Original Article

Microbial dynamics in primary and refractory apical periodontitis: Next-generation 16S rRNA sequencing reveals selective shifts and reduced diversity

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Abstract

Context: Apical periodontitis (AP) is an inflammatory condition caused by root canal infection. Primary AP (PAP) arises from untreated necrotic pulp and is typically polymicrobial, whereas persistent or refractory AP (RAP) occurs after root canal treatment (RCT) and may reflect selective microbial survival.

Aim: The study aimed to compare the root canal microbiomes in PAP and persistent AP using 16S rRNA gene sequencing in a standardized mandibular molar cohort.

Settings and Design: Comparative, observational, cross-sectional study conducted at a tertiary care dental institution.

Materials and Methods: Intracanal samples were collected from 45 systemically healthy adults (30 RAP, 15 PAP) with chronic asymptomatic mandibular molars (Periapical Index 2–4). Samples were processed using Illumina MiSeq sequencing targeting the V3–V4 region. Bioinformatics analysis was performed using QIIME 2 and DADA2 pipelines.

Statistical Analysis Used: Alpha diversity was analyzed using the Mann–Whitney *U*-test. Beta diversity was assessed using permutational multivariate analysis of variance (PERMANOVA). Differential abundance was evaluated using DESeq2 with Benjamini–Hochberg false discovery rate (FDR) correction.

Results: RAP exhibited significantly reduced alpha diversity ($P \leq 0.003$) and a distinct microbial community structure compared with PAP (PERMANOVA, $P \leq 0.003$). Persistent cases showed enrichment of Gram-positive taxa, particularly *Enterococcus faecalis* (38.5% vs. 2.1%; Log2 fold change +4.2, FDR < 0.001), whereas primary cases demonstrated higher abundance of anaerobic Gram-negative taxa such as *Fusobacterium nucleatum* and *Porphyromonas gingivalis*.

Conclusions: Persistent AP is associated with reduced microbial diversity and enrichment of Gram-positive organisms, particularly *E. faecalis*. These findings may reflect selective survival of resistant taxa and/or secondary colonization following RCT.

Keywords: 16S rRNA sequencing; Apical periodontitis; *Enterococcus faecalis*; persistent apical periodontitis; root canal microbiome

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INTRODUCTION

Apical periodontitis (AP) is a common inflammatory disease of the periapical tissues caused by polymicrobial infection of the root canal system. Primary AP (PAP) arises from untreated necrotic pulp and is characterized by diverse anaerobic microbial communities, including *Fusobacterium nucleatum* and *Porphyromonas gingivalis*, which contribute to tissue destruction and lesion progression.[1,2] In contrast, persistent or refractory apical periodontitis (RAP) develops after root canal treatment (RCT) and is a major cause of endodontic failure. Persistent infections are attributed to the selective survival of resistant microorganisms, particularly Gram-positive species such as *Enterococcus faecalis*, within biofilms and anatomically complex canal systems despite chemomechanical preparation and obturation procedures.[3-5]

Radiographic diagnosis using the Periapical Index (PAI) enables standardized assessment of apical pathology, with scores ≥2 indicating the presence of AP and scores 2–4 commonly reflecting chronic asymptomatic lesions.[6] Earlier culture-based and polymerase chain reaction (PCR) investigations identified microbiological differences between primary and posttreatment infections, frequently implicating Gram-positive taxa, such as *E. faecalis* in persistent disease.[1,6,7]

With the advent of next-generation sequencing (NGS), broader and more detailed characterization of the endodontic microbiome has become possible. Recent literature has demonstrated high microbial diversity and anaerobic predominance in PAP, whereas posttreatment infections often exhibit reduced diversity and enrichment of specific resistant taxa.[4,8,9] However, many available studies have focused primarily on primary infections, evaluated longitudinal antimicrobial phases within small cohorts, or described persistent microbiota without standardized anatomical restrictions or adequately powered group comparisons.[7-10] Therefore, direct comparisons between persistent and PAP using robust differential abundance analyses remain limited.

A clearer understanding of microbial diversity patterns and taxonomic shifts between primary and persistent diseases is clinically important, as refractory AP continues to necessitate retreatment or surgical intervention. Identifying the ecological signatures associated with posttreatment persistence may support the development of targeted retreatment strategies aimed at resistant taxa.

Distinguishing between true persistence of resistant organisms and secondary reinfection via coronal microleakage remains challenging in cross-sectional designs; the present study therefore aimed to compare the root canal microbiomes in patients with persistent and PAP using 16S rRNA gene sequencing within a standardized mandibular molar cohort. By evaluating microbial diversity, community structure, and differentially abundant taxa using complementary statistical approaches, this study sought to characterize the ecological differences associated with posttreatment persistence.

MATERIALS AND METHODS

Study design and ethical considerations

This comparative, observational, cross-sectional study collected intracanal samples at a single time point during clinical intervention. The study was restricted to mandibular molars to standardize anatomical variability and minimize confounding factors affecting microbial ecology. Ethical approval was obtained from the Institutional Ethics Committee of Sri Siddhartha Dental College and Hospital, Sri Siddhartha Academy of Higher Education, Tumkur, Karnataka, India (Approval No.: SSDCHIEC/2024/46 dated January 8, 2024), and written informed consent was obtained from all participants. The study adhered to the Declaration of Helsinki and followed STROBE guidelines for observational studies.[11] Participants were recruited between January 2024 and December 2025 at a tertiary care dental institution.

Sample size determination

The sample size was calculated using G*Power (version 3.1.9.7; Heinrich Heine University Düsseldorf, Germany) based on differences in *E. faecalis* abundance between RAP and PAP groups.[1] Assuming a moderate-to-large effect size (Cohen's $d = 0.8$), alpha of 0.05, power of 80%, and a 2:1 allocation ratio, the minimum required sample size was 26 RAP and 13 PAP cases. To account for potential attrition and improve statistical robustness, 45 participants were included (30 RAP, 15 PAP). *Post hoc* power exceeded 90% for primary outcomes.

Participant selection

Systemically healthy adults aged 18–65 years without antibiotic use in the preceding 3 months were included. RAP cases comprised mandibular molars with prior RCT completed ≥1 year earlier and radiographic evidence of AP (PAI 2–4).[1,12] PAP cases included untreated necrotic teeth with AP (PAI 2–4).[1] Exclusion criteria included systemic disease, pregnancy, recent antimicrobial or probiotic use, periodontal pockets >4 mm, acute symptoms, open apices, root fractures, and teeth unsuitable for rubber dam isolation.

Clinical sampling procedure

Sampling was performed under strict aseptic conditions using validated protocols. Patients rinsed with 1% hydrogen peroxide for 30 s prior to isolation. Teeth were isolated using a rubber dam and disinfected with 3% hydrogen

peroxide followed by 2.5% sodium hypochlorite, with subsequent neutralization using sodium thiosulfate. Sterility was verified using control paper points tested by PCR with universal 16S rRNA primers.

Working length was determined using an apex locator (Root ZX II; J. Morita, Kyoto, Japan). In RAP cases, existing restorations and Gutta-percha were removed aseptically. Intracanal samples were collected using five sterile paper points (size 20, 0.02 taper; Dentsply Sirona, Switzerland), each placed for 1 min. Samples were transferred to RNAlater solution (Thermo Fisher Scientific, USA) and stored at −80°C.

DNA extraction and sequencing

Genomic DNA was extracted using a GeneMATRIX Tissue and Bacterial DNA Purification Kit (Roboklon GmbH, Germany) with bead-beating to enhance lysis of Gram-positive organisms. DNA quality and concentration were assessed using a NanoDrop spectrophotometer. The V3–V4 regions of the 16S rRNA gene were amplified using barcoded primers and sequenced on the Illumina MiSeq platform (Illumina Inc., USA) using 2 × 250 bp paired-end reads.[13]

Bioinformatics analysis

Sequence data were processed using QIIME 2 Development Team, USA.[14] Demultiplexed paired-end reads were quality filtered and denoised using the DADA2 plugin, with forward and reverse reads truncated at 240 bp and 200 bp, respectively, maximum expected errors set to 2 for both forward and reverse reads, and chimera removal performed using the consensus method. Amplicon sequence variants (ASVs) were inferred without clustering. Taxonomic classification was performed using a pretrained Naive Bayes classifier against the SILVA reference database (version 138).[15] A phylogenetic tree was constructed for downstream diversity analyses using MAFFT for multiple sequence alignment and FastTree for tree generation within QIIME 2 (see supplementary methods).[16]

Alpha and beta diversity analyses were conducted on rarefied data (10,000 reads per sample) to account for variation in sequencing depth. Beta diversity metrics included Bray–Curtis dissimilarity and weighted and unweighted UniFrac distances. Differential abundance analysis was performed using raw ASV count data in DESeq2, applying median-of-ratios normalization, and Benjamini–Hochberg false discovery rate (FDR) correction.[17] All analyses were conducted using default parameters unless otherwise specified, and the complete bioinformatics workflow and command-line parameters are available from the corresponding author upon reasonable request.

Statistical analysis

Alpha diversity indices were compared between groups using the Mann–Whitney *U*-test following confirmation of nonnormality by the Shapiro–Wilk test. Beta diversity differences were assessed using permutational multivariate analysis of variance (PERMANOVA; 999 permutations) based on Bray–Curtis dissimilarity and weighted and unweighted UniFrac distance metrics, with homogeneity of dispersion evaluated using PERMDISP.

Differential abundance analysis was performed using DESeq2 as described in the bioinformatics section, with Benjamini–Hochberg FDR correction applied for multiple comparisons. Demographic variables were analyzed using the Mann–Whitney U test or Chi-square test as appropriate. All statistical analyses were performed using R software (version 4.3.1; R Foundation for Statistical Computing, Vienna, Austria) and all tests were two-tailed with statistical significance set at $P < 0.05$.

RESULTS

A total of 45 participants were included in the final analysis, comprising 30 patients with RAP and 15 with PAP. No samples were excluded due to contamination, inadequate DNA extraction, or sequencing failure. Demographic and clinical characteristics are summarized in Table 1. The two groups were comparable with respect to age ($P = 0.45$) and sex distribution ($P = 0.12$). In the RAP group, the mean duration since prior RCT was 4.8 ± 2.1 years. Apical radiolucency was present in all cases, while coronal leakage (26.7%) and sinus tracts (20%) were observed only in RAP cases. No multivariable adjustment was performed; however, coronal leakage and sinus tract presence, observed exclusively in the RAP group, represent potential confounders and were considered during interpretation of the findings.

Alpha diversity analysis demonstrated significantly reduced microbial richness and diversity in RAP compared with PAP [all $P \leq 0.003$; Table 2], indicating a less diverse microbial ecosystem in persistent disease. Beta diversity analysis further revealed significant differences in overall microbial

Table 1: Demographic and clinical characteristics of participants with persistent apical periodontitis and primary apical periodontitis

Characteristic	RAP (n=30)	PAP (n=15)	P
Age (years), mean±SD	45.2±12.5	42.1±8.5	0.45
Sex (male/female)	14/16	10/5	0.12
Time since prior RCT (years), Mean±SD	4.8±2.1 years	NA	-
Presence of coronal leakage, *n* (%)	8 (26.7)	NA	-
Presence of apical radiolucency, *n* (%)	30 (100)	15 (100)	-
Sinus tract presence, *n* (%)	6 (20)	0	-

Values are presented as mean±SD for continuous variables and number (percentage) for categorical variables. Comparisons between groups were performed using the Mann–Whitney *U*-test for the continuous variables and Chi-square test for the categorical variables. NA: Not applicable, SD: standard deviation, RAP: Persistent or refractory apical periodontitis, PAP: Primary apical periodontitis, RCT: randomized control trail

community composition between groups across Bray–Curtis and UniFrac metrics (PERMANOVA, all $P \leq 0.003$), with moderate effect sizes [pseudo-R^2: 0.15–0.21; Table 3]. Homogeneity of dispersion was confirmed ($P > 0.05$).

At the phylum level [Figure 1], RAP samples were predominantly composed of Firmicutes and Actinobacteria, whereas PAP samples exhibited a more heterogeneous distribution, with greater representation of Bacteroidetes, Proteobacteria, and Fusobacteriota.

Differential abundance analysis [Supplementary Table 1] identified significant enrichment of Gram-positive taxa in RAP, particularly *E. faecalis*, *Olsenella uli*, *Actinomyces radicidentis*, and *Parvimonas micra*. In contrast, PAP samples demonstrated higher abundance of anaerobic Gram-negative taxa, including *F. nucleatum* and *P. gingivalis*. No significant difference was observed for *Streptococcus* spp.

DISCUSSION

The present study demonstrated distinct microbial profiles between PAP and persistent AP in a standardized mandibular molar cohort using 16S rRNA sequencing. Primary cases showed higher microbial richness and diversity with predominance of anaerobic Gram-negative taxa, while persistent cases exhibited significantly reduced alpha diversity and enrichment of Gram-positive organisms, particularly *E. faecalis*. These findings align with previous NGS studies.[1,4,5,9,18,19]

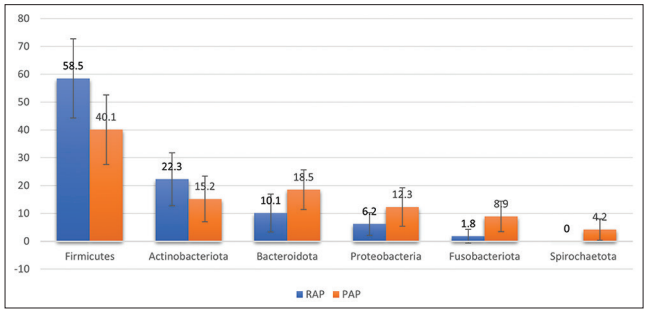

Figure 1: Relative abundance of dominant bacterial phyla in persistent (refractory apical periodontitis) and primary apical periodontitis. Bars represent mean relative abundance (%) ±standard deviation in each group. RAP: Persistent or refractory apical periodontitis, PAP: Primary apical periodontitis

PAP displayed a heterogeneous polymicrobial community dominated by Bacteroidetes, Fusobacteria, and Spirochaetes, consistent with anaerobic profiles reported in systematic reviews and metagenomic analyses of untreated necrotic pulps.[1,18,19] In contrast, persistent cases were characterized by predominance of Firmicutes and Actinobacteria, along with marked enrichment of *E. faecalis* (38.5% vs. 2.1%), *O. uli*, *A. radicidentis*, and *P. micra*. This shift toward reduced diversity and Gram-positive dominance in posttreatment infections has been consistently observed in sequencing-based literature.[4,20,21] Our results corroborate earlier findings that *E. faecalis* is significantly more abundant in secondary/persistent infections compared to primary ones.[1,3,5]

The enrichment of resilient Gram-positive taxa in refractory cases may reflect selective survival following chemomechanical preparation and/or secondary colonization, potentially facilitated by coronal leakage in some teeth. Mechanisms such as biofilm formation, stress response adaptations, and metabolic versatility likely contribute to persistence within treated canals.[20,22] Keystone taxa in posttreatment lesions have also been suggested to maintain ecological stability.[23,24] However, the cross-sectional design limits causal inferences. Clinically, the dominance of *E. faecalis* and other Gram-positive organisms in persistent AP suggests that conventional retreatment protocols may require targeted antimicrobial strategies against these resistant taxa.

Table 2: Alpha diversity indices in persistent and primary apical periodontitis

Diversity measure	RAP (n=30) (mean±SD)	PAP (n=15) (mean±SD)	Mann–Whitney U	P	95% CI (RAP to PAP)
Observed ASVs (Richness)	45.2±15.3	78.5±22.1	82.5	<0.001	−45.8 to −20.8
Shannon Index (Diversity)	2.1±0.6	3.4±0.8	85.5	<0.001	−1.8 to −0.9
Chao1 Index	62.5±18.2	102.3±30.5	79.0	<0.001	−55.1 to −24.5
Simpson Index	0.65±0.12	0.82±0.09	112.0	0.003	−0.23 to −0.09

Alpha diversity metrics (Observed ASVs, Shannon index, Chao1 index, Simpson index) are presented as mean±SD and compared between groups using two-tailed Mann–Whitney U-tests following confirmation of nonnormal distribution (Shapiro–Wilk test). Ninety-five percent CI are reported for group differences. SD: standard deviation, CI: Confidence intervals, RAP: Persistent or refractory apical periodontitis, PAP: Primary apical periodontitis, ASVs: Amplicon sequence variants

Table 3: Beta diversity indices in persistent and primary apical periodontitis

Beta diversity metric	Pseudo-F	Pseudo-R^2	$P^{\S}$
Bray–Curtis dissimilarity (abundance-weighted)	4.82	0.18	0.001
Weighted UniFrac (phylogenetic, abundance-weighted)	3.91	0.15	0.003
Unweighted UniFrac (phylogenetic, presence/absence)	5.24	0.21	0.001

Beta diversity differences were assessed using PERMANOVA; 999 permutations based on Bray–Curtis and weighted/unweighted UniFrac distance metrics. Pseudo-F statistics and pseudo-R^2 values are reported. Homogeneity of dispersion was verified using PERMDISP. A two-tailed P<0.05 was considered statistically significant. PERMANOVA: Permutational multivariate analysis of variance

Limitations

The cross-sectional design precludes causal inference. Importantly, coronal leakage (26.7%) and sinus tracts (20%), observed exclusively in the RAP group, may have facilitated secondary microbial ingress and represent important structural confounders. These factors may contribute to the observed microbial shifts, and therefore the enrichment of Gram-positive taxa should be interpreted as reflecting a combination of persistence and reinfection dynamics rather than selective survival alone. Paper-point sampling primarily captured planktonic and luminal bacteria and may underestimate deeper dentinal tubule or extraradicular biofilms. In addition, 16S rRNA sequencing provides only taxonomic profiles without functional insights. Future longitudinal studies with deeper sampling and shotgun metagenomics are warranted.

CONCLUSIONS

This study identified distinct microbial signatures in PAP and persistent AP in a standardized mandibular molar cohort. Primary cases exhibited greater microbial diversity and predominance of anaerobic taxa, whereas persistent cases were characterized by significantly reduced diversity and enrichment of Gram-positive organisms, particularly *E. faecalis*. These ecological differences may reflect selective survival of resistant taxa following chemomechanical preparation and/or secondary colonization, potentially facilitated by coronal leakage in some cases. Although causal relationships cannot be established owing to the cross-sectional design, the findings highlight microbial patterns that may inform targeted retreatment strategies for persistent AP.

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Conflicts of interest

There are no conflicts of interest.

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Supplementary Table 1: Differentially abundant taxa between persistent and primary apical periodontitis identified by DESeq2 analysis

Taxon (Genus/Species)	Mean RA (%) - RAP	Mean RA (%) - PAP	Log2FC	Adjusted *P* (FDR)	95% CI for Log2FC
Enterococcus faecalis	38.5	2.1	4.2	<0.001	3.1 to 5.3
Olsenella uli	10.2	1.5	2.8	0.002	1.5 to 4.1
Parvimonas micra	8.9	4.2	1.1	0.045	0.2 to 2.0
Fusobacterium nucleatum	2.5	12.8	−2.4	0.010	−3.5 to −1.3
Porphyromonas gingivalis	0.8	6.5	−3.1	0.007	−4.8 to −1.5
Streptococcus spp.	5.5	8.1	−0.6	0.350	−1.5 to 0.3
Actinomyces radicidentis	4.5	0.5	3.2	0.001	1.8 to 4.6

Mean RA (%) is presented for each group. Log2FC represents enrichment in RAP relative to PAP. Adjusted p-values were calculated using the Benjamini–Hochberg FDR method, with FDR-adjusted $P<0.05$ considered statistically significant. Ninety-five percent CI are reported for log2FC estimates. Differential abundance analysis was performed using raw ASV counts with median-of-ratios normalization and dispersion estimation in DESeq2. RA: Relative abundance, Log2FC: Log2 fold change, PAP: Primary apical periodontitis, RAP: Persistent or refractory apical periodontitis, FDR: False discovery rate, CI: Confidence interval, ASVs: Amplicon sequence variants

SUPPLEMENTARY METHODS

Supplementary Methods: Complete QIIME 2 bioinformatics pipeline and command-line parameters

Microbial Dynamics in Primary and Refractory Apical Periodontitis: Next-Generation 16S rRNA Sequencing Reveals Selective Shifts and Reduced Diversity

Overview of the bioinformatics workflow

Sequence data were processed using QIIME 2 (version 2023.9). The pipeline encompassed the following sequential steps: (1) import and demultiplexing, (2) quality filtering and denoising via DADA2, (3) taxonomic classification using a SILVA 138 Naive Bayes classifier, (4) phylogenetic tree construction, and (5) diversity analyses. All commands were executed in a Linux environment with QIIME 2 installed via Conda.

Software environment

QIIME 2 installation and activation

```
# Install QIIME 2 2023.9 via Conda

conda env create -n qiime2-2023.9 \

--file https://data.qiime2.org/distro/core/qiime2-2023.9-py38-linux-conda.yml

# Activate the environment

conda activate qiime2-2023.9

# Verify installation

qiime info
```

Step 1 – Data import

Import paired-end demultiplexed reads

Illumina MiSeq paired-end reads (2 × 250 bp) targeting the V3–V4 hypervariable region of the 16S rRNA gene were imported into QIIME 2 artifact format. Reads were already demultiplexed by the sequencing facility.

```
# Import demultiplexed paired-end FASTQ files

qiime tools import \

--type 'SampleData[PairedEndSequencesWithQuality]' \

--input-path manifest.tsv \
```

--output-path demux-paired-end.qza \

--input-format PairedEndFastqManifestPhred33V2

Note: manifest.tsv is a tab-separated file with columns: sample-id, forward-absolute-filepath, reverse-absolute-filepath. This file maps each of the 45 sample IDs (30 RAP, 15 PAP) to their respective paired FASTQ files.

Visualize sequence quality

Generate quality summary visualization

qiime demux summarize \

--i-data demux-paired-end.qza \

--o-visualization demux-summary.qzv

View in QIIME 2 View

qiime tools view demux-summary.qzv

Step 2 – Quality filtering and denoizing (DADA2)

DADA2 parameters

Quality filtering and denoizing were performed using the DADA2 plugin within QIIME 2. The following parameters were applied based on quality score inspection of the V3–V4 amplicons:

Parameter	Value	Rationale
--p-trunc-len-f	240 bp	Truncation length for forward reads; retains high-quality bases across all samples
--p-trunc-len-r	200 bp	Truncation length for reverse reads; removes lower-quality 3′ end bases
--p-max-ee-f	2	Maximum expected errors for forward reads
--p-max-ee-r	2	Maximum expected errors for reverse reads
--p-trunc-q	2	Truncate reads at first base with quality score ≤2
--p-chimera-method	Consensus	Chimera removal using consensus approach across samples
--p-n-threads	8	Number of CPU threads for parallel processing
--p-n-reads-learn	1,000,000	Number of reads used to learn error rates (default)

DADA2 command

Run DADA2 denoising

qiime dada2 denoise-paired \

--i-demultiplexed-seqs demux-paired-end.qza \

--p-trunc-len-f 240 \

--p-trunc-len-r 200 \

--p-max-ee-f 2 \

--p-max-ee-r 2 \

--p-trunc-q 2 \

--p-chimera-method consensus \

--p-n-reads-learn 1000000 \

--p-n-threads 8 \

--o-table feature-table.qza \

--o-representative-sequences rep-seqs.qza \

--o-denoising-stats denoising-stats.qza \

--verbose

Inspect denoising statistics

Visualize denoising statistics

qiime metadata tabulate \

--m-input-file denoising-stats.qza \

--o-visualization denoising-stats.qzv

Summarize feature table

qiime feature-table summarize \

--i-table feature-table.qza \

--m-sample-metadata-file metadata.tsv \

--o-visualization feature-table-summary.qzv

View representative sequences

qiime feature-table tabulate-seqs \

--i-data rep-seqs.qza \

--o-visualization rep-seqs.qzv

Step 3 – Taxonomic classification

Classifier training (pretrained SILVA 138 classifier)

Taxonomic classification was performed using a Naive Bayes classifier pre-trained on the SILVA reference database (version 138), trimmed to the V3–V4 region (primers 341F/806R). The pre-trained classifier was obtained from the QIIME 2 data resources portal.

Download pre-trained SILVA 138 V3-V4 classifier

wget https://data.qiime2.org/2023.9/common/silva-138-99-seqs-515-806.qza \

-O silva-138-99-classifier.qza

Alternatively, train a custom classifier on V3-V4 primers:

qiime feature-classifier fit-classifier-naive-bayes \

--i-reference-reads silva-138-99-seqs-341-806.qza \

--i-reference-taxonomy silva-138-99-tax.qza \

```
# --o-classifier silva-138-99-v34-classifier.qza
```

Classify representative sequences

```
# Classify ASV sequences against SILVA 138

qiime feature-classifier classify-sklearn \

--i-classifier silva-138-99-classifier.qza \

--i-reads rep-seqs.qza \

--p-n-jobs 8 \

--p-confidence 0.7 \

--o-classification taxonomy.qza \

--verbose

# Visualize taxonomy

qiime metadata tabulate \

--m-input-file taxonomy.qza \

--o-visualization taxonomy.qzv

# Bar plots of taxonomic composition

qiime taxa barplot \

--i-table feature-table.qza \

--i-taxonomy taxonomy.qza \

--m-metadata-file metadata.tsv \

--o-visualization taxa-barplot.qzv
```

Step 4 – Phylogenetic tree construction

A phylogenetic tree was constructed for UniFrac-based diversity analyses using MAFFT for multiple sequence alignment and FastTree for tree inference, both implemented within the QIIME 2 phylogeny plugin.

```
# Full phylogenetic pipeline (align, mask, tree, root)

qiime phylogeny align-to-tree-mafft-fasttree \

--i-sequences rep-seqs.qza \

--p-n-threads 8 \

--o-alignment aligned-rep-seqs.qza \

--o-masked-alignment masked-aligned-rep-seqs.qza \
```

```
--o-tree unrooted-tree.qza \

--o-rooted-tree rooted-tree.qza \

--verbose
```

Step 5 – Alpha and beta diversity analyses

Rarefaction depth selection

Rarefaction depth was set at 10,000 reads per sample to standardize sequencing effort across all 45 samples, retaining all samples with adequate sequencing depth. Rarefaction curves were inspected to confirm saturation at this depth.

```
# Generate rarefaction curves to select appropriate depth

qiime diversity alpha-rarefaction \

--i-table feature-table.qza \

--i-phylogeny rooted-tree.qza \

--p-max-depth 10000 \

--p-steps 20 \

--m-metadata-file metadata.tsv \

--o-visualization alpha-rarefaction.qzv
```

Core diversity metrics

```
# Compute all core diversity metrics at rarefaction depth 10,000

qiime diversity core-metrics-phylogenetic \

--i-phylogeny rooted-tree.qza \

--i-table feature-table.qza \

--p-sampling-depth 10000 \

--m-metadata-file metadata.tsv \

--output-dir core-metrics-results/\

--p-n-jobs-or-threads 8

# Outputs include:

# rarefied_table.qza

# faith_pd_vector.qza (Faith's PD)

# observed_features_vector.qza

# shannon_vector.qza (Shannon entropy)

# evenness_vector.qza (Pielou's J)
```

```
# bray_curtis_distance_matrix.qza

# unweighted_unifrac_distance_matrix.qza

# weighted_unifrac_distance_matrix.qza
```

Alpha diversity group significance (Mann–Whitney U-test)

```
# Alpha diversity significance — Shannon index

qiime diversity alpha-group-significance \

--i-alpha-diversity core-metrics-results/shannon_vector.qza \

--m-metadata-file metadata.tsv \

--o-visualization shannon-group-significance.qzv

# Repeat for Faith's PD

qiime diversity alpha-group-significance \

--i-alpha-diversity core-metrics-results/faith_pd_vector.qza \

--m-metadata-file metadata.tsv \

--o-visualization faithpd-group-significance.qzv

# Repeat for observed features

qiime diversity alpha-group-significance \

--i-alpha-diversity core-metrics-results/observed_features_vector.qza \

--m-metadata-file metadata.tsv \

--o-visualization observed-features-significance.qzv
```

Beta diversity – PERMANOVA (999 permutations)

```
# Beta diversity significance — Bray-Curtis (PERMANOVA)

qiime diversity beta-group-significance \

--i-distance-matrix core-metrics-results/bray_curtis_distance_matrix.qza \

--m-metadata-file metadata.tsv \

--m-metadata-column Group \

--p-method permanova \

--p-pairwise \

--p-permutations 999 \

--o-visualization bray-curtis-permanova.qzv
```

```
# Unweighted UniFrac PERMANOVA

qiime diversity beta-group-significance \

--i-distance-matrix core-metrics-results/unweighted_unifrac_distance_matrix.qza \

--m-metadata-file metadata.tsv \

--m-metadata-column Group \

--p-method permanova \

--p-pairwise \

--p-permutations 999 \

--o-visualization unweighted-unifrac-permanova.qzv

# Weighted UniFrac PERMANOVA

qiime diversity beta-group-significance \

--i-distance-matrix core-metrics-results/weighted_unifrac_distance_matrix.qza \

--m-metadata-file metadata.tsv \

--m-metadata-column Group \

--p-method permanova \

--p-pairwise \

--p-permutations 999 \

--o-visualization weighted-unifrac-permanova.qzv

# PERMDISP (homogeneity of dispersion test)

qiime diversity beta-group-significance \

--i-distance-matrix core-metrics-results/bray_curtis_distance_matrix.qza \

--m-metadata-file metadata.tsv \

--m-metadata-column Group \

--p-method permdisp \

--p-permutations 999 \

--o-visualization bray-curtis-permdisp.qzv
```

Step 6 – Differential abundance analysis (DESeq2)

Differential abundance analysis was performed using raw (non-rarefied) ASV count data in R (version 4.3.1) with the DESeq2 package. The QIIME 2 feature table was first exported and converted to the format required by DESeq2.

Export feature table from QIIME 2

```
# Export ASV feature table as BIOM format

qiime tools export \

--input-path feature-table.qza \

--output-path exported-feature-table/

# Convert BIOM to TSV (for import into R)

biom convert \

-i exported-feature-table/feature-table.biom \

-o exported-feature-table/feature-table.tsv \

--to-tsv

# Export taxonomy

qiime tools export \

--input-path taxonomy.qza \

--output-path exported-taxonomy/
```

DESeq2 analysis in R

```
# ── Load libraries ──────────────────────────────────────────────────────────

library (DESeq2)

library (phyloseq)

library (ggplot2)

# ── Import data ─────────────────────────────────────────────────────────────

asv_table <- read.csv('asv_table.csv', row.names = 1)

taxonomy <- read.csv('taxonomy.csv', row.names = 1)

metadata <- read.csv('metadata.csv', row.names = 1)

# ── Build phyloseq object ──────────────────────────────────────────────────

OTU <- otu_table (as.matrix (asv_table), taxa_are_rows = TRUE)

TAX <- tax_table (as.matrix (taxonomy))

META <- sample_data (metadata)

ps <- phyloseq (OTU, TAX, META)

# ── Convert to DESeq2 object ──────────────────────────────────────────────
```

```
# RAP is set as reference (baseline) group

ps$Group <- relevel (factor (ps$Group), ref = 'RAP')

dds <- phyloseq_to_deseq2(ps, ~ Group)

# ── Run DESeq2 with default median-of-ratios normalization ──────────

dds <- DESeq (dds,

test = 'Wald',

fitType = 'parametric',

sfType = 'ratio',

betaPrior = FALSE)

# ── Extract results: PAP vs RAP ──────────────────────────────────────

res <- results (dds,

contrast = c('Group', 'PAP', 'RAP'),

alpha = 0.05,

pAdjustMethod = 'BH') # Benjamini-Hochberg FDR

# ── Filter significant ASVs (FDR < 0.05) ─────────────────────────────

res_sig <- subset (res, padj < 0.05)

res_sig <- res_sig[order (res_sig$log2FoldChange, decreasing = TRUE), ]

# ── Export results ──────────────────────────────────────────────────

write.csv (as.data.frame (res_sig), 'DESeq2_significant_results.csv')

write.csv (as.data.frame (res), 'DESeq2_all_results.csv')
```

Metadata file format

The metadata file (metadata.tsv) used throughout the QIIME 2 pipeline followed QIIME 2 formatting standards (TSV format, first column named #SampleID). The file contained the following columns:

Column	Type	Values/Description
#SampleID	Categorical	RAP_01 – RAP_30, PAP_01 – PAP_15
Group	Categorical	RAP or PAP (primary grouping variable)
Age	Numeric	Patient age in years (range: 26.1–71.5)
Gender	Categorical	M (male) or F (female)

Summary of key analytical parameters

Analysis Step	Tool/Plugin	Key Parameters
Import	Qiime tools import	Type: SampleData[PairedEndSequencesWithQuality]; Format: PairedEndFastqManifestPhred33V2
Denoising	DADA2 (denoise-paired)	trunc-len-f=240; trunc-len-r=200; max-ee-f=2; max-ee-r=2; chimera-method=consensus
Taxonomic classification	Feature-classifier classify-sklearn	SILVA 138 Naive Bayes; confidence=0.7
Phylogenetic tree	Phylogeny align-to-tree-mafft-fasttree	MAFFT alignment + FastTree; 8 threads
Rarefaction depth	Diversity core-metrics-phylogenetic	Sampling-depth=10,000 reads/sample
Alpha diversity significance	diversity alpha-Group-significance	Mann–Whitney U test (via Kruskal–Wallis in QIIME 2)
Beta diversity significance	Diversity beta-group-significance	PERMANOVA, 999 permutations; PERMDISP
Differential abundance	DESeq2 (R 4.3.1)	Wald test; Benjamini–Hochberg FDR; contrast: PAP versus RAP

Reproducibility Note: All QIIME 2 commands were executed in a controlled Conda environment (qiime2-2023.9). The processed ASV count table used for all statistical analyses is available from the corresponding author upon reasonable request.