CROSS-SECTIONAL STUDY

Microbiota of Endodontically Infected Primary and Permanent Teeth

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Abstract: ***Purpose:*** *Differences in the endodontic microbiome of permanent and primary teeth during the mixed dentition period are still unknown. The purpose of this study was to examine bacterial diversity in endodontically infected primary and permanent teeth using 16S rRNA gene sequencing and the QIIME 2 (Quantitative Insights Into Microbial Ecology 2) bioinformatics pipeline.* ***Methods:*** *Microbial samples from endodontically infected primary (N equals 15) and permanent (N equals 15) maxillary or mandibular molar teeth were subjected to next-generation sequencing analysis based on examination of the hypervariable V3 to V4 region of the 16S rRNA gene. Statistical analysis was performed using R software.* ***Results:*** *Of 1,664,926 reads and 2,237 operational taxonomic units, 14 phyla, 89 families, and 236 genera were identified. Firmicutes were the most commonly detected phyla in both endodontically infected primary and permanent root canals. Bacteroides and Proteobacteria were more common in primary teeth, whereas Actinobacteria and Verrucomicrobia were more common in permanent teeth. The overall canal microbiota composition was similar in endodontically infected primary and permanent teeth (P=0.338).* ***Conclusions:*** *This study provides a comprehensive assessment of microbiota composition in endodontically infected primary and permanent teeth and gives a deeper insight into the origin of the root canal infections.* *(Pediatr Dent 2021;43(2):102-8.E17-E18) Received March 20, 2020 / Last Revision October 31, 2020 / Accepted November 2, 2020.*

KEYWORDS: ENDODONTIC INFECTION, MICROBIAL FLORA, PRIMARY TEETH

Endodontic infections are the cause of inflammation of pulp tissue which may be limited to pulp and/or spread to periapical tissues. These infections are caused by multiple bacterial species, prominently dominated by anaerobic bacteria. The number of microorganisms per canal ranges from 10^2 to 10^8.[1,2]

The success rate of root canal therapies is associated with having in-depth knowledge of the pathogenic factors in the root canal system.[3] Traditionally, the endodontic microbiome has been identified by culture-based (phenotype-based) techniques. Since phenotypic characteristics are dynamic and can alter under stress or during evolution, studies using this technique can fail to manifest bacterial diversity in polymicrobial infections accurately.[4] Moreover, this technique is challenging and time-consuming due to the anaerobic growth requirements of bacteria.[5] Also, researcher bias or lack of experience and inability to cultivate approximately 40 percent to 55 percent of bacteria are other limitations of the culture-based technique.[1,4,6] Polymerase chain reaction (**PCR**), Sanger sequencing, 454 pyrosequencing technologies, and fragment length polymorphism analysis have been used to determine bacterial diversity.[7,8] However, these techniques have been associated with relatively high reading error rates. Also, they have deficiencies in the detection of low abundant species within a complex microbial community.[9]

Microbiome-based next-generation sequencing (**NGS**) has been widely used in recent years to identify bacterial diversity using the 16S ribosomal RNA (**rRNA**) gene and provide a better understanding of the pathogenesis.[1,10,11] This technique is an accurate, rapid, and reliable means of identifying bacteria that have not been defined using phenotype-based tests.[3,6] Metagenomic studies are usually performed by analyzing the 1500-2000 base length prokaryotic 16S rRNA gene. This marker gene is found in all prokaryotes and has both highly conserved and variable regions. The variable regions, indicated as V1-V9 (V3-V4 are most targeted regions), are specific for detection in phylogenetic classifications. This technique allows the detection of large-scale bacterial communities, including cultivable/non-cultivable, low abundance taxa, and even unspecified bacterial community.[12]

Although many studies have investigated microbiota in permanent teeth,[7,13,14] there are few studies in primary teeth.[5,15] As such, it is still unclear whether there are differences in endodontic microbiomes during the mixed dentition period when both dentition types can be seen.

Therefore, the purpose of this study was to investigate bacterial diversity in endodontically infected primary and permanent teeth using 16S rRNA gene sequencing and the QIIME 2 bioinformatics pipeline.

Methods

The study protocol was approved by the Ethics Committee of Mersin University, Mersin, Turkey, and performed in accordance with the ethical principles of the World Medical Association Declaration of Helsinki. Additionally, written informed

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consent was obtained from the parents of all children from whom canal samples were taken.

Samples were obtained from 30 children (15 females and 15 males aged 4 to 13 years) who attended the Department of Pediatric Dentistry, Faculty of Dentistry, Mersin University, for the treatment of primary or permanent teeth with extensive caries. The study consisted of two groups, including primary and permanent teeth: 15 primary and 15 permanent endodontically infected maxillary or mandibular molar teeth. The experimental design of the study was as shown in Figure 1.

The selected teeth had intact roots or less than one-third of physiological root resorption as well as clinical crowns that permitted effective rubber dam isolation. All teeth had profound dentin caries, which cause spontaneous pain complaints. However, no mobility, fistula, pus discharge, gingival swelling, periapical abscess, or internal resorption was observed. The exclusion criteria were as follows: conditions that alter microbial flora, such as marginal periodontitis; a history of pharmacological treatment, antibiotics, or systemic and/or topical fluoride intake within the last two months; and a history of cancer, diabetes, or immunodeficiency disorders. Prior to the procedure, demographic data and clinical characteristics such as age, gender, dentition type, tooth number, and location were recorded for each patient (Table 1).

Sample collection. After applying a topical anesthetic, the tooth was anesthetized with four percent articaine with 1:200,000 epinephrine. Then, the tooth was cleaned with a pumice and rubber dam isolation was performed. For tooth disinfection, 30 percent hydrogen peroxide and 2.5 percent sodium hypochlorite solution were used for 30 seconds each. Subsequently, a five percent sodium thiosulfate solution was used to inactivate the disinfectant agents. Cavity preparation and root canal access were carried out with sterile high-speed diamond burs (Komet Dental, Gebr. Brasseler GmbH & Co. KG, Lemgo, Germany) under water cooling. Samples were taken from the largest root canal using the paper point method under strict aseptic conditions. A minimum of four sterile paper points (#25 size) was placed to the same level in the root canal, and the root canal content was absorbed. If the canal was dry, the sample was taken after a small amount of sterile saline solution was delivered into the canal using a 27-G syringe (Ultradent, South Jordan, Utah, USA). Each paper point remained in the canal for at least 30 seconds. Finally, paper points were placed into Eppendorf tubes (Isolab, Istanbul, Turkey) and refrigerated at -80 degrees Celsius within 10 minutes.

DNA extraction. All samples were shipped overnight on dry ice packs to Diagen Biotechnologic Systems Corp. (Ankara, Turkey) for DNA extraction. Bacterial DNA was extracted from samples using the GeneMATRIX Tissue and Bacterial DNA Purification Kit (Roboklon, Berlin, Germany) according to the manufacturer's recommendations.

PCR amplification, library construction, and sequencing. The bacterial 16S rRNA gene V3-V4 region was amplified according to the Illumina 16S Metagenomic Sequence Library Protocols using the forward primer (5'-TCG TCG GCA GCG TCA GAT GTG TAT AAG AGA CAG CCT ACG GGN GGC WGC AG-3') and reverse primer (5'-GTC TCG

Table 1. DEMOGRAPHIC DATA OF THE CHILDREN AND CHARACTERISTICS OF THE TEETH IN THIS STUDY

Sample Id	Age of child (yrs)	Sex	Tooth type	Tooth number	Jaw
1	6	Female	Primary	J	Maxillary
2	13	Female	Permanent	19	Mandibular
3	5	Female	Primary	L	Mandibular
4	7	Male	Primary	S	Mandibular
5	6	Female	Primary	B	Maxillary
6	5	Female	Primary	S	Mandibular
7	8	Male	Primary	K	Mandibular
8	8	Male	Primary	K	Mandibular
9	8	Female	Permanent	19	Mandibular
10	10	Female	Permanent	19	Mandibular
11	12	Female	Permanent	30	Mandibular
12	11	Male	Permanent	19	Mandibular
13	7	Female	Primary	B	Maxillary
14	5	Male	Primary	B	Maxillary
15	7	Male	Primary	L	Mandibular
16	12	Male	Permanent	19	Mandibular
17	7	Female	Primary	S	Mandibular
18	13	Male	Permanent	30	Mandibular
19	10	Male	Permanent	30	Mandibular
20	13	Male	Permanent	14	Maxillary
21	8	Male	Primary	J	Maxillary
22	11	Female	Permanent	19	Mandibular
23	7	Female	Primary	K	Mandibular
24	8.5	Female	Permanent	14	Maxillary
25	4	Male	Primary	T	Mandibular
26	5	Male	Primary	K	Mandibular
27	10	Male	Permanent	19	Mandibular
28	8	Female	Permanent	30	Mandibular
29	12	Female	Permanent	30	Mandibular
30	13	Male	Permanent	3	Maxillary

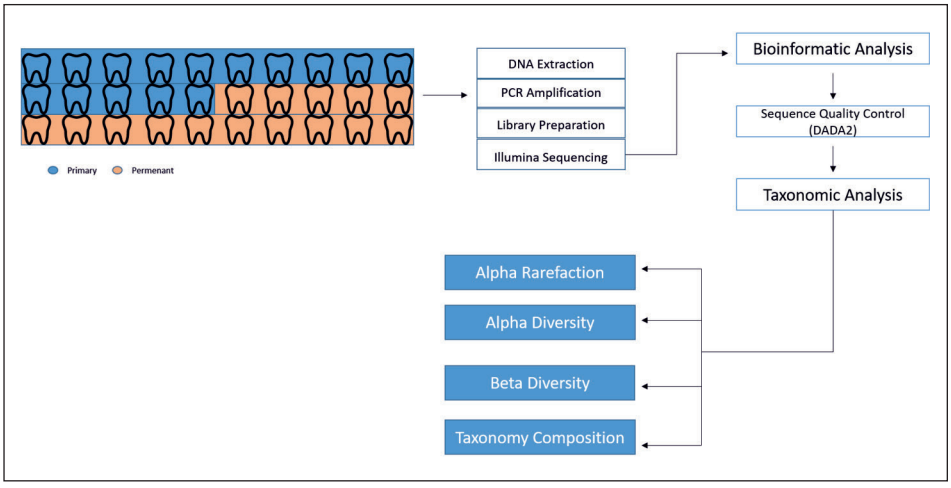

Figure 1. Experimental design of the study. DNA extraction was performed from 30 samples (15 permanent teeth, 15 primary teeth). The library was created and sequenced on the MiSeq platform. Rarefaction curves, alpha-beta diversity, and taxonomy composition were obtained using bioinformatics analysis.

TGG GCT CGG AGA TGT GTA TAA GAG ACA GGA CTA CHV GGG TAT CTA ATC C-3').[14]

The following PCR conditions were used for amplification: initial denaturation at 95 degrees Celsius for three minutes, followed by 25 cycles of denaturation at 55 degrees Celsius for 30 seconds, annealing at 52 degrees Celsius for 30 seconds, elongation at 72 degrees Celsius for 30 seconds, and extension at 72 degrees Celsius for 30 seconds. Final elongation was at 72 degrees Celsius for five minutes. The PCR products were separated by two percent agarose gel electrophoresis and purified using Agencourt AMPure XP (Beckman Coulter Genomics, Brea, Calif., USA). The library was constructed and sequenced on the MiSeq platform (Illumina Inc., San Diego, Calif., USA).

Bioinformatics analysis. Raw sequences of 30 samples were obtained in the Casava 1.8 paired end demultiplexed fastq format. First, the raw data were imported as QIIME 2 artifact. Following, the open-source R package DADA2 (version 1.14.0) was used to filter out errors, chimeras, and low-quality reads.

After amplicon sequence variants were obtained with the q2-phylogeny plug-in, a rooted phylogenetic tree was created to analyze alpha and beta diversity metrics. Bacterial abundance (proportion of microbiome) across groups was examined using the Wilcoxon rank-sum test using a conventional statistical significance threshold ($P<0.05$). Observed operational taxonomic units (**OTUs**), Shannon's index, Faith's phylogenetic diversity, and Evenness were calculated and plotted as alpha diversity metrics; weighted UniFrac, unweighted UniFrac, Bray Curtis, Jaccard, UniFrac distance-based nonmetric multidimensional scaling, and principal component analysis as beta diversity metrics were calculated and plotted as beta diversity metrics. Statistical analysis was performed using R software (R Core Team, version 3.6.1, 2019).

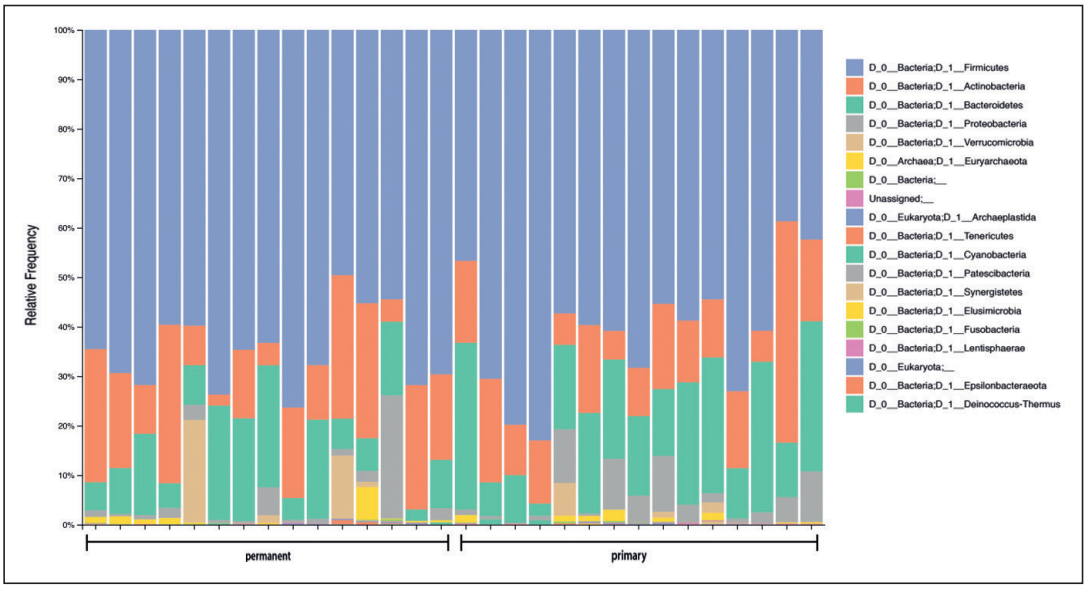

Figure 2. Diversity and abundance of dental pulp microbiota in endodontically infected permanent and primary teeth at the phylum level (over one percent of the microbiome). The phylum in the samples included Firmicutes, Actinobacteria, Bacteroides, Proteobacteria, Verrucomicrobia, and Euryarcheota, respectively, from high to low. Firmicutes were the main abundant phylum in both groups. The second dominant phylum was Actinobacteria in permanent teeth followed by Bacteroidetes in primary teeth. Proteobacteria were more dominant in primary teeth than permanent teeth.

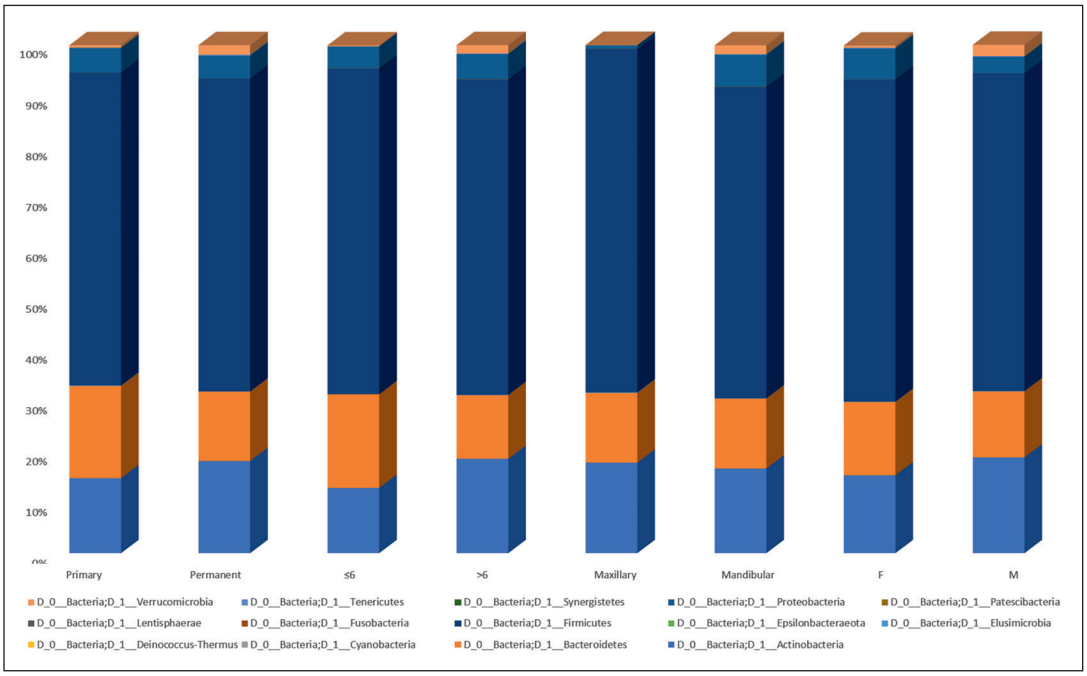

Figure 3. Bar graphs of phyla abundance between primary/permanent, younger than six years old/older than six years, maxillary/mandibular, female/male groups. Firmicutes were the most abundant phylum in all groups. Bacteroides were the phylum in notable abundance in primary teeth and among those younger than six years old. Verrucomicrobia abundancy was similar in permanent teeth, older than six years, mandibular teeth, and males. Proteobacteria were low abundant in maxillary teeth.

Results

Of the 30 children in the study, 15 were female and 15 were male. Further, seven participants were no older than six years old and 23 were older than six years. Sampled teeth consisted of eight (26.7 percent) primary first molars, seven (23.3 percent) primary second molars, and 15 (50 percent) permanent first molars. Eight of these teeth were maxillary and 22 were mandibular. The demographic data of the children and the characteristics of the teeth in the study are shown in Table 1.

Table 2. ABUNDANCE* OF SELECTED BACTERIAL TAXA

Bacterial taxa	Gram staining/ oxygen requirements	Tooth type		*P*-value†	Age		*P*-value†	Jaw		*P*-value †	Sex		*P*-value†
		Primary	Permanent		≤6 years	>6 years		Maxillary	Mandibular		Female	Male	
Bifidobacterium	Gr +/Anaerobic	0.06	0.11	0.09	0.10	0.04	0.21	0.08	0.09	0.78	0.08	0.08	0.76
Atopobiaceae; __	Gr +/Facultative anaerobic	0.00	0.01	**0.04**	0.01	0.00	0.41	0.01	0.00	0.59	0.00	0.01	0.55
Collinsella	Gr +/Anaerobic	0.07	0.05	0.10	0.06	0.07	0.49	0.07	0.06	0.67	0.05	0.06	0.52
Eggerthellaceae; __	Gr +/Anaerobic	0.00	0.01	0.93	0.01	0.00	**0.01**	0.01	0.01	0.52	0.01	0.01	0.10
Bacteroides	Gr -/Anaerobic	0.07	0.04	0.31	0.04	0.09	0.07	0.06	0.05	0.61	0.07	0.03	0.25
Barnesiella	Gr -/Anaerobic	0.00	0.00	0.36	0.00	0.01	0.31	0.00	0.00	0.24	0.00	0.00	0.57
Alloprevotella	Gr -/Anaerobic	0.00	0.01	**0.04**	0.00	0.00	**0.04**	0.00	0.0	0.47	0.00	0.00	0.62
Prevotella 9	Gr -/Anaerobic	0.06	0.02	0.46	0.00	0.00	1.00	0.00	0.00	0.74	0.00	0.00	0.83
Alistipes	Gr -/Anaerobic	0.01	0.01	0.66	0.01	0.02	0.64	0.01	0.01	0.80	0.01	0.01	0.38
Rikenellaceae RC9 gut group	Gr -/Anaerobic	0.01	0.00	0.43	0.00	0.00	0.41	0.00	0.00	0.96	0.01	0.00	0.27
Parabacteroides	Gr -/Anaerobic	0.01	0.01	**0.03**	0.01	0.02	**0.01**	0.01	0.01	0.15	0.01	0.01	0.35
Enterococcus	Gr +/Facultative anaerobic	0.00	0.01	0.22	0.01	0.00	0.62	0.00	0.01	0.17	0.01	0.0	0.08
Lactobacillus	Gr +/Facultative anaerobic or microaerophilic	0.03	0.07	0.35	0.05	0.04	0.71	010	0.03	0.24	0.02	0.07	0.12
Weissella	Gr +/Facultative anaerobic	0.01	0.00	0.95	0.00	0.03	0.68	0.00	0.01	0.72	0.00	0.01	0.32
Streptococcus	Gr +/Facultative anaerobic	0.04	0.04	0.44	0.04	0.03	0.54	0.03	0.04	0.85	0.04	0.04	0.76
Christensenellaceae R-7 group	Gr -/Anaerobic	0.01	0.01	0.56	0.01	0.01	0.23	0.01	0.01	0.25	0.01	0.01	0.74
Clostridiaceae 1; __	Gr +/Anaerobic	0.01	0.01	0.36	0.00	0.00	0.34	0.00	0.00	0.53	0.00	0.00	0.20
Blautia	Gr +/Anaerobic	0.06	0.08	0.07	0.07	0.06	0.71	0.08	0.06	0.26	0.07	0.06	0.98
[Eubacterium] hallii group	Gr +/Anaerobic	0.01	0.02	0.21	0.02	0.01	0.57	0.02	0.01	0.66	0.01	0.01	0.42
Lachnospiraceae; __	Gr -/Anaerobic	0.11	0.10	0.98	0.10	0.14	0.12	0.12	0.11	0.28	0.11	0.10	0.66
Peptococcus	Gr +/Anaerobic	0.00	0.00	0.12	0.00	0.00	0.11	0.00	0.00	0.08	0.00	0.00	**0.00**
Peptostreptococcaceae; __	Gr +/Anaerobic or Facultative aerobic	0.03	0.02	0.15	0.02	0.02	0.90	0.02	0.02	0.74	0.02	0.02	0.14
Butyricicoccus	Gr +/Anaerobic	0.01	0.01	0.29	0.01	0.01	0.90	0.01	0.01	0.89	0.01	0.01	**0.10**
Faecalibacterium	Gr +/Anaerobic	0.09	0.04	0.42	0.05	0.09	0.51	0.10	0.05	**0.04**	0.06	0.06	0.76
Ruminococcaceae NK4A214 group	Gr +/Anaerobic	0.01	0.00	0.72	0.00	0.01	0.59	0.00	0.01	0.29	0.01	0.00	0.45
Ruminococcaceae UCG-002	Gr +/Anaerobic	0.01	0.01	0.85	0.01	0.01	0.75	0.01	0.01	0.57	0.01	0.01	0.58
Ruminococcaceae UCG-005	Gr +/Anaerobic	0.00	0.01	0.43	0.01	0.00	0.28	0.00	0.01	0.11	0.01	0.00	0.83
Ruminococcaceae UCG-013	Gr +/Anaerobic	0.01	0.01	0.63	0.01	0.01	0.44	0.01	0.01	0.64	0.01	0.00	0.88
Ruminococcaceae UCG-014	Gr +/Anaerobic	0.00	0.01	0.44	0.01	0.01	0.47	0.01	0.01	1.00	0.01	0.01	0.18
Ruminococcus 1	Gr +/Anaerobic	0.01	0.01	0.88	0.00	0.01	0.50	0.01	0.00	0.25	0.01	0.00	0.95
Ruminococcus 2	Gr +/Anaerobic	0.02	0.03	0.28	0.03	0.01	**0.03**	0.02	0.02	0.80	0.03	0.01	0.62
Subdoligranulum	Gr -/Anaerobic	0.02	0.02	0.35	0.02	0.02	0.16	0.02	0.02	0.93	0.02	0.02	0.52
[Eubacterium] co-prostanoligenes group	Gr +/Anaerobic	0.02	0.01	0.66	0.01	0.02	0.81	0.01	0.02	0.25	0.02	0.01	0.72
Ruminococcaceae; __	Gr +/Anaerobic	0.01	0.02	0.19	0.02	0.01	0.54	0.01	0.02	0.33	0.02	0.01	0.55
Clostridiales; __; __	Gr +/Anaerobic	0.00	0.00	0.35	0.00	0.00	0.51	0.00	0.00	0.40	0.01	0.00	0.33
Catenibacterium	Gr +/Anaerobic	0.01	0.01	0.61	0.01	0.01	0.59	0.00	0.01	**0.02**	0.01	0.01	0.72
Erysipelatoclostridium	Gr +/Anaerobic	0.01	0.00	0.84	0.00	0.01	0.28	0.00	0.00	0.69	0.01	0.00	0.07
Erysipelotrichaceae UCG-003	Gr +/Facultative anaerobic or anaerobic	0.01	0.01	0.32	0.01	0.01	0.24	0.01	0.01	0.06	0.01	0.00	0.49
Faecalitalea	Gr +/Anaerobic	0.00	0.01	0.18	0.00	0.00	0.88	0.01	0.00	0.73	0.00	0.00	**0.00**
Holdemanella	Gr +/Anaerobic	0.02	0.03	0.12	0.03	0.00	**0.02**	0.02	0.03	0.25	0.03	0.01	0.22
Dialister	Gr -/Anaerobic	0.03	0.01	0.11	0.02	0.03	0.66	0.02	0.02	0.66	0.02	0.02	0.74
Escherichia-Shigella	Gr -/Facultative anaerobic	0.03	0.04	0.41	0.04	0.03	0.81	0.01	0.05	0.08	0.05	0.02	0.84
Akkermansia	Gr -/Anaerobic	0.01	0.02	0.87	0.02	0.00	0.85	0.00	0.02	0.72	0.00	0.02	0.30

* Abundance is presented as the proportion of taxa in relation to the total OTUs identified in a given sample.

† *P*-value derived from Wilcoxon rank-sum test. Bold numbers signify statistically significant associations ($P<0.05$).

‡ The symbols indicate the taxonomic level of bacteria. "No symbol" defines genera taxonomic level, "_ "defines the family taxonomic level and" _; _" defines the order taxonomic level.

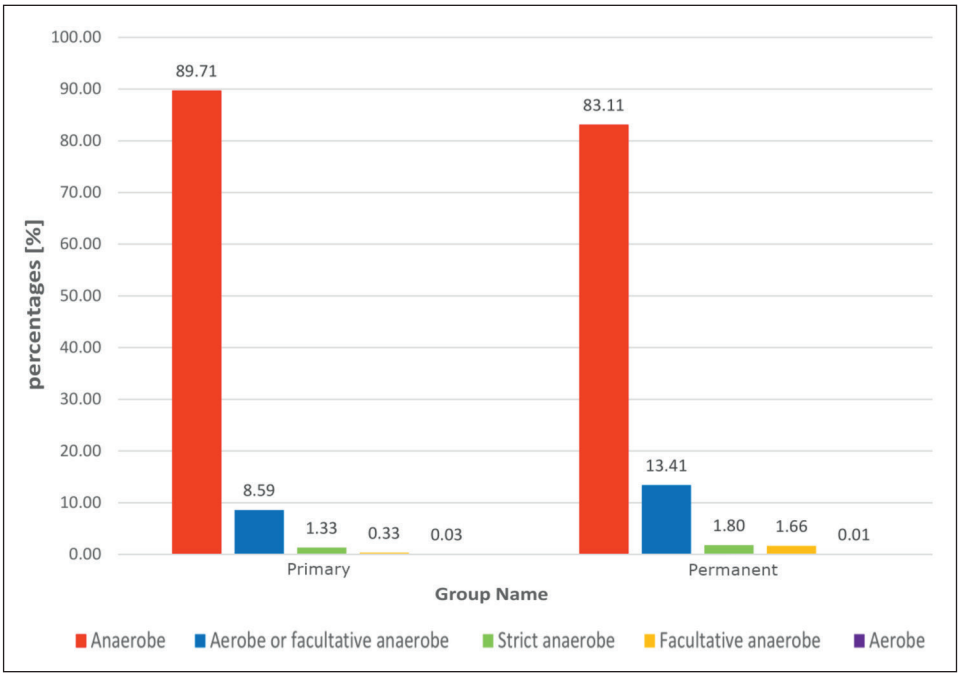

Figure 4. Bacteria percentages based on the oxygen requirement in endodontically infected primary and permanent teeth. Anaerobic bacteria were dominant in both groups, with a higher rate in primary teeth. Aerobe or facultative anaerobe bacteria, strict anaerobe, and facultative anaerobe bacteria were more abundant in permanent teeth.

Results of 2,754,191 raw sequences of Illumina MiSeq paired-end sequencing revealed a positive presence of bacterial DNA in all root canal contents of the primary and permanent teeth. Further, 1,664,926 sequence reads were obtained after removing dropped reads and chimeric reads. In total, 2,237 OTUs (969 OTUs in primary teeth, 819 OTUs in permanent teeth, and 449 OTUs in both permanent and primary teeth) at 97 percent similarity were assigned to 14 phyla, 89 families, and 236 genera.

The rarefaction curves constructed from OTUs, indicating the bacterial richness of individual samples, reached a saturation plateau (see **Supplemental Electronic Data–sFigure 1**) and were found to be similar in primary and permanent teeth (see **Supplemental Electronic Data–sFigure 2**).

There was no statistically significant difference in species richness and evenness in primary and permanent teeth canals (Observed OTUs, Plelou's evenness, and Shannon index, $P>0.05$). However, a phylogenetic difference was found (Faith's phylogenetic diversity, $P=0.04$). Principal Coordinate Analysis (**PCoA**) also confirmed that the canal microbiota composition was similar between the endodontically infected (initial stage) primary and permanent teeth groups.

The abundance of the different phyla detected in each sample is presented in Figure 2. The most abundant phyla of all samples were Firmicutes (61.4 percent), Actinobacteria (16.4 percent), Bacteroides (15.9 percent), and Proteobacteria (4.7 percent). The abundance of the different phyla detected in groups (dentition type, age, jaw, and gender) is presented in Figure 3. Specifically, Firmicutes was the most commonly detected phyla in both endodontically infected primary (61.4 percent) and permanent (61.4 percent) root canals. Further, Bacteroides (18.1 percent) and Proteobacteria (4.8 percent) were more common in primary teeth, whereas Actinobacteria (18.1 percent) and Verrucomicrobia (1.9 percent) were more common in permanent teeth.

When the endodontic microbiome was examined at the genus level, most genera were in relatively low abundance. Abundant bacterial taxa greater than 0.4 percent are shown in Table 2. *Lachnospiraceae* (11.0 percent), *Faecalibacterium* (9.0 percent), *Collinsella* (7.0 percent), *Bacteroides* (7.0 percent), *Prevotella* (6.0 percent), and *Bifidobacterium* (6.0 percent) were abundant in primary teeth root canals, whereas *Bifidobacterium* (11.0 percent), *Lachnospiraceae* (10.0 percent), *Blautia* (8.0 percent), *Lactobacillus* (7.0 percent), and *Collinsella* (5.0 percent) were abundant in the permanent teeth root canal. Moreover, *Atopobiaceae* and *Alloprevotella* were significantly higher in permanent teeth root canals ($P=0.04$), while and *Parabacteroides* was significantly higher in primary teeth root canals ($P=0.03$).

Alloprevotella, *Ruminococcus 2,* and *Holdemanella* were significantly higher in six-year-olds and younger children, whereas *Parabacteroides* was significantly higher in children older than six years. *Faecalibacterium* was significantly higher in maxillary teeth, while *Catenibacterium* was significantly higher in mandibular teeth. *Peptococcus* was significantly higher in females, whereas *Faecalitalea* was significantly higher in males (Table 2).

Analysis of group similarities (ANOSIM) test, Principal component analysis (PCA), and PCoA showed no statistically significant differences in primary and permanent teeth endodontic microbiomes (R=0.011, $P=0.338$). According to order taxonomic level heat map analysis, *Clostridiales* were the most abundant taxa in both permanent and primary teeth. *Bacteroidales* were abundant in permanent teeth, while *Coriobacteriales*, *Bifidobacteriales*, *Lactobacillales*, and *Erysipelotrichales* were abundant in primary teeth (see **Supplemental Electronic Data–sFigure 3**).

Anaerobes were predominantly abundant in both primary and permanent teeth. However, compared to permanent teeth, it was observed that the rate of anaerobes was higher, and the rate of aerobe or facultative anaerobes was lower in primary teeth (Figure 4). Likewise, children six years old or younger had higher anaerobe rate and lower aerobe or facultative anaerobe rate than children older than six years.

Discussion

This study investigated the bacterial diversity of primary and permanent teeth endodontic microbiome using 16S rRNA gene sequencing to identify each phylum and genera, even if at low abundance.[11] The question of bacterial diversity in primary and permanent teeth has been assessed numerous times and has clinical implications but has not been well answered previously. This study investigated primary and permanent root canal microbiota using Illumina sequencing. Amplicon sequence of the hypervariable regions of the 16S rRNA gene was used to detect more than 2,000 OTUs in endodontic infections of primary and permanent teeth using Illumina MiSeq sequencing. Compared to previous studies, the detection of a higher number of

OTUs could be due to the large sample size and the technique's ability to detect rare taxa.[11,17,18] Since the number of OTUs has been reported to decrease significantly as the degree of disease increases, the initial stage endodontic infection of the samples is thought to have an impact on the detection of a high number of OTUs.[19]

The technique used for sample collection is important to accurately determine microbial communities. As such, different sampling methods have been used in different studies. In this study, the paper point technique was preferred, which has been used in several studies investigating endodontic canal microbiota,[10,11,15,17,18] because it is a simple and easy method and does not require extraction of the examined teeth. However, this technique has some limitations for identifying the total microbiome in the root canal system. Specifically, microorganisms that colonize in the dentinal tubules cannot be obtained with paper points. Since microorganisms that colonize in the dentinal tubules may differ from species found in the main canals,[11] this sampling method has been criticized for underestimating the actual microbial population. As an alternative, cryopulverization has some advantages to detect as many endodontic microbial communities as possible. However, this procedure requires tooth extraction; thus, it is not appropriate for cases that can be treated.[20] Consequently, since no technique is ideal for endodontic microbiological sampling, a sample collection method appropriate for the purpose of the study should be selected.

In the present study, the authors focused on the bacterial community in primary and permanent teeth canals with irreversible pulpitis to investigate microbial richness and diversity. It was observed that microbial richness and evenness were similar in both groups, but there was a phylogenetic diversity. The main abundant phylum was Firmicutes. This result is in agreement with previous microbial community profile studies conducted in different populations.[18,21-23] However, second abundant phylum were found to differ in primary and permanent teeth. Firmicutes were followed by Actinobacteria in permanent teeth and Bacteroidetes in primary teeth. A recent study has shown that both Firmicutes and Actinobacteria were highly abundant in reversible pulpitis. However, in symptomatic irreversible pulpitis, the abundance of Firmicutes was increased. The researchers stated that the decrease of Actinobacteria and the increase of Firmicutes may indicate the progression of the infection.[24] The phylum differences in the present study are thought to be related to the rate of infection progression.

In previous studies, Keskin et al.[14] and Sanchez-Sanhueza et al.[11] found Proteobacteria to be the most abundant phylum in primary or secondary/persistent endodontic infections, while Hong et al.[25] and Tzanetakis et al.[10] reported that Bacteroidetes was the most abundant phyla in their studies. In another study, Firmicutes, Proteobacteria, and Actinobacteria were reported to be the most dominant phyla.[13] However, all of these studies were performed only at permanent teeth. In a study of microbiomes in infected primary teeth, Proteobacteria was the most abundant phylum, followed by Firmicutes and Bacteroides.[17] However, some of the teeth had pus discharge, gingival swelling, periapical abscess, and root resorption. The present study is the first examining microbiota of initial stage irreversible endodontic infections.

Few studies have evaluated the primary teeth canal microbiota.[17,26,27] In these studies, *Fusobacterium*, *Prevotella*, and *Porphyromonas* were the most commonly isolated genera. In a study by Güven et al.,[5] *F. nucleatum* was the most frequently isolated bacteria, followed by *P micra* and *P intermedia* in the endodontic microbiota of abscessed primary teeth. Yun et al.[17] evaluated teeth at different stages of endodontic infection and found that *Lactobacilli* was abundant at the initial stage of endodontic infection. As the infection progresses, nutritional and environmental changes occur within the canal. These changes cause differentiation in endodontic microbiota, thus the microbiome in an initial endodontic infection differs from the microbiome in a severe endodontic infection.[28] For instance, *Fusobacterium* has been associated with more severe endodontic infection.[17]

In this study, *Lactobacillus* was present in both primary and permanent teeth while *Fusobacterium* was absent. Since teeth at initial stage endodontic infections were included in the study, the absence of *Fusobacterium* was consistent. Apart from the severity of the infection, other factors can cause differentiation of the microbiome; consider that primary teeth have shorter roots, more accessory canals present in the furcation area, a thinner enamel and dentin layer, and a larger pulp volume. Particularly, shorter roots cause more fistulization incidence adjacent to the crown surface.

Oral bacterial composition is affected by age and become more diverse.[29] However, there is not much information about the composition of the flora in different age groups. Burcham et al.[30] investigated oral microbiota diversity in adults and children. They stated that the bacterial composition was more diverse in youth microbiomes than adults and adult oral microbiomes were mainly affected by oral health habits, while youth microbiomes were affected by sex and weight. Also, in another microbiota study conducted in 24 adult patients in need of retreatment, there was no significant difference in bacterial richness and evenness between age groups.[11] In the present study, bacterial richness and evenness were similar in different age groups, jaws, and sex. In terms of bacterial diversity, Alloprevotella, Ruminococcus, and Holdemanella were significantly higher in younger (younger than six years) and Parabacterioides was significantly higher in older subjects (older than six years).

Nonetheless, this study had some limitations. First, by using the paper point sampling method, samples could not be taken from the accessory, lateral canals, or dentin tubules. In addition, only the initial stage endodontically infected primary and permanent teeth were included in the study. Therefore, these results do not provide information about microbiota at different stages of endodontic infection with different symptoms (pus discharge, gingival swelling, periapical abscess, et cetera). Also, with a study organizing an adequate number of samples at each age range, a more detailed examination of the development/composition of microorganisms can be obtained. Finally, all potential exposures and confounding variables that could affect the microbiome could not be controlled in this study (e.g., individual diet, habitat, et cetera).

Conclusions

1. A higher number of operational taxonomic units were found in infected primary teeth pulp than permanent teeth.
2. Although microbial richness and evenness of the endodontically infected primary and permanent teeth were found to be quite similar, there were some phylogenetic differences.
3. These findings indicate that antibiotics effective for anaerobic bacteria should be considered in both primary and permanent endodontic infections when considering antimicrobial therapy.

4. As the next generation of microbiome-based sequencing tests become more economical and faster, more effective results and good prognosis could be obtained with the treatments based on microbiota analysis in clinical dentistry practice.
5. Further studies with a larger sample size, including various local, systemic, and environmental factors that may affect microbial flora and with different clinical and radiographic symptoms, should be performed.

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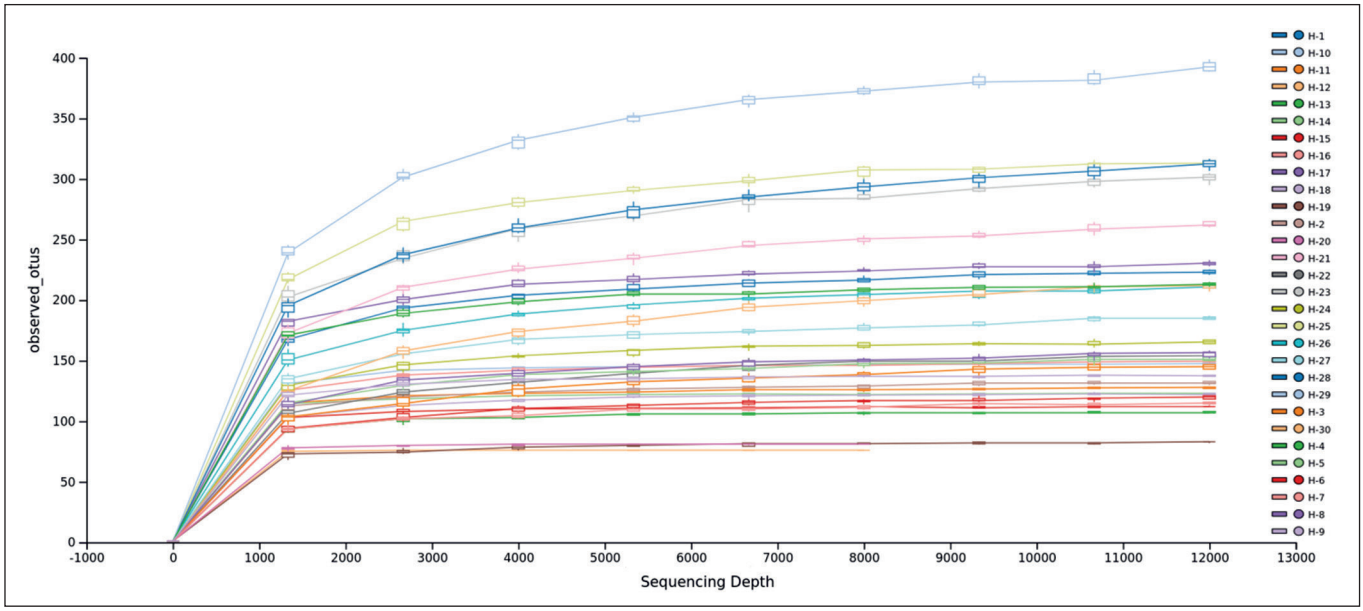
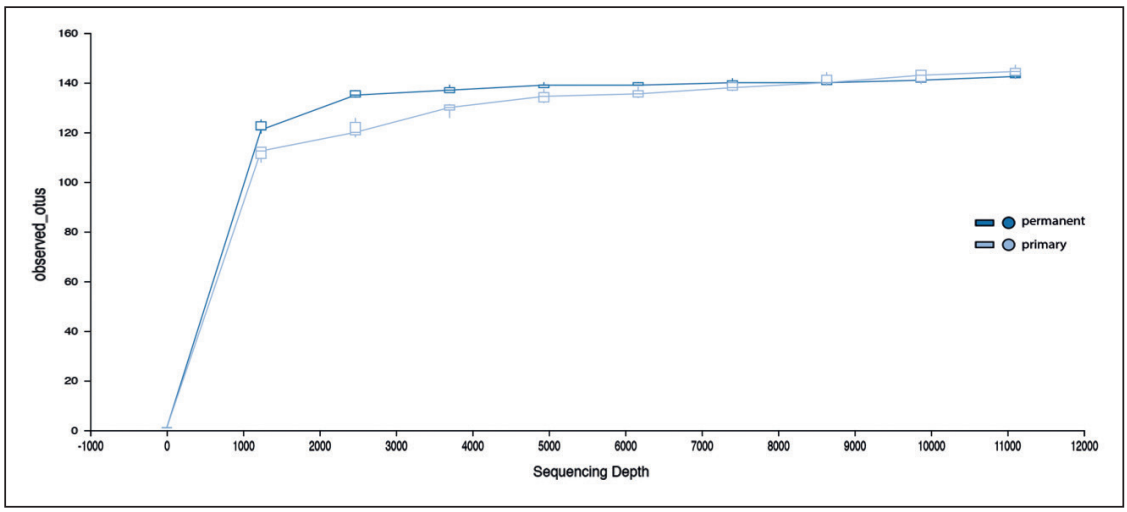
Supplemental Electronic Data

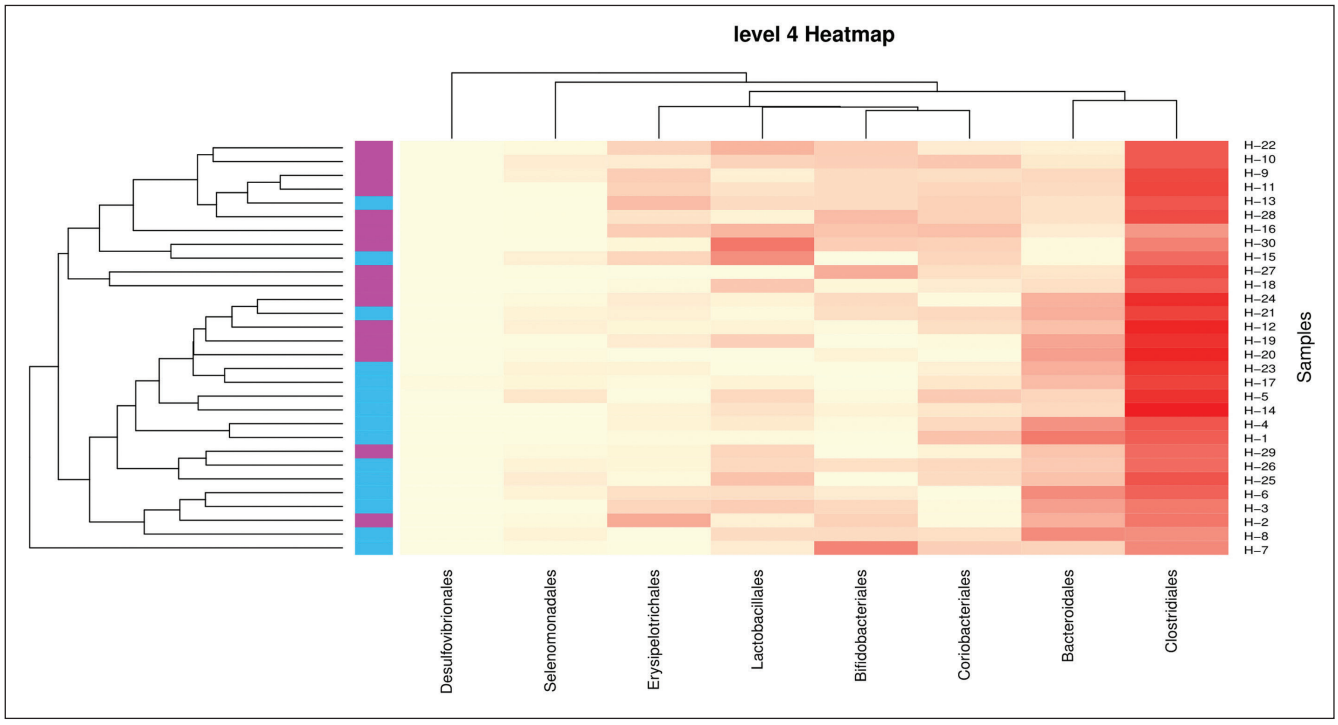
Supplemental Figures



sFigure 1. Rarefaction curves of two groups. Permanent and primary teeth show similar curves that express the similarity of bacterial richness. Parallel curves demonstrate that the number of readings is sufficient for analysis.



sFigure 2. Rarefaction curves indicating the bacterial richness of each sample. Rarefaction curves of 16S rRNA gene sequences for each sample were calculated for operational taxonomic units (OTUs) at 97 percent similarity. In the graph, the X-axis represents the number of readings; the Y-axis represents the number of OTUs. Each color indicates a sample. Parallel curves demonstrate that the number of readings is sufficient for analysis.



sFigure 3. Gradient heatmap with an order-level dendrogram (over one percent of the microbiome). The most abundant genera were assessed by hierarchical clustering to evaluate relationships across 30 samples. Purple represents primary teeth, and blue represents permanent teeth. Clostridiales were the most abundant taxa in both permanent and primary teeth. Bifidobacteriales, Lactobacillales, and Erysipelotrichales were more abundant in primary teeth.