Microbiome Analysis of Three Sample Types Obtained From Mixed Dentition Children With Different Caries Statuses: A Cross-Sectional Survey

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Research Article

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Abstract

Background: Both supragingival plaque and saliva are commonly sampled for analysis of microbial communities. However, it remains unclear whether saliva can replace supragingival plaque for microbial studies of caries. There are limited studies on whether the microbiota of healthy first permanent molar (FPM) differs in different caries statuses of deciduous teeth. Herein, the PacBio Sequel platform was used to identify microbiome between three types of oral samples. Additionally, we compared the microbiota between children with caries and healthy children in the same kind of microhabitat.

Methods: In total, 30 children (aged 7–9 years) were enrolled in this study; 15 of them had dental caries. DNA was extracted from supragingival plaques of deciduous molars, supragingival plaques of maxillary FPMs, and saliva, and the v1–v9 regions of 16S rRNA was amplified. Additionally, PacBio sequencing and bioinformatic analyses were performed.

Results: The salivary microbial alpha diversity were lower than that of the supragingival plaque on the teeth, but there was no difference between deciduous teeth and FPMs. Nonmetric multidimensional scaling analysis showed that the saliva samples were mainly scattered in the left side, whereas most plaque samples were distributed on the right. In the same [microhabitat](#), there was no difference in microbial alpha and beta diversity between children with caries and healthy children. For the samples of the deciduous teeth, *Streptococcus mutans*, *Propionibacterium acidifaciens*, and *Veillonella dispar* were more abundant in the children with caries than healthy children, and the first two bacteria showed a positive correlation. For the samples of the FPMs, *Selenomonas noxia* was more abundant in healthy children than children with caries. But no differentially abundant microorganism were identified between the saliva subgroups.

Conclusion: The microbial composition and structure of supragingival plaque was different from that of saliva, and supragingival plaque was found to be the best candidate for studying caries etiology. *Streptococcus mutans*, *Veillonella dispar*, and *Propionibacterium acidifaciens* are highly associated with the existence of deciduous caries. The microbiota of the supragingival plaque on the healthy FPMs resemble when the caries status of the deciduous teeth was different, except for the abundance of *Selenomonas noxia*.

Background

Caries, a localized damage to the hard tooth tissue caused by acidic byproducts of bacterial fermentation of free sugars, is the one of the most common and preventable diseases in children [1–3]. Caries not only affects masticatory function, aesthetics, and school performance but also reduces the quality of life of children [4]. Moreover, progression of caries can cause pain [5] and potentially life-threatening complications, such as odontogenic brain abscesses [6, 7] and deep neck-space infections [8]. Once cavitated caries occurs, dental destruction progresses and becomes irreversible. Thus, studies of caries etiology are particularly important.

The biological basis of caries is alteration of the microbial community, i.e., imbalance of the oral microbiome [9, 10]. Acidogenic and aciduric bacteria become abundant in a low-pH environment and are involved in the occurrence and development of caries [11]. Several studies based on the 16S rRNA gene sequencing have demonstrated that children with caries have a different oral microbiome from healthy children [12–15]. However, these results were obtained using next-generation sequencing (NGS) technology, which has some limitations. Although NGS technology is a high-throughput, low-cost approach, it yields relatively short reads [16]; therefore, NGS is insufficient for sequencing the complete 16S rRNA gene (total length: ~1500 bp), and only the integrant V region of the 16S rRNA gene is sequenced. Teng et al. found that the selection of the specific V region affected the analysis results of oral microbial diversity [17]. Third-generation sequencing (TGS) platform has greatly improved these limitations.

Pacific Biosciences (PacBio) sequencing is one of the representatives of TGS [16]. Firstly, subsequent development of PacBio RSII and PacBio Sequel sequencers have greatly improved accuracy compared with PacBio RS sequencer [18]. The accuracy was improved through circular consensus sequencing (CCS) to 99.9999%, similar to or higher than NGS. In addition, Myer et al. found the TGS platform (PacBio RSII) achieved a better phylogenetic resolution than the NGS platform (MiSeq) [19]. However, few studies have reported the oral microbiome in the PacBio sequencing, i.e., salivary microbial communities in children using the PacBio RSII sequencer [20] and microbial communities of dental plaques from young adults using the PacBio Sequel sequencer [21]. To date, no study has investigated the microbiome from multiple regions in the oral cavity in children using PacBio sequencing.

Saliva and supragingival plaque on tooth surface are the most used samples in studying caries. However, whether saliva can replace supragingival plaque to study caries remains controversial. Additionally, children with mixed dentition have both primary and permanent teeth. Both cross-sectional and longitudinal studies have shown that caries of a deciduous tooth is a risk factor for the first permanent molar (FPM) to be carious [22–24]. We determined whether the microbial community of noncarious FPMs is different when the caries status of the deciduous teeth differs.

To compare microbiome between three types of oral samples and confirm whether saliva samples could be used in microbial studies of caries, to characterize the oral microbiome in children with and without caries using optimal samples, and to compare the microbial community of the supragingival plaque of healthy FPMs from children with different caries statuses, we performed PacBio sequencing to determine the oral microbiome from supragingival plaque and saliva in children with and without deciduous caries and who had mixed dentition.

Methods

Subjects

Thirty children with mixed dentition were enrolled in this study. All subjects were recruited from the same primary school in Guangzhou, China, to reduce the impact of environmental factors, such as fluoride

concentration in drinking water, on the microbiota. The maxillary FPMs of all children had fully erupted without decay cavities, and deep pits and fissures were observed. The exclusion criteria in this study were as follows: having active bacterial or viral infections in other parts of the body; having received antibiotic treatment in the past 3 months; having other oral diseases; and wearing a movable or fixed orthodontic appliance.

The diagnostic criteria for caries were based on the fifth edition of the Oral Health Surveys: Basic Methods published by World Health Organization [25]. Accordingly, the decayed, missing, and filled tooth (dmft) index and the decayed, missing, and filled tooth surface (dmfs) index were used to record caries status in all children. The dmft scores of healthy individuals were 0 (dmft = 0), indicating they had no caries, whereas subjects with caries had three or more deciduous teeth, with caries in their oral cavities (dmft $\geq$ 3). According to the results of oral examination, 15 children without caries were enrolled in the healthy group (H group), whereas 15 children with caries were enrolled in the caries group (C group). All examinations and sampling were performed by Y , who had previously been trained in the diagnosis of caries and in appropriate sampling procedures.

Sample collection

Children were not allowed to brush their teeth the night before and in the morning of the day of sampling, and water and food were withheld prior to sampling. Supragingival plaque was scraped with a sterile metal excavator and placed in a sterile Eppendorf tube containing 0.5 mL Tris-EDTA buffer. Dental plaque from the buccal and lingual surfaces and the caries lesions in deciduous molars was collected from individuals with caries (CD subgroup), and dental plaque from the buccal, lingual, and occlusal surfaces of deciduous molars was collected from healthy individuals (HD subgroup). The dental plaque from the buccal, lingual, and occlusal surfaces of FPMs of all children was sampled as the CP subgroup and HP subgroup from C group and H group, respectively. Two milliliters of non-stimulating saliva was collected from the bottom of each child's oral cavity with a disposable sterile pipette and then placed in a sterile centrifuge tube (CS and HS subgroups from the C and H groups, respectively). Every subgroup contained 15 samples, and a total of 90 samples were included. Following collection, samples were placed in a foam incubator with dry ice, and all samples were immediately transported to the laboratory and stored at -80 °C. The FPMs of all subjects were treated with pit and fissure sealant after sampling.

DNA isolation and amplification

The total bacterial DNA was extracted using an E.Z.N.A. Bacterial DNA Kit (Omega, USA) according to the manufacturer's protocol. The quality of DNA was evaluated via agarose gel electrophoresis, and the DNA concentration was measured by determining the absorbance ratios A260/A280 and A260/A230 using a Nanodrop spectrophotometer (Thermo Scientific, Waltham, MA, USA). The total DNA was stored at -80 °C until use.

The barcode sequence was inserted into the forward primer (5'-AGAGTTTGATCCTGGCTCAG-3') to distinguish each sample, and the v1– v9 regions of the 16S rRNA gene were amplified in a 25- μ L

amplification system containing 5 µL of 5× reaction buffer, 5 µL of 5× GC buffer, 2 µL dNTPs, 1 µL forward primer, 1 µL reverse primer (5'-GGTTACCTTGTTACGACTT-3'), 2 µL DNA template, 8.75 µL ddH₂O, and 0.25 µL Q5 high-fidelity DNA Polymerase (NEB, USA). The PCR conditions were as follows: initial denaturation at 98 °C for 2 min; 25–30 cycles of denaturation at 98 °C for 15 s, annealing at 55 °C for 30 s, and extension at 72 °C for 30 s; and a final extension at 72 °C for 5 min. The PCR products were examined using 2% agarose gel electrophoresis and extracted with an AxyPrep DNA Gel Extraction kit (Axygen, USA). A Quant-iT PicoGreen dsDNA Assay Kit (Thermo Fisher, Waltham, MA, USA) was used to quantify the obtained PCR products on a microplate reader (BioTek, VT, USA). Based on the fluorescence quantitative results, the products from each sample were adjusted to equal concentrations and then mixed.

DNA library construction and sequencing

DNA library was constructed using a SMRTbell Express Template Prep Kit 2.0 (Pacific Biosciences, CA, USA), and each constructed library was combined with a primer and DNA polymerase to form a primer/template/polymerase, using a DNA Polymerase Binding Kit P4 (Pacific Biosciences). All complexes were loaded onto the SMRT Cell using a MagBead kit (Pacific Biosciences), and the MagBead was combined with the complex to travel over the zero-mode waveguide (ZMW). Sequencing was performed using a DNA Sequencing Kit 2.0 (Pacific Biosciences) on a PacBio Sequel Sequencer (Pacific Biosciences). The biotin-modified DNA polymerase bound to streptavidin at the bottom of the ZMW, anchoring the complex to the bottom of the ZMW. The DNA polymerase read the single-stranded circular DNA template several times to produce polymerase sequences.

Quality filtration of sequences

Several subreads were obtained by removing sequencing adapters from one polymerase sequence. The CCS approach was used to read subreads for at least three fully passes, thus producing CCS sequences with predicted accuracy of 99% and allocating the CCS sequences into the corresponding samples according to the barcode sequence. The QIIME (v1.8.0) software [26] was used to filter low-quality sequences that met the following conditions: sequence length being less than 500 bp; sequence having one or more fuzzy base N; primer of 5' terminal of sequence having more than 5 mismatched bases; and sequence containing more than 8 of the same continuous bases. USEARCH (v5.2.236) [27] via the QIIME (v1.8.0) software was used to check and eliminate chimeric sequences. Finally, high-quality sequences for each sample were obtained and used for subsequent analysis.

The 16S rRNA sequence analysis

Using the sequence alignment tool UCLUST [28] via the QIIME (v1.8.0) software, high-quality sequences were clustered into operational taxonomic units (OTUs) [29] at 97% similarity. To ensure the accuracy of the subsequent analysis, OTUs whose abundance was lower than 0.001% of abundance of the total OTUs were removed [30]. An OTU table was then constructed based on the number of sequences contained in each OTU for each sample. The sequence with the highest abundance in each OTU was

selected as the representative sequence. By comparing representative OTU sequences with reference sequences in the Human Oral Microbiome Database (HOMD) [31], the taxonomic information for each OTU was obtained, allowing us to determine the taxonomic composition of each sample at the phylum, class, order, family, genus, and species levels.

Based on the OTU table, the QIIME software was used to calculate the alpha diversity of each sample using Simpson, Chao1, ACE, and Shannon indices and rarefaction curves. Species accumulation curves and rank abundance curves were drawn with the R software. Chao1 and ACE indices were used to evaluate community richness, whereas Shannon and Simpson indices were used to evaluate both community richness and evenness.

In the taxonomic analysis, we used GraPhlAn [32] to construct and plot a taxonomic tree for determining the predominant microbiome in all samples. Nonmetric multidimensional scaling (NMDS) analysis was used to evaluate the community structure. The linear discriminant analysis effective size (LEfSe) method [33] was used to identify microbial biomarkers in each subgroup. Co-occurrence analysis was performed to demonstrate the interactions of the microbiota.

Statistical analysis

Independent sample t test was used to compare the ages of the two groups of children. Chi-square test was used for gender. Different statistical methods were conducted based on whether the alpha diversity indices could satisfy both normality and homogeneity of variance among subgroups. One-way ANOVA or Kruskal-Wallis H test were used for the comparison of alpha diversity indices between every three subgroups, and Bonferroni test or paired comparison was used for further pair comparison. The abovementioned tests were completed using SPSS (V20.0) software. Adonis analysis was applied for statistical testing of sample groupings in a distance matrix. The LEfSe method was used to compare the microbial abundances among subgroups. Spearman’s correlation coefficients among microbes were calculated using the co-occurrence analysis, performed in the 'Wu Kong' platform (<https://www.omicsolution.org/wkomics/main/>). $p < 0.05$ was considered statistically significant.

Result

Subjects, groups, and sequencing data

Thirty children underwent oral examinations in this study (Table 1), half of which were in the C group, while the other half were in the H group. Three samples were collected from every child, totaling 90 samples. According to sample type collected (saliva and supragingival plaque of the FPMs and deciduous molars, respectively), each group consisted of three subgroups, including CS, CP, CD, HS, HP, and HD subgroups. Thus, 15 samples were obtained from each subgroup.

Table 1. Demographic and clinical characteristics of the 30 Chinese children.

Group	Subgroup	Age* (months)	Sex (male/female)	dmft*	dmfs*
Caries (n = 15)	CD, CP, CS	97.31 ± 3.22	10/5	4.07 ± 0.96	4.87 ± 1.60
Healthy (n = 15)	HD, HP, HS	97.91 ± 3.86	9/6	0	0

dmft: the number decayed, missing, or filled teeth.

dmfs: the number of decayed, missing, or filled tooth surfaces.

*Data are expressed as mean ± standard deviation.

After filtering out low-quality sequences, 418,094 high-quality sequences were obtained, with 4,645 sequences per sample. The average length of sequences was 1,510 bp, 98.39% of which were distributed between 1,401 and 1,600 bp (Figure 1). A total of 2,495 OTUs were obtained.

Sequencing depth and sample size

Rarefaction curves showed a gentle shape, indicating that the current sequencing depth in our study was sufficient, which could adequately reflect the microbial diversity (Figure 2A, B). The blue-shaded areas in the species accumulation curve represent confidence intervals of OTUs determined, whereas the upper and lower lines of the yellow rectangle represent quartiles of OTUs. With the increase of sample size, the blue-shaded areas and the height of yellow rectangle first increased and then gradually decreased. When the sample size was 90, the species accumulation curve was saturated (Figure 2C), indicating that the quantity of OTUs would not increase with the addition of new samples; this suggested that the sample size was sufficient for this study. The steep rank abundance curve suggested that species evenness was low across all samples (Figure 2D).

Predominant taxon

A total of 11 phyla, 19 classes, 28 orders, 52 families, 96 genera, and 370 species of bacteria were identified, and the number of taxa in 6 subgroups at each taxonomic level is shown in Table 2. We mainly focused on three taxonomic levels, namely phylum, genus, and species. At the phylum level, Proteobacteria (29.9%) had the highest relative abundance, followed by Firmicutes (25.8%), Bacteroidetes (20.3%), Fusobacteria (12.0%), Actinobacteria (8.0%), and TM7 (3.2%; Figure 3A, B), accounting for 99.15% and 99.21% of the oral microbiota in children with and without caries, respectively. At the genus level, *Neisseria* (12.2%), *Streptococcus* (10.3%), *Prevotella* (7.0%), *Leptotrichia* (6.8%), *Capnocytophaga* (6.5%), and *Selenomonas* (6.2%) had the highest relative abundances (Figure 4A, 5A). Of the 370 species of bacteria, the following 8 species showed high relative abundances: *Neisseria flava* (6.5%), *Selenomonas noxia* (4.2%), *Veillonella dispar* (4.2%), *Haemophilus parainfluenzae* (3.6%), *Aggregatibacter* sp. HMT 458 (3.4%), *Streptococcus mitis* (3.2%), *Corynebacterium matruchotii* (3.1%), and *Lautropia mirabilis* (3.1%; Figure 4B, 5B). The microbes with the highest relative abundances were

generally dominant. The phylum Proteobacteria, phylum Firmicutes, phylum Bacteroidetes, phylum Fusobacteria, and genus *Neisseria* were analyzed as the dominant microbiota by the GraPhlAn tool. (Figure 6)

Table 2. The number of taxa in six subgroups at each taxonomic level.

Subgroup	Phylum	Class	Order	Family	Genus	Species
CD	11	19	26	43	77	302
CP	9	17	24	39	65	255
CS	9	17	24	41	75	281
HD	11	19	25	41	71	276
HP	10	17	23	39	69	256
HS	11	19	26	48	83	280

Spatial variation of microbiota

A total of 1,894 OTUs were obtained in the C group, and CD, CS, and CP subgroups shared 574 OTUs (Figure 7A), which accounted for 30.31% of the OTUs in the C group; 1,770 OTUs were obtained in the H group, and HD, HS, and HP subgroups shared 554 OTUs, which accounted for 31.30% of the OTUs in the H group (Figure 7B).

The alpha diversity indices were calculated based on OTU data. For samples of different microniches, the microbial alpha diversity indices were different. In children with caries, the Simpson, Chao1, ACE and Shannon indices of CS subgroup were lower than those in CD and CP subgroups ($p < 0.05$, Figure 8A, B). Among children without caries, the Simpson, Chao1, and ACE indices of HS subgroup were lower than those in the HD subgroup ($p < 0.01$, Figure 8C, D), and the Shannon index was lower than that in the HD and HP subgroups ($p < 0.05$, Figure 8D). However, there was no significant difference between deciduous and permanent teeth in children with or without caries ($p > 0.05$, Figure 8). These results indicated that the microbial richness and evenness of non-stimulating saliva were lower than that of supragingival plaque, especially in deciduous teeth.

The LEfSe analysis method was used to analyze the taxonomic biomarkers among subgroups, and the threshold of the logarithmic LDA score for discriminative features was set at 4.0. In similar oral environments (caries or caries-free), the abundance of the same microorganism in different spatial locations was different ($p < 0.05$). In children with caries, *Prevotella*, *Lactobacillus*, *Streptococcus mutans*, and *Propionibacterium acidifaciens* were taxonomic biomarkers in the CD subgroup; *Corynebacterium* and *Corynebacterium matruchotii* were taxonomic biomarkers in the CP subgroup; and *Streptococcus* was a taxonomic biomarker in the CS subgroup (Figure 9A). In children without caries, *Eubacterium* [XIVa]

[G-1], *Leptotrichia*, and *Eubacterium* [XIVa][G-1] *saburreum* were taxonomic biomarkers in the HP subgroup; *Haemophilus* and *Haemophilus parainfluenzae* were taxonomic biomarkers in the HS subgroup (Figure 9B); and no differences in the abundant features were found in the HD subgroups.

To evaluate the similarities and differences of the microbial community structures among subgroups, NMDS analysis was performed based on the Weighted UniFrac distances. As shown in Figure 10A, saliva samples (CS and HS subgroups) were primarily scattered in the left side, whereas most plaque samples (CD, HD, CP, and HP subgroups) were distributed on the right. Since the samples of deciduous teeth and PFMs were almost distributed in the same area (Figure 10B), the samples of deciduous teeth and PFMs with the same disease status (caries or caries-free) were mixed to represent the supragingival plaque in Adonis analysis; the subgroup CD plus CP was CDP, and HD plus HP was HDP. Adonis analysis demonstrated a significant difference among CS, HS, CDP, and HDP subgroups ($p < 0.001$).

Comparison of microbial communities between children with and without caries

For children with different caries statuses, there was no significant difference in microbial alpha diversity in the same microhabitat (CD vs HD, CP vs HP, and CS vs HS). Additionally, NMDS analysis showed no significant separation was found in the samples between two subgroups (Figure 10A). In the LEfSe analysis, the phylum Fusobacteria was more abundant in the HD subgroup than in the CD subgroup, whereas the phylum Firmicutes was more abundant in the CD subgroup than in the HD subgroup (Figure 9C). At the genus level, *Streptococcus* and *Veillonella* were biomarkers in the CD subgroup, whereas *Corynebacterium*, *Selenomonas*, and *Fusobacterium* were biomarkers in the HD subgroup (Figure 9C). At the species level, *Streptococcus mutans*, *Veillonella dispar*, and *Propionibacterium acidifaciens* were biomarkers in CD subgroup (Figure 9C). In contrast, *Selenomonas noxia* and *Corynebacterium matruchotii* were the taxonomic biomarkers of HD subgroup (Figure 9C). Compared with the CP subgroup, the HP subgroup had a high abundance of the phylum Fusobacteria and *Leptotrichia*, *Selenomonas*, and *Selenomonas noxia*. (Figure 9D). However, we found no taxonomical biomarkers to distinguish between the CS and HS subgroups based on our analysis.

We selected species with the top 100 relative abundances and calculated the Spearman's rank correlation coefficients for co-occurrence analysis, which was used to evaluate interactions among the microbes in each subgroup. There were more abundant microbes that showed strong associations in the caries subgroups than healthy subgroups (CD vs HD, CP vs HP, and CS vs HS; Figures 11–13). *Streptococcus mutans* and *Propionibacterium acidifaciens*, which are both caries-associated taxa, were positively correlated in the CD and CS subgroups (Figures 11, 13).

Discussion

Caries is the most common disease of the oral cavity, which is mainly caused by oral microbiota. The 16S rRNA gene sequencing is a momentous tool for identifying microorganisms. Compared with NGS platforms, SGS technology such as PacBio sequencing enables more accurate taxonomic resolution of microbial communities owing to sequencing of full-length 16S rRNA genes [34]. In this study, the PacBio

Sequel platform was used to sequence and analyze the v1–v9 region of the 16S rRNA of microorganisms, which were classified into species, thereby obtaining a total of 370 species. Previous studies have shown that the resolution of identifying microorganisms using the NGS platform, which analyzes part of the high-variation region (V region) of the microbial 16S rRNA gene, usually reaches only the genus level [12, 14]. Therefore, we used the PacBio Sequel platform that could identify the microbial communities at a more precise resolution to explore the microbial etiology of caries better.

The results showed that in the same microhabitat, the microbial richness and evenness of healthy children and children with caries were similar, which was consistent with the previous findings [20, 35, 36]. However, Belstrom et al. found that the alpha diversity of oral microbiota of healthy individuals was higher than that of patients with caries. Their study differed from that of ours in two aspects [37]. Firstly, children in the caries group in their study had more severe caries with an average dmfs index of 57.1, which was much higher than that in our study. It has been shown that with the increase of tooth decay, the microbial alpha diversity decreased [14]. Secondly, the sample type collected by Belstrom et al. [37] was stimulating saliva, which is different from that used in our study. The severity of caries and sample type collected may influence the result of microbial alpha diversity, which needs to be verified by more in-depth studies. However, our study showed that microbial richness and evenness of saliva was lower than that of supragingival plaque in children with or without caries, which was consistent with previous studies [38, 39], suggesting that the tooth surface provided a more ideal place for microbial growth and reproduction. Taken together, differences of microbial richness and evenness in different microniches were more significant, but not in different caries statuses, suggesting that the differences in microbial diversity in spatial sites should be taken into account when studying microbial diversity.

Both supragingival plaque and saliva are commonly sampled for analysis of caries. However, there are considerable differences between the supragingival plaque and saliva in terms of the microbiome [12, 39, 40], while it remains unclear whether saliva can replace plaque for microbial studies of caries. The results of this study showed that the microbial alpha and beta diversities between saliva and deciduous molars were different, whether in children with caries or in healthy children. In addition, the LEfSe method showed several taxonomic biomarkers in supragingival plaque samples of deciduous molars, but not in saliva samples, of children with caries and healthy children. Therefore, our findings supported that non-stimulating saliva was unsuitable for studying caries-related microorganisms. Hurley et al. found that the salivary microbiota was not sufficiently specific to identify caries risk in children [12]. In contrast, Eriksson et al. showed that the salivary microbiota was associated with cross-sectional caries prevalence [41]. We found that non-stimulating saliva was used in our study and that of Hurley et al. [12], whereas stimulating saliva was used by Eriksson et al. [41], suggesting that different types of saliva samples may affect the experimental results.

At the genus level, *Fusobacterium*, a type of asaccharolytic bacteria [42], was more abundant in healthy children than children with caries in case of deciduous teeth plaque in our study. Similarly, a previous study demonstrated that the asaccharolytic *Fusobacterium* showed higher bacterial activity on healthy surfaces than on caries lesions, suggesting that *Fusobacterium* may influence tooth health [43].

Furthermore, we identified *Streptococcus* and *Veillonella* as two microbial biomarkers in deciduous molar plaque of children with caries, compared with the corresponding subgroup. Consistent with this, Dzidic et al. found that *Streptococcus* and *Veillonella* inhabited the oral cavity during the first 3–6 months of life, designating these bacteria as early colonizers [44]. Indeed, these bacteria are generally found in children with ECC [12, 20, 45], which was also found in the decayed deciduous teeth in our study.

At the species level, *Propionibacterium acidifaciens* not only was more abundant in healthy children than children with caries in terms of deciduous teeth plaque but was also identified as a caries-specific bacterium, absent in healthy children, and was detected in 13 children with caries in this study.

Propionibacterium acidifaciens showed a higher abundance in individuals with caries in previous studies [46–48]. In 2009, Downes and Wade isolated *Propionibacterium acidifaciens* from the human oral cavity for the first time [49]. Additionally, Obata et al. found that *Propionibacterium acidifaciens* could combine strongly with collagen (dentine contains approximately 20% organic matter, including collagen) and could produce acid in a low-pH environment; these features could contribute to the occurrence of dentine caries [50].

Selenomonas noxia was the second most abundant species in all samples. LEfSe analysis also showed that *Selenomonas noxia* had a higher abundance in the deciduous teeth plaque of healthy children than children with caries, consistent with a study by Preza et al. [51]. *Selenomonas noxia*, a known periodontal pathogen, was isolated, cultured, and identified by Moore and his team in human periodontal pockets in 1987 [52]. Moreover, *Selenomonas noxia* is responsible for the loss of attachment in periodontitis [53] and is present at higher levels in patients with periodontitis than in healthy individuals [54, 55]. Accordingly, the increased or decreased abundance of *Selenomonas noxia* may lead to different oral diseases. Additionally, *Selenomonas noxia* was identified as a microbial biomarker in the FPM plaque of healthy children in our study. The prevention of FPM damage is critical. Indeed, FPMs are essential for establishing occlusion and are the most important teeth for mastication. However, since the FPMs are the earliest permanent teeth to erupt, they are the most prone to caries. Moreover, the prevalence of caries in the FPMs is closely related to the presence of deciduous caries. In 1959, Bruszt et al. recorded the caries statuses of 97 children at 5 and 11 years of age and found that 94.6% of children with deciduous caries had permanent caries 6 years later [56]. In recent years, both cross-sectional and longitudinal studies have shown that deciduous tooth caries is a risk factor for caries in permanent molars (particularly FPMs) [22–24]. However, no microbiological studies have evaluated the correlations between deciduous and permanent molar caries. Our findings revealed that reduction of *Selenomonas noxia* occurs in healthy FPMs of children with deciduous caries, suggesting that it could be associated with increased caries risk in FPMs in children with deciduous caries.

Of the hundreds of oral bacteria, *Streptococcus mutans* is most frequently associated with dental caries and is the primary pathogenic bacteria of caries due to its ability to colonize the oral cavity, form a firm biofilm on the surface of the teeth, survive and reproduce in an acidic environment, and continue to produce acid [57, 58]. In our study, *Streptococcus mutans* was a microbial biomarker in deciduous molar plaque of children with caries, compared with healthy children, indicating that *Streptococcus mutans* was

closely associated with caries. However, neither all children with caries tested positive for *Streptococcus mutans*, nor were all healthy children negative for *Streptococcus mutans*. We found that *Streptococcus mutans* was present in 14 children with caries and that *Veillonella dispar*, another microbial biomarker in deciduous teeth plaque of children with caries, was present in *Streptococcus mutans*-negative individuals, suggesting that caries was not caused by a single bacterium but by a combination of multiple bacteria.

Co-occurrence analysis showed potential interactions among the oral microbiota. Compared with healthy subgroups, there were more abundant correlation pairs of microorganisms in the subgroups of the caries group, indicating that the microbial community had more complex relationships in children with caries. *Streptococcus gordonii* and *TM7[G-1] bacterium HMT 347* were positively correlated in the CD subgroup but showed no correlation in the HD subgroup. *Neisseria subflava* and *TM7[G-1] bacterium HMT 347* were negatively correlated in the HD subgroup but showed no correlation in the CD subgroup. These findings suggested that the same organisms interacted differently in different environments (caries or caries-free). Notably, in a previous study, biofilm formation by *Propionibacterium acidifaciens* was found to be inhibited by *Streptococcus mutans* [50]. However, *Streptococcus mutans* and *Propionibacterium acidifaciens* showed a positive correlation in the co-occurrence analysis in our study, contradictory to the previous findings.

Conclusion

In this study, the composition and structure of oral microbial community were analyzed from the perspectives of different microniches and different caries statuses. In the same caries status, the distribution of microbial is spatially different, which should be taken into account during sampling when studying oral microbiota. In the same sample type, *Streptococcus mutans*, *Veillonella dispar*, and *Propionibacterium acidifaciens* were highly correlated with caries of deciduous teeth, whereas no taxonomic biomarkers were found in saliva samples, suggesting that dental plaque was more representative than saliva for identifying the caries-related microorganisms. Further studies are warranted to determine the reason for the correlation between *Streptococcus mutans* and *Propionibacterium acidifaciens*. Additionally, *Selenomonas noxia* was more abundant in the supragingival plaque of FPMs in healthy children, compared with that of the caries children. To the best of our knowledge, this study is the first to provide a reference on the microbiological differences in healthy FPMs in different caries statuses, thereby establishing a microbial basis for prevention of caries in FPM.

List Of Abbreviations

NGS, next-generation sequencing; PCR, polymerase chain reaction; FPM, first permanent molar; CCS, circular consensus sequencing; ZMW, zero-mode waveguide; OTU, operational taxonomic unit; HOMD, Human Oral Microbiome Database; NMDS, Nonmetric multidimensional scaling; LDA, linear discriminant analysis; LEfSe, linear discriminant analysis effective size; PacBio, Pacific Biosciences; ECC, early childhood caries; rRNA, ribosomal RNA; TGS, third-generation sequencing

Declarations

Ethics approval and consent to participate

This study was performed according to Declaration of Helsinki and approved by the ethical committee of Stomatological Hospital, Southern Medical University (approval number: 2019-07). The parents or legal guardians of all participants provided written informed consent prior to the study.

Consent for publication

Not applicable

Availability of data and materials

Data is available upon request. Contact e-mail: 1021374711@qq.com.

Conflict of interest

The authors declare that they have no conflict of interest.

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Author contributions

GQ conceptualized the study and provided expert advice in designing the experiments and in organizing, writing, and revising the manuscript. XY, XC, and SY planned the experiments. XY and LH conducted the experiments. XY analyzed the data, created the figures, and wrote the manuscript.

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Figures

Figure 1

Length distributions of high-quality sequences.

Figure 2

Curve charts for 90 samples. Each curve represents one sample. (A, B) Rarefaction curves. (C) Species accumulation curves. (D) Rank abundance distribution curves.

Figure 3

Predominant microbes at the phylum level. (A) Heat map analysis in 90 samples. Blue indicates high relative abundance and yellow indicates low relative abundance. (B) Bar graph analysis in 6 subgroups. The larger the corresponding bar area, the higher the relative abundance.

Figure 4

Heat maps show the microbes by relative abundance in 90 samples, including the top 50 taxa (A) at the genus level and (B) at the species level.

Figure 5

Bar graphs show the top 20 taxa in each subgroup (A) at the genus level and (B) at the species level.

Figure 6

A taxonomic tree showing the hierarchical relationships of all taxa arranged from phylum to species. Each node represents a microbiota, and the size of the node corresponds to the average relative abundance of the taxa. Twenty dominant taxa were identified in the diagram, with letters having the shadows of the same color as the corresponding node. p, phylum; c, class; o, order; f, family; g, genus.

Figure 7

Venn Diagram representing the number of OTUs among every three subgroups. The numbers in each circle show the number of OTUs found in each subgroup, and the overlap represents the shared OTUs. (A) CD, CS, and CP subgroups. (B) HD, HS, and HP subgroups.

Figure 8

Comparison of alpha diversity indices among subgroups. (A, B) CD, CP, and CS subgroups. (C, D) HD, HP, and HS subgroups.

Figure 9

LEfSe analysis among subgroups. LEfSe analyses identified taxonomic biomarkers among: (A) the CD, CP and CS subgroups; (B) the HD, HP and HS subgroups; (C) the CD and HD subgroups; (D) the CP and HP subgroups.

Figure 10

NMDS analysis based on Weighted UniFrac distances. Each sample is represented by a square/triangle/circle. (A) CD, CP, CS, HD, HP, and HS subgroups. (B) CS, CDP, HS and HDP subgroups.

Figure 11

Microbial interactions in supragingival plaque on the deciduous teeth ($|r| > 0.8$ and $p < 0.05$). (A) CD subgroup. (B) HD subgroup.

A **B** **Figure 12**

Microbial interactions in supragingival plaque on the FPMs ($|r| > 0.8$ and $p < 0.05$). (A) CP subgroup. (B) HP subgroup.

A **B** **Figure 13**

Microbial interactions in saliva ($|r| > 0.8$ and $p < 0.05$). (A) CS subgroup. (B) HS subgroup.