome in teeth with necrotic pulp and open-apex teeth after apexification, regeneration, and revascularization

protocols (126). Zahran et al. (2022) investigated the microbial community profile before and post-instrumentation with 2.5% NaOCl (123).

Similar to previous investigations (115,123,124), we sequenced the 16S rRNA gene in the V3–V4 region. While these studies used operational taxonomic units (OTUs) analysis, we used ASV analysis. Previous studies have reported that OTUs can cause misassignment of query sequences to taxa (149) and possibly overestimate the bacterial diversity and microbial abundance in the infection (150,151). In the ASVs method, sequences are conditional to a quality control analysis, which removes low-quality samples, and the potential errors are denoised by computational methods (152). Additionally, the sequence errors in the ASVs method are controlled up to one nucleotide difference, having higher sensitivity to biological variations (153). We found higher ASV counts in s2 than in s1, with a total of 348 ASVs shared. The presence of slightly higher ASV counts in s2 may be due to the impact of the CMP using 2.5% NaOCl. These ASVs might have been present in low abundance in s1, and the change in the root canal environment and the reduction in selection pressure at s2 might have enriched these species. In addition, the difference in ASVs between the two sampling time points might come from viable and nonviable bacteria that persisted in the canal concerning the isthmus and its ramifications.

The most abundant phyla at s1 were Bacteroidetes (gram-negative bacteria), followed by Firmicutes (gram-positive bacteria) and Actinobacteria. Bacteroidetes and Firmicutes are two of the most abundant phyla reported in endodontic infections (79,85,103,124,126,161). After CMP with 2.5% NaOCl, we found a significant reduction in the Bacteroidetes phylum. Such findings suggest the high susceptibility of some

Bacteroidetes to CMP using 2.5% NaOCl. With the Bacteroidetes drop, Firmicutes became the most abundant phylum. de Castro Kruly et al. (2022) and Fouad et al. (2022) found Firmicutes and Bacteroidetes among the three most abundant phyla after treatment with 2.5% NaOCl + Ca (OH)₂ intracanal medication and 1.25% or 5% NaOCl, respectively (124,126). In a systematic review and meta-analysis, Nardello et al. (2022) confirmed that the most abundant bacterial species resistant to CMP belonged mainly to Firmicutes, followed by Bacteroidetes and Actinobacteria (161). The high relative abundance of Firmicutes after treatment found here and in previous studies could be related to the ability of some gram-positive bacterial species to invade the dentinal tubules and to form biofilms, which can firmly adhere to dentine, thus becoming less susceptible to the CMP. Moreover, cutting the dentine during mechanical instrumentation might have increased the recovery of bacterial species present in deeper layers of the dentine and inside the dentinal tubules not reached in s1.

qPCR analysis has been widely used to quantitatively determine the effectiveness of CMP using 2.5% NaOCl to treat PEI (123,189,192–194). Here, the qPCR analysis showed a significant reduction in bacterial DNA counts after CMP using 2.5% NaOCl. The high effectiveness of NaOCl in disinfecting the root canal system found here is consistent with previous investigations (123,124,192–194).

The important features of a bacterial community in a niche are characterized by the number of species present and their numerical composition (201). Here, we used Chao1, Shannon, and Simpson alpha indexes to investigate the impact of CMP using 2.5% NaOCl on bacterial alpha diversity by comparing s1 and s2 sampling times. Previous studies also evaluated the impact of various treatments on bacterial alpha diversity using different alpha

diversity indexes (123,124,126). The Chao richness estimator (Chao1 index), which gives more weight to low-abundance species (201), indicated no significant difference in alpha diversity between s1 and s2. The Shannon and Simpson alpha diversity indexes, which give deeper insight into the community composition, considering both richness and evenness (201), were higher at s2. These findings suggest that while the absolute bacterial load was markedly lower in s2 compared to s1, s2 presented significantly higher ecological diversity and evenness. Fouad et al. (2022) suggested that the increase in bacterial alpha diversity after treatment may be associated with the reduction in bacterial load (126). The increase in alpha diversity in s2 could be linked to the presence of a lower amount of DNA, such as in s2. This circumstance allows for the detection of low-abundance microorganisms, especially those belonging to rare taxa. Of note, Chao1, which indicates bacterial abundance, was not significantly different between s1 and s2 but diversity related indices Shannon and Simpson reflected a more equitable species distribution in s2, plausibly owing to the loss of dominant taxa susceptible to the debridement process. Therefore, while a low amount of DNA can enable the detection of low-abundance taxa due to reduced competition from dominant taxa, it is important to consider the limitations of detection methods, the biological relevance of rare taxa, and the potential biases introduced by low DNA concentrations in microbial analysis. In addition to alpha diversity indexes, we used the beta diversity analysis to investigate the similarity in bacterial community composition at the two sampling points. Our data showed significantly distinct clustering patterns in s1 and s2, highlighting the impact of CMP using 2.5% NaOCl in the bacteriome present in PEI. Additionally, our results confirmed that individual features play a role in shaping the bacteriome in endodontic infections, showing distinct clustering patterns among them.

We applied ALDEx2 (anova-like differential expression tool for high throughput sequencing data) (202) to identify differential relative abundance taxa between s1 and s2 at all 7 taxonomic levels. At the phylum level, Proteobacteria were differentially abundant between s1 and s2. The differentially abundant genera between s1 and s2 were Dialister, Mogibacterium, Prevotella, Olsenella, and Actinomyces, which were differentially enriched in s1. At the species level, Slackia_exigua and Dialister_invisus were differentially enriched at s1, while Stenotrophomonas_unclassified, Enterococcus_unclassified, and Actinomyces_unclassified were enriched in s2. Interestingly, Enterococcus_unclassified, rarely detected in s1, was enriched in s2. We could speculate that Enterococcus_unclassified was highly abundant after CMP using 2.5% NaOCl for various reasons, including the ability of Enterococcus to invade dentinal tubules (203), limited ability of the PP sampling to reach deeper layers of the dentine, and lower susceptibility to CMP using 2.5% NaOCl.

Several additional limitations must be considered when interpreting the results of the 16s rRNA-based endodontic microbiome analysis. The selection of 16s rRNA regions and primers for amplification (204), reference database (205), ASV versus OUT-based, ASV versus OUT-based methods, where ASVs are considered more sensitive for determining ecological patterns (153), alongside clinical and wet laboratory methodological variations. The need to ensure data comparability across multiple microbiome studies has been recognized and calls for greater standardization and sequence data sharing in the domain of endodontic microbiome study (206). Finally, the present study was exploratory and included a convenience sample of patients from a single site. Owing to a lack of effect size data, a priori sample size estimation was not attempted. Of

note, the ADONIS2 test for community beta diversity comparison showed an R-squared value of .06 ($F = 2.30$, $p = .012^*$) indicating a small effect size. In contrast, when patient ID was tested as a clustering factor an R-squared value of .69 was observed, suggesting the dominant community differentiating factor was inter-individual variation. Of note, has been shown that beta diversity metrics are the most sensitive indicators of community differences (207) future studies may be designed using these metrics as effect size measures.

3.5 Conclusion

The present study showed a quantitative and qualitative impact of CMP using 2.5% NaOCl in the bacteriome present in teeth with PEI with AP. The qPCR analysis showed a significant reduction in bacteria after treatment. Our findings revealed a distinct community composition and increased alpha diversity after CMP using 2.5% NaOCl, despite a dramatic decrease in bacterial abundance and intra- individual similarity in pre- and post-treatment bacteriome. Differential enrichment of *Stenotrophomonas_unclassified*, *Enterococcus_unclassified*, and *Actinomyces_unclassified* at s2 suggests lower effectiveness of CMP using 2.5% NaOCl against these specific taxa. Future clinical studies should be conducted for the analyses of whole-microbiome aspects, including such as fungi, viruses, genes, host immune response, and environment analyses.

CHAPTER FOUR

Conclusion and Future Work

AP is a prevalent oral disease that significantly threatens tooth survival. It is destructive and affects the tissues surrounding the root apex, including the bone, cementum, and periodontal ligament. Effective management of AP requires thorough disinfection of the root canal system to minimize bacterial load. By reducing bacterial load to the lowest possible level, inflammation can be diminished, creating an environment conducive to periapical tissue regeneration and supporting the tooth structure.

Over the past few decades, significant advancements have been made in endodontic technology. These developments include cone beam computed tomography (CBCT), dental operating microscopes, flexible titanium instruments, rotary files, apex locators, and irrigation delivery devices. These technological advances have enhanced our diagnostic capabilities, enabled us to manage more complex cases, and reduced treatment duration. However, despite these advancements, reported success rates for root canal treatment have not shown proportional improvement relative to the advancements in endodontic technology.

A review article by Bergenholtz and Spangberg (2004) suggested that the strong focus on developing technological tools in endodontics may have diverted attention from addressing the primary issues underlying endodontic disease. While our knowledge of endodontic microbiology is expanding, there are still several unresolved and controversial issues, including the composition and behavior of the root canal microbiota. Therefore, it seems that improving treatment outcomes and the success rates of root canal treatment

requires further understanding of the composition and diversity of endodontic microbiology.

The studies presented in this dissertation were intended to investigate the diversity and composition of the bacteriome in PEIs with AP and the impact of patient-related factors and root canal disinfection on the endodontic bacteriome using HTS on the Illumina MiSeq Platform.

Illumina MiSeq is a high-throughput sequencing platform that offers substantially greater sequencing coverage with lower sequencing error rates and lower cost compared to other sequencing platforms. To the best of our knowledge, this research is the first to describe the bacteriome of PEI with AP before and after disinfection using the Illumina MiSeq platform. By employing high-throughput sequencing (HTS) both before and after disinfection, we were able to identify the most persistent bacteria following root canal disinfection. Furthermore, we were the first to investigate the association between the bacteriome in PEI with AP and patient demographic factors, as well as clinical and radiographic findings using HTS on the Illumina MiSeq Platform. This research filled a gap in the understanding of the endodontic bacteriome and its response to disinfection, providing valuable insights into the bacteria associated with PEIs with AP.

In Chapter 2, we characterized the bacteriome associated with PEI and AP, revealing significant inter-individual variations and identifying a core bacteriome consisting of 24 prevalent species. Notably, taxa such as *Dialister invisus*, *Slackia exigua*, *Prevotella oris*, *Parvimonas micra*, and *Fusobacterium nucleatum* subsp. *vincentii* were prominent within the diseased state. Clustering analysis unveiled distinct community types driven by specific

bacterial enrichments, with discriminant species associated with age, gender, smoking, symptomatology, and lesion size.

In Chapter 3, we investigated the quantitative and qualitative impact of CMP using 2.5% NaOCl on the bacteriome of teeth with PEI and AP. Our findings demonstrated a significant reduction in bacterial abundance post-disinfection, accompanied by changes in community composition and increased alpha diversity. Notably, certain taxa such as *Stenotrophomonas* unclassified, *Enterococcus* unclassified, and *Actinomyces* unclassified showed differential enrichment following CMP, suggesting varying effectiveness against specific microbial groups.

The findings from this research have significant potential clinical applications and implications. By identifying the most persistent bacteria following root canal therapy, we can improve disinfection protocols and develop more effective antimicrobial strategies to target these resilient microbial groups. Understanding the association between the bacteriome and patient demographic factors, as well as clinical and radiographic findings, can lead to more personalized treatment approaches, potentially improving patient outcomes. The enhanced knowledge of the bacterial composition and diversity in PEI with AP also supports the development of new diagnostic tools and therapeutic interventions, ultimately contributing to better management of endodontic infections, reduce postoperative complications and preservation of tooth structure.

Future studies should broaden their scope to investigate broader aspects of the root canal microbiome, including fungal and viral components, genetic analyses, host immune responses, and environmental factors. Additionally, it is crucial to uncover how identified microorganisms actively contribute to disease pathogenesis and treatment outcomes

through metagenomic approaches. Understanding the roles and interactions of the detected microorganisms from metagenomic analyses will advance our knowledge of endodontic infections and inform the development of optimal endodontic materials and disinfection protocol.

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