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Comparative Microbial Profiles of Caries and Black Extrinsic Tooth Stain in Primary Dentition

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Keywords
Supragingival plaque · Extrinsic black tooth stain · Early childhood caries · 16S rRNA · Oral microbiota

Abstract
Extrinsic black tooth stain (BS) is a common oral disease associated with lower caries experience in preschool children, although the microbiotic features contributing to the low risk of caries in this group remain elusive. In this study, we aimed at identifying the dominant bacteria in dental plaque to indicate the incidence of caries in the primary dentition. Subjects were divided into 3 groups based on the clinical examination: group CF, children without pigment who had no caries lesions or restorations (n = 18); group CS, children who were diagnosed with severe early childhood caries (n = 17); and group BS, children with pigment (black extrinsic stain) without caries or restorations (n = 15). The total microbial genomic DNA was extracted and subjected to bacterial 16S ribosomal RNA gene sequencing using an Illumina HiSeq platform. The differential dominant bacteria were determined using Wilcoxon rank-sum testing and linear discriminant analysis effect size (LEfSe). Co-occurrence network analysis was performed using sparse correlations for compositional data, calculation and functional features were predicted using PICRUSt. Interestingly, our results showed that the relative abundance of *Pseudopropionibacterium*, *Actinomyces*, *Rothia*, and *Cardiobacterium* was from high to low and that of *Porphyromonas* was low to high in the BS, CF, and CS groups, consistent with the clinical incidence of caries in the 3 groups. Moreover, an increased level of *Selenomonas_3*, *Fusobacterium*, and *Leptotrichia* was associated with high caries prevalence. We found that the interactions among genera in the BS and CS plaque communities are less complex than those in the CF communities at the taxon level. Functional features, including cofactor and vitamin metabolism, glycan biosynthesis and metabolism, and translation, significantly increased in caries plaque samples. These bacterial competition- and commensalism-induced changes in microbiota would result in a change of their symbiotic function, finally affecting the balance of oral microflora.

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Introduction

Dental caries is a biofilm-mediated, diet-modulated, multifactorial, non-communicable and dynamic disease resulting in the net mineral loss of the hard dental tissues. It has become a major public health problem due to its high prevalence, economic impact, and negative influence on the life quality of the affected individuals [Kassebaum et al., 2015]. Early childhood caries (ECC) is defined by the presence of one or more decayed (non-cavitated or cavitated lesions), missing (due to caries), or filled tooth in any deciduous dentition in a child younger than 6 years [Seow, 2018]. Untreated dental caries in the primary teeth affects >600 million children worldwide [Pitts et al., 2019].

The comprehensive understanding of ECC requires the characterisation of both the composition and the function or metabolic activity of the supragingival biofilm according to the clinical manifestation [Hajishengallis et al., 2017]. A reduced microbial richness could be reportedly observed before the caries onset [Hao et al., 2015]. A high abundance of several genera, including *Streptococcus*, *Granulicatella*, *Porphyromonas*, and species, such as *Streptococcus mutans*, *Scardovia wiggsiae*, *Parascardovia denticolens,* and *Lactobacillus salivarius*, had been described in the supragingival plaques of children diagnosed with ECC through 16S ribosomal RNA (rRNA) gene sequencing-based pyrosequencing analysis [Fakhruddin et al., 2019].

Extrinsic black tooth stain (BS) could be characterised as pigmented, dark lines parallel to the gingival margin or as an incomplete coalescence of dark dots rarely extending beyond the cervical third of the crown firmly attached to the tooth surface [Ronay and Attin, 2011]. Black extrinsic discolouration in primary dentition is a common clinical and aesthetic problem during childhood. Recently, more and more epidemiological studies worldwide described a negative relationship between the degree of staining and caries severity [Żyła et al., 2015; de Rezende et al., 2019]. However, there have only been a few studies focusing on the comparative microbial profiles of the supragingival plaque from black stain, caries, and healthy controls. Therefore, it remains largely unknown whether there is any significant divergence within the microbial communities associated with caries and black stain and whether the predominant bacterial species related to pigmentation could potentially reverse the occurrence of caries.

Subjects, Materials, and Methods

Patient Recruitment

A total of fifty-five children aged 2–6 years with full deciduous teeth participated in this study. All parents of children agreed to allow the participation of their children in the study with informed consent. The exclusion criteria were (1) systemic diseases, (2) visually detectable enamel or dentin hypoplasia, (3) a history of antibiotics or anti-inflammatory drug treatment within the preceding 2 weeks prior to the study, and (4) a history of fluoride treatment within the preceding month prior to the study.

Fifty children were examined at the Department of Paediatric Dentistry at the School of Stomatology of Peking University. Caries status and the decayed, missing, or filled teeth (dmft) index were scored by a single chief dentist according to the World Health Organisation (WHO, 2013) caries diagnostic criteria. The black extrinsic tooth stain was evaluated based on the presence of pigmented dark lines parallel to the gingival margin or an incomplete coalescence of dark dots rarely extending beyond the cervical third of the crown.

Subjects were divided into 3 groups based on the clinical examination: group CF, healthy control group, that is, children without pigment who had no caries lesions or restorations (n = 18); group CS, children who were diagnosed with severe ECC (n = 17); and group BS, children with pigment (black extrinsic stain) without caries or restorations (n = 15). We collected all plaque samples between 9:00 and 10:00 a.m. All participants were instructed not to brush their teeth in the morning and not to drink or eat for 4 h prior to sample collection.

Sampling

Each supragingival plaque specimen was pooled from the labial smooth surfaces of all teeth by using an individual sterile dental excavator. Plaque samples were immediately released from the excavator by agitation into a sterile, labelled 1.5-mL centrifuge tube (Axygen, USA) containing 1 mL TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA). All samples were frozen at −80°C prior to DNA isolation.

DNA Extraction and Sequencing

The total bacterial DNA was extracted and purified using a commercial TIANamp Bacteria DNA Kit (Tiangen Biotech, Beijing, China) following the manufactures' protocol. The quality and concentration of the extracted DNA were measured using a NanoDrop 8000 spectrophotometer (Thermo Fisher Scientific, Carlsbad, CA, USA). The DNA integrity was verified by electrophoresis using a 1.8% agarose gel. The V3-V4 region of the bacterial 16S rRNA was PCR-amplified using the cycling parameters and the universal primers as follows: 98°C for 2 min initiation, then 30 cycles of 98°C for 30 s, 50°C for 30 s, 72°C for 60 s, and 72°C for 5 min; 338F: 5′-ACTCCTACGGGAGGCAGCA-3′ and 806R: 5′-GGACTACHVGGGTWTCTAAT-3′.

All PCR products were sequenced using an Illumina HiSeq 2500 Sequencing platform according to standard protocols. Primary FastQ files were divided into multiple files for processing and quality-filtering using Trimmomatic v0.33 software. In addition, chimeric sequences were recognised and deleted using UCHIME v4.2 software. The remaining sequences were then aligned with the SILVA database (V.132). Operational taxonomic units (OTUs) were clustered with a similarity cut-off of 97% using UPARSE [Edgar, 2013].

Table 1. Alpha diversity indices of different groups

Group	Shannon	Simpson	Chao	ACE
CS	3.09±0.43	0.10±0.05	165.24±25.44	166.36±32.81
CF	2.89±0.64	0.13±0.07[c]	157.95±40.53[b]	162.44±36.47[b]
BS	3.25±0.39	0.08±0.04	204.12±16.16[a]	203.43±13.15[a]

Each value is represented in mean ± SD. Similarity between the 3 groups in bacterial composition. BS, black tooth stain. [a] Represents an extreme difference between BS, CF, and CS ($p < 0.001$). [b] Represents an extreme difference between BS and CF ($p < 0.001$). [c] Represents a significant difference between BS and CF ($p = 0.03$).

Bioinformatics and Statistical Analysis

The bioinformatics analysis was conducted using QIIME. The alpha diversity indices of Chao1, ACE, and Shannon and Simpson were calculated using Mothur software (version v.1.30). Samples from different groups were compared using the Wilcoxon rank-sum test. Any two-group comparisons were assessed using the Mann-Whitney test. A Venn diagram was constructed to define the core microbiome and the special taxa in each group. The Kruskal-Wallis test was used to compare the relative abundances of the microbial taxa between the 3 groups, and the *p* values were rectified by the Benjamini-Hochberg false discovery rate (BH FDR). Beta diversity analysis was performed by nonmetric multi-dimensional scaling and unweighted pair-group method with arithmetic mean based on the unweighted UniFrac distances. Linear discriminant analysis (LDA) effect size (LEfSe) was conducted to define the biomarkers of the 3 groups. The threshold on the logarithmic LDA score for the distinguishing features was set to 4.0. We performed co-occurrence analysis through sparse correlations for compositional data calculation according to the abundance and variation of each taxon in each sample using Python software. Microbial functions were predicted using PICRUSt (v1.0.0) software following the online protocol and aligned to the Kyoto Encyclopedia of Genes and Genomes (KEGG) database.

Results

Plaque samples were collected from 18, 15, and 17 children without pigmentation and caries (CF), with pigmentation but without caries (BS), and diagnosed with severe ECC (CS) aged 3.58 ± 0.33, 4.30 ± 0.94, and 4.11 ± 0.64 years on average, respectively. The χ^2 analysis and the Kruskal-Wallis test indicated no significant differences between the groups in terms of gender and age. The mean number of decayed, missing due to caries, or filled teeth was 14.29 ± 2.11.

The Illumina HiSeq sequencing yielded a total of 2,056,307 effective sequences, which were acquired from 50 samples. On average, 41,126 reads were obtained per sample for analysis. The mean sequence length was 422 bp, with the longest and the shortest being 428 and 415 bp, respectively. The qualified sequences were clustered at an identity of 97%. A total of 271 OTUs were obtained. Good's coverage index of the generated OTUs reached up to 99.94%. The rarefaction curves reached a plateau, which suggested that the sequencing data volume was of sufficient size.

We observed no significant difference between the CS and CF groups (Table 1) in community richness and diversity. However, when the BS group was compared with the CF, the indices of ACE were significantly different ($p < 0.001$), as was the Chao analysis ($p < 0.05$), which indicated that the plaque community from the BS group exhibited higher bacterial richness than that of the CF group. As for the microbiota diversity, the BS group exhibited lower Simpson and Shannon indices than the CF

Fig. 1. Comparative microbial profiles of CF, CS, and BS in children aged 2–6 years. The distributions of the top 10 predominant bacteria across the BS, CF, and CS groups at the phylum level (**A**-**a**) and genus level (**A**-**b**). **B** Venn diagram at the species level in each group. **C** NMDS based on unweighted UniFrac distances at the OTU level. The index of stress was <0.2, indicating reliability of the analysis. Each sample is represented by a dot. The distance between the dots represents the degree of difference. The closer the sample, the higher the similarity. **D** ANOSIM analysis based on unweighted UniFrac distances at the genus level. The closer the *R* value is to 1, the higher the difference between groups than that within groups. $p < 0.05$ indicates high reliability of the test. *Y*-axis represents beta distance. **E** Mann-Whitney test bar plot on the predominant genus. * $p < 0.05$; ** $p < 0.001$. **F** Network analysis showing the interactions between predominant genera (relative abundance >1%) (|SparCC threshold| >0.1 and $p < 0.05$. Bacterial interaction of BS (**F**-**a**); bacterial interaction of CS (**F**-**b**); bacterial interaction of CF (**F**-**c**). The nodes with different colours represent the genera. The size of the node is proportional to the abundance. The thickness of lines represents the strength of correlation and the colour of lines represents positive (orange) and negative (green) correlations. NMDS, nonmetric multidimensional scaling; BS, black tooth stain; SparCC, sparse correlations for compositional data; OTU, operational taxonomic unit.

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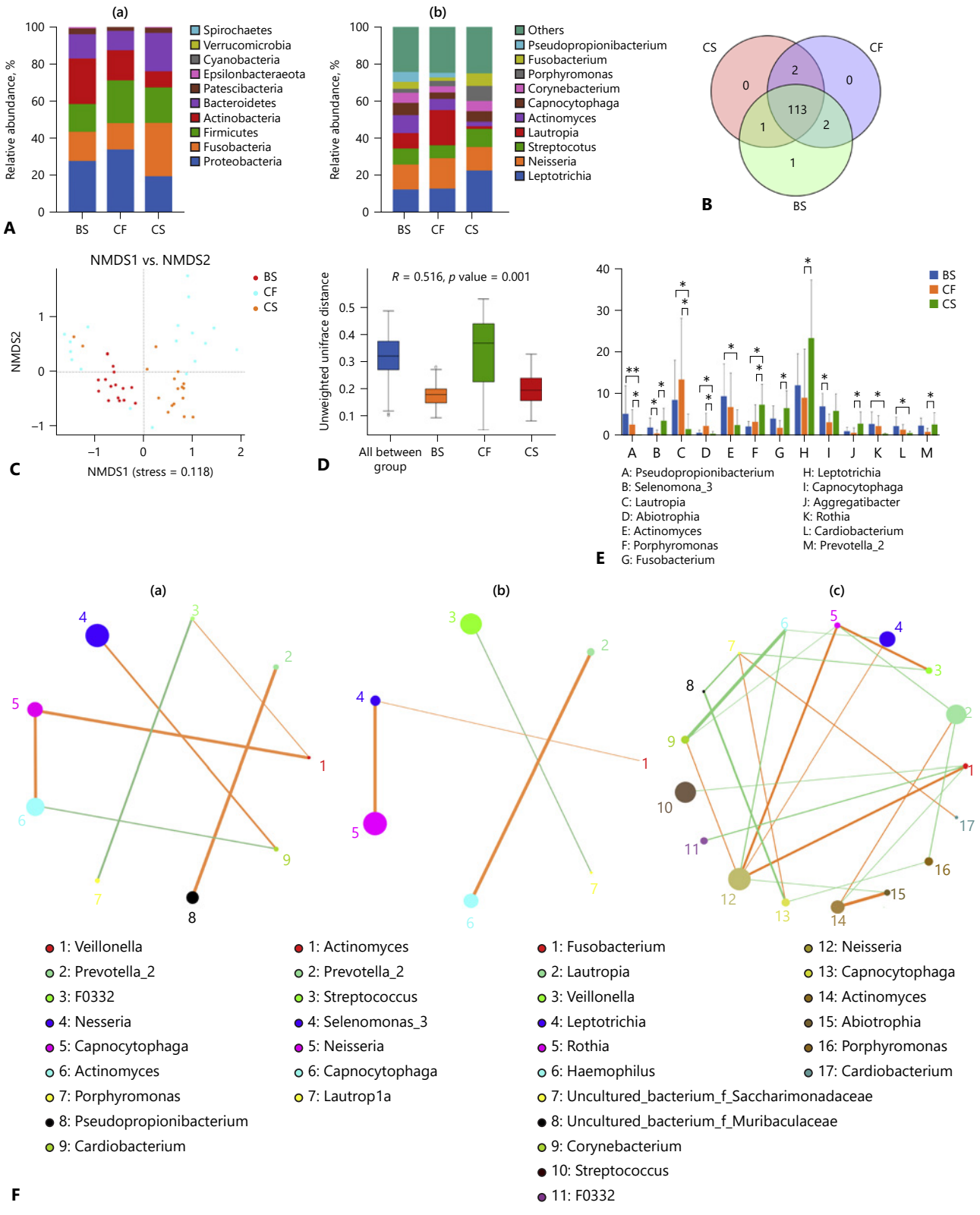

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group, but only the Simpson indices between the 2 groups were significantly different ($p < 0.05$) (Table 1).

A total of 10 phyla, 17 classes, 31 orders, 55 families, 107 genera, and 116 species were detected from all samples. The 6 most abundant phyla were *Proteobacteria* (26.90%), *Fusobacteria* (19.70%), *Firmicutes* (18.94%), *Actinobacteria* (16.12%), *Bacteroidetes* (14.72%), and *Patescibacteria* (2.80%), together comprising 99.17% of the total sequences. The most prevalent genera were *Leptotrichia* (15.55%), *Neisseria* (14.38%), *Streptococcus* (10.75%), *Lautropia* (7.33%), *Actinomyces* (5.77%), *Capnocytophaga* (5.16%), *Corynebacterium* (4.73%), *Porphyromonas* (4.46%), *Fusobacterium* (4.13%), and *Pseudopropionibacterium* (2.37%), which represented 74.64% of the total sequences (Fig. 1A).

We identified 117, 117, and 116 species in the BS, CF, and CS groups, respectively (Fig. 1B). Among them, 113 species were uniform, accounting for 95% of all species detected. *Uncultured_bacterium_f_Lentimicrobiaceae* was only detected in the BS group, which were assigned to *Bacteroidetes*, although there were only 3 samples with a mean relative abundance of 0.24% among fifteen samples.

The samples from the BS group appeared to cluster. In contrast, samples from the CF group were scattered. The finding indicated that patients diagnosed with black stain could have special and steady bacterial community structures, whereas bacterial microbiome structures from healthy children are more variable (Fig. 1C).

The analysis based on the phylogenetic tree demonstrated that most samples clustered within groups and cross-existed between the 3 groups. Sample CF7 clustered with the samples from the CS group. Samples CS1 and CS3 clustered with the samples from the CF group. Sample CF8 clustered with the samples from the BS group. These children still need further inspection using longitudinal studies (online suppl. Fig. S1; for all online suppl. material, see www.karger.com/doi/10.1159/000517006).

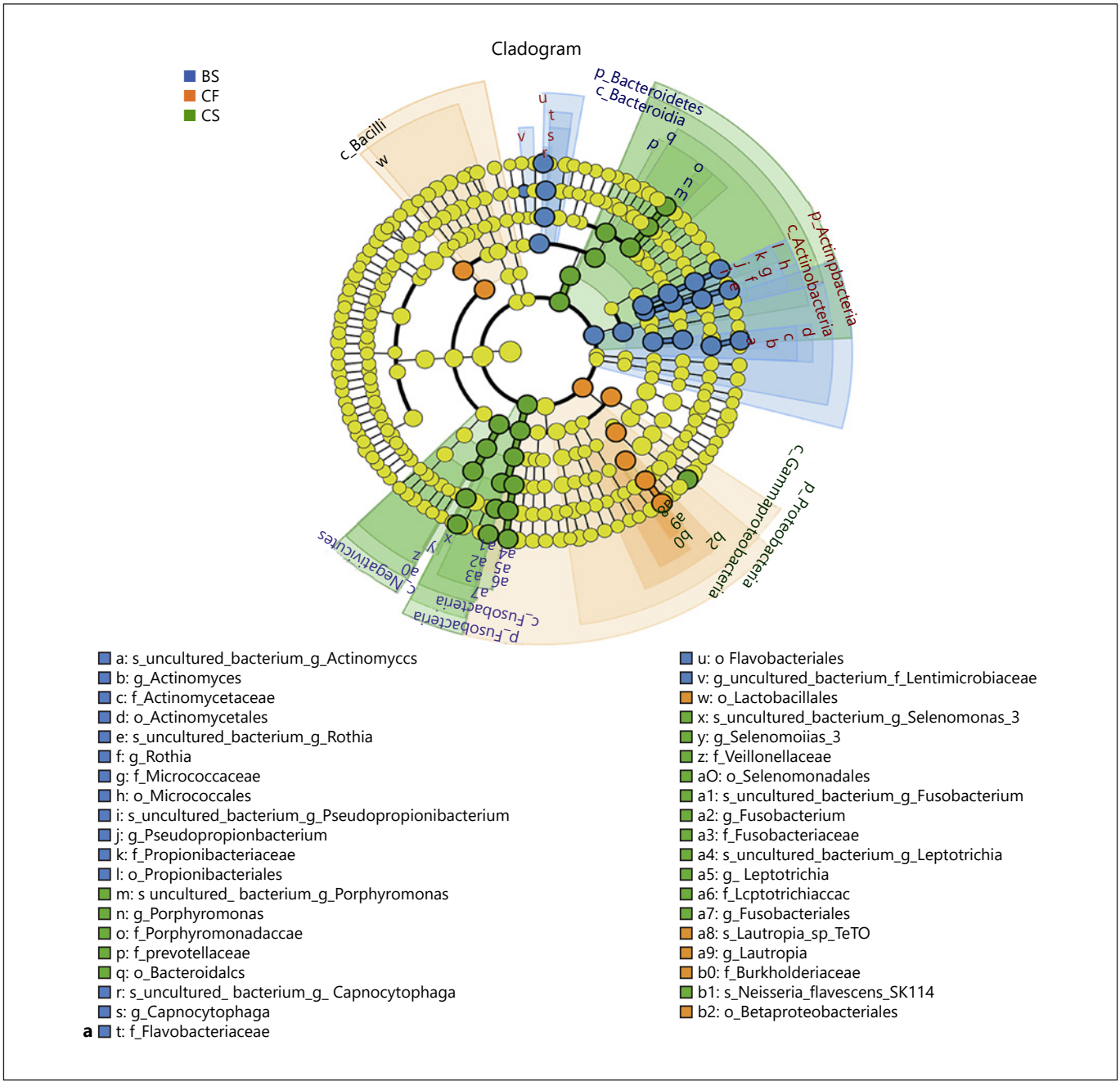
Based on the analysis described earlier, we removed CS1, CS3, CF7, and CF8 from the 3 groups to identify the dominant bacteria. The ANOSIM showed that the differences between the groups were significantly higher than those within the groups (Fig. 1D).

We compared the genera in all residual samples that had a relative abundance of >1% and identified thirteen genera that displayed significant differences between the 3 groups ($p < 0.05$) (Fig. 1E). Samples from the caries group exhibited the highest abundance of *Porphyromonas* and the lowest of *Pseudopropionibacterium*, followed by the healthy group, with significant differences between these 2 groups. We also observed a significantly decreasing trend of *Actinomyces*, *Rothia*, and *Cardiobacterium* in the caries group, followed by the healthy control, although we observed no significant difference between the 2 groups. Moreover, *Selenomonas_3*, *Fusobacterium*, *Leptotrichia*, *Aggregatibacter*, and *Prevotella_2* presented the highest levels in the caries group, followed by the samples from the black stain group. In addition, compared with samples from the healthy group, the black stain group possessed a significantly higher abundance of *Selenomonas_3* and *Capnocytophaga*, indicating that the 2 taxa could be linked to the black stain. The healthy group presented the highest abundance of *Lautropia* and *Abiotrophia*. The bacterial community composition varied in the case of the different disease statuses.

The LEfSe highlighted the potential biomarkers of different groups (Fig. 2). At the genus level, *Actinomyces*, *Rothia*, *Pseudopropionibacterium*, *Capnocytophaga*, and *g_uncultured_bacterium_f_Lentimicrobiaceae* were significantly enriched in the BS group, while *Porphyromonas*, *Selenomonas_3*, *Fusobacterium*, and *Leptotrichia* resulted to be potential caries biomarkers. *Lautropia* was elected to be associated with the healthy status (LDA > 4, $p < 0.05$).

Next, we performed a co-occurrence analysis using sparse correlations for compositional data calculation to identify interactions among the genera in the different groups according to the abundance and variation of each taxon in each sample. The interactions of the predominant genera (>1%) are shown in Figure 1F. More various and complex bacterial correlations were found in plaque communities from the healthy samples. A total of 9, 17, and 7 genera presented a positive bacterial correlation in BS, CF, and CS, respectively. Among them, *Actinomyces* and *Capnocytophaga* presented a positive relationship with the BS group; *Actinomyces* was a positive link to *Selenomonas_3* in the CS group. In healthy plaque samples, the communities displayed a more complex interaction among taxa. In the CF group, *Capnocytophaga* was negatively associated with *Porphyromonas* and *Lautropia*, and *Actinomyces* was positively related with *Lautropia* and negatively related to *Fusobacterium*.

The samples from the 3 groups showed similar KEGG profiles, indicating similar microbial functional features. The most abundant functions were global and overview maps (43.43%), carbohydrate metabolism (9.18%), amino acid metabolism (6.59%), metabolism of cofactors and vitamins (4.51%), and energy metabolism (4.22%) (Fig. 3a). The proportions of all functional features presented no differences between the BS and CF groups. We



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then highlighted the difference of the predominant functional features (mean proportions >1%) between the CS and CF groups in Figure 3b. The CS group showed significantly higher proportions in functional features, including cofactor and vitamin metabolism, glycan biosynthesis and metabolism, translation, replication and repair, nucleotide metabolism, cellular community prokaryotes, and terpenoid and polyketide metabolism. At the same time, we detected the predominant functional features of the previously identified genera (Fig. 3c). The results showed that *Leptotrichia*, *Pseudopropionibacterium*, and *Actinomyces* exhibited higher carbohydrate metabolism, while *Pseudopropionibacterium* presented a higher level of amino acid metabolism and *Leptotrichia* a higher membrane transport. *Porphyromonas* demonstrated higher proportions of cofactor, vitamin and nu-

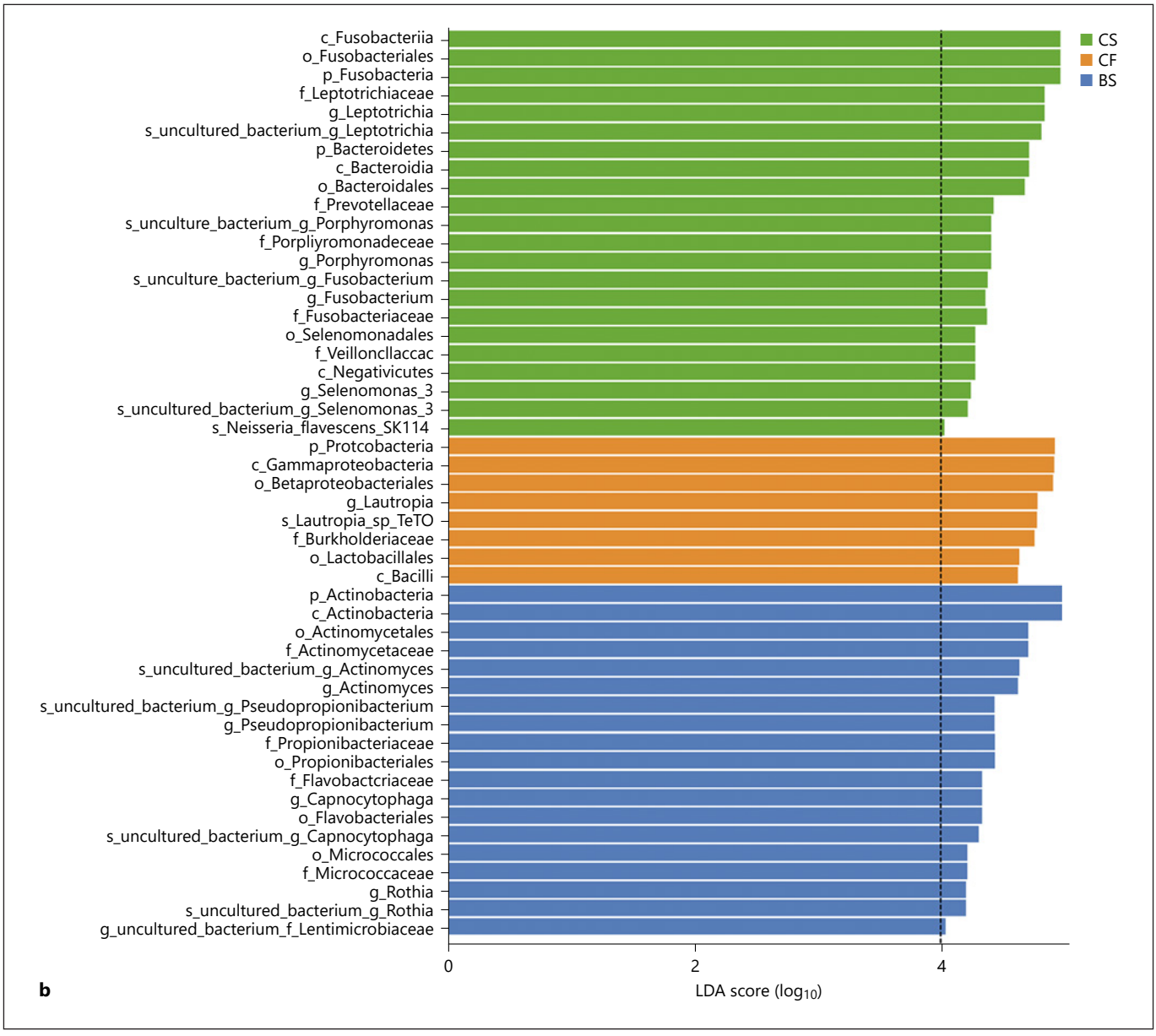

Fig. 2. Cladogram and LDA. **a** Cladogram for taxonomic representation of significant differences between the 3 groups. The coloured nodes from inner to the outer circles represent taxa from phyla to the genus level. The significantly different taxa were signified by the 3 groups with different colours. **b** Histogram of the LDA scores for differently abundant features between the 3 groups. The length represents the impact. The threshold on the logarithmic LDA score for discriminative features was set to 4.0. LDA, linear discriminant analysis.

cleotide metabolism, translation, replication, and repair. *Lautropia* presented higher levels of signal transduction, xenobiotics biodegradation, and metabolism. The aforementioned information indicated that special functional features could be highlighted in the cariogenic plaque communities.

Discussion/Conclusion

We identified *Leptotrichia*, *Neisseria*, *Streptococcus*, *Lautropia*, *Actinomyces*, *Capnocytophaga*, *Corynebacterium*, *Porphyromonas*, *Fusobacterium*, and *Pseudopropionibacterium* as the predominant genera in this research, in good agreement with previous studies [Wade, 2013;

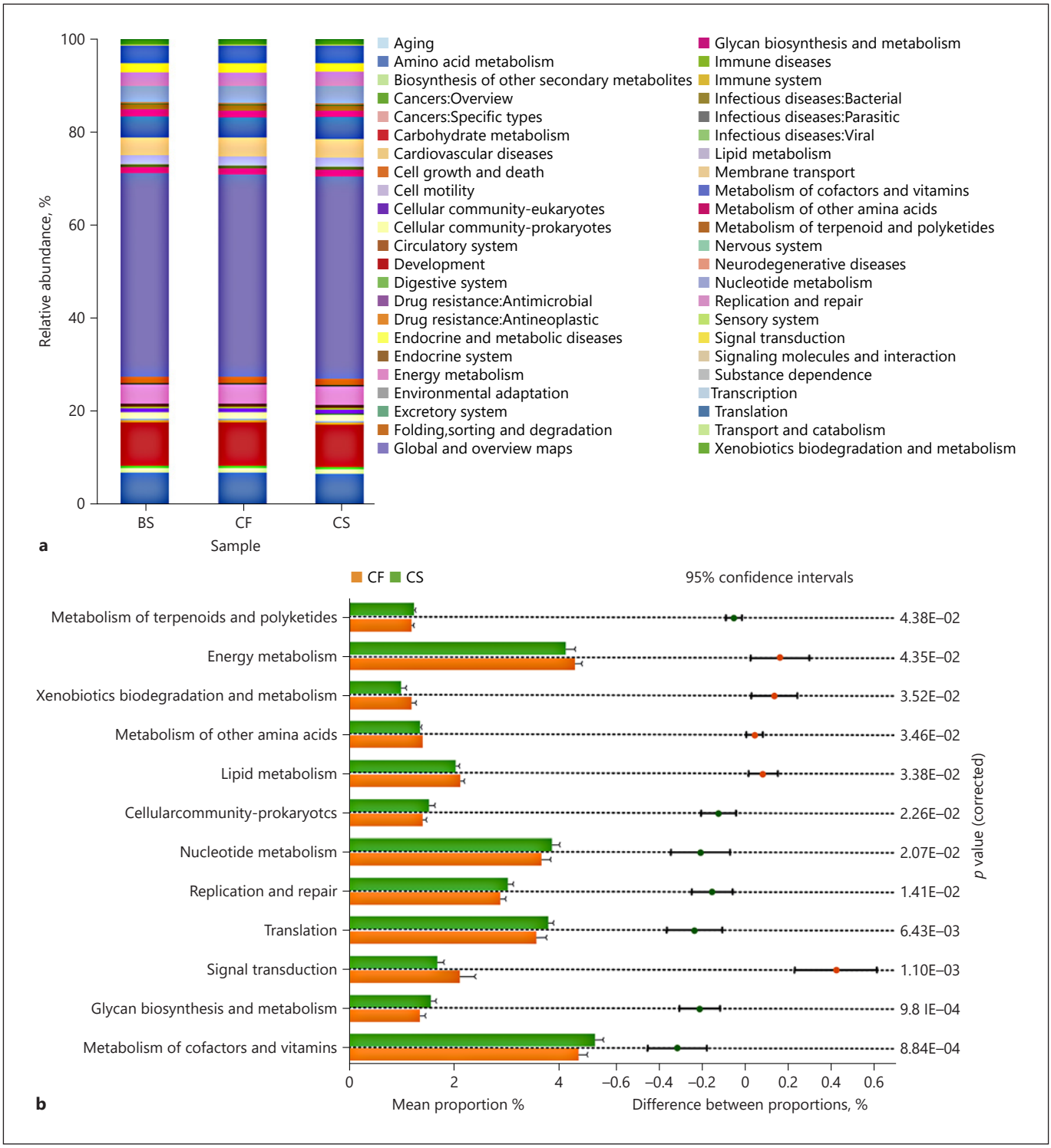

Fig. 3. Function prediction by PICRUSt. **a** Compositions of the KEGG function in the 3 groups. **b** The different functional features between CF and CS groups. **c** The proportions of functional features in special genera.

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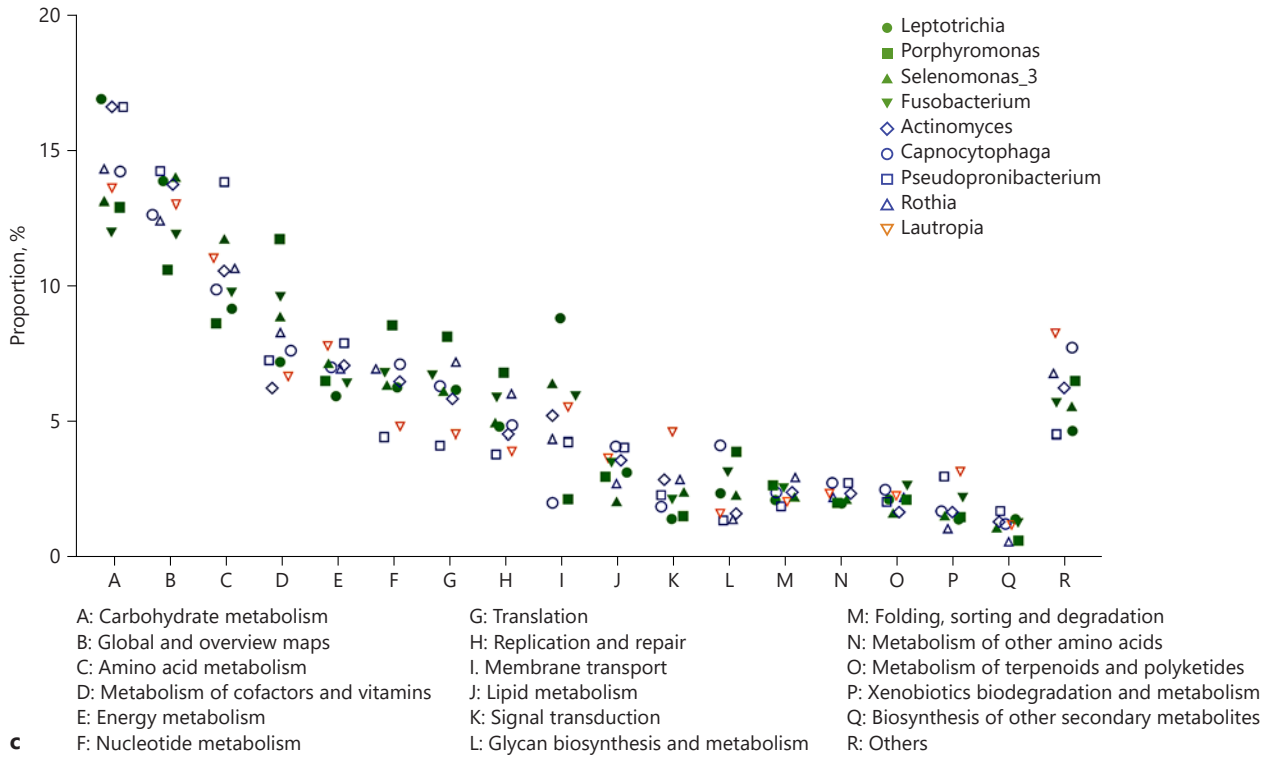

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Welch et al., 2016; Xu et al., 2018b], indicating the presence of a relatively steady bacterial community composition in the oral cavity. Based on the analysis using the Wilcoxon rank-sum test and the LEfSe results, we concluded that the relative abundance of *Pseudopropionibacterium* significantly exhibited a high to low trend and that of *Porphyromonas* a low to high trend in the BS, CF, and CS groups, consistent with the clinical incidence of caries in the 3 groups. Furthermore, we observed an increased level of *Selenomonas_3*, *Fusobacterium*, and *Leptotrichia* associated with high caries prevalence. In contrast to *Pseudopropionibacterium*, *Actinomyces*, *Rothia*, and *Cardiobacterium* that were also prone to the black stain, *Lautropia* was rather associated with the healthy status.

Children with poor caries status reportedly exhibited a higher abundance of *Porphyromonas* in their dental plaques. *Porphyromonas* is reportedly associated with primary endodontic infections and acute periradicular abscess [Ma et al., 2015; Nelson-Filho et al., 2018]. *Leptotrichia* species are typically found in dental plaques and are associated with dental caries [Luo et al., 2012; Eribe and Olsen, 2017]. Our functional feature analysis also showed that *Leptotrichia* exhibited a high carbohydrate metabolism. Carbohydrate has long been regarded as the "arch-criminal" of dental caries. Bacteria can produce organic acid and exopolysaccharides through carbohydrate metabolism. *Fusobacterium* is enriched under the status of caries occurrence [Kanasi et al., 2010; Jiang et al., 2011], although *Fusobacterium* and *Leptotrichia* have also been found to be enriched in adults without caries experience. However, this contradiction could be explained with age, different dentition stages, and a higher level of *Corynebacterium* in the previous study [Schoilew et al., 2019]. *Selenomonas* reportedly ferments glucose and utilises lactate in rumen bacteria and is reportedly associated with coronal caries in young children [Hespell et al., 2006; Gross et al., 2010]. In this study, we detected the mutually reinforcing relationship between *Selenomonas_3* and *Actinomyces* in the caries group, which might play a complex role in caries.

Actinomyces belongs to the resident oral microbiota of supragingival dental plaques. Some species can produce hydrogen sulphide, associated with the extrinsic black stain formation [Żyła et al., 2015]. A negative relationship

has been reported between *Actinomyces* and oral *Candida*, and mutualism between *Actinomyces* and *Capnocytophaga* in black stain samples [Xiao et al., 2018a]. *Actinomyces* and *Capnocytophaga* are both known to be early colonising bacteria in the oral cavity, and the abundance of *Actinomyces* reportedly increased in the saliva samples of children with the recurrence of ECC [Zhu et al., 2018]. *Rothia dentocariosa* and *Rothia mucilaginosa* are part of the normal flora in the human oral cavity. Previous studies described that *Rothia* significantly decreased the prevalence of dental caries as well [Xu et al., 2018a]. However, we did not find any divergence between the caries and the healthy groups in this study. Therefore, testifying the relationship between caries and the 3 species would require more evidence and further studies.

Pseudopropionibacterium is a new reclassification of the genus *Propionibacterium* proposed by Scholz and Kilian [2016]. In previous reports, *Propionibacterium* was described to exhibit a high abundance in dental caries lesions [Wolff et al., 2013; Obata et al., 2014], although the relationship between *Pseudopropionibacterium* and caries has not been described. In this study, we further detected higher levels of amino acid metabolism in *Pseudopropionibacterium* through functional prediction using PICRUSt. Amino acid metabolism is well-known for its contribution to dental plaque acid neutralisation and can thus in part account for caries inhibition [He et al., 2016].

Lautropia was prone to be detected in supragingival dental plaques from healthy children in a previous study [Shaddox et al., 2012]. Due to the high level of complexity and variety of the dental plaque microbiota in the healthy population and the low relative abundance of *Lautropia*, we have no sufficient evidence to indicate the relationship between the special genus and health.

In the present study, we also found that *uncultured_bacterium_f_Lentimicrobiaceae* was only detected in the black stain group. *Uncultured_bacterium_f_Lentimicrobiaceae* was assigned to *Bacteroidetes* that encompasses several strains regarded to conduce to pigmentations, such as *Prevotella* and *Tannerella* [Żyła et al., 2015; Sun et al., 2016]. Furthermore, *Bacteroidetes* reportedly elaborate a few thousand enzyme combinations to break down glycan, which is the main energy source of cariogenic bacteria [Lapébie et al., 2019]. In addition, we found no difference in community diversity between the 3 groups, but the samples from the black stain group exhibited highest richness. The bacterial richness and evenness similarity between the caries and healthy plaques were in good agreement with certain previously described results [Yang et al., 2012; Xu et al., 2014; Jiang et al., 2016; de Jesus et al., 2020], although other studies have reported the opposite [Xiao et al., 2016]. However, the higher indices of richness in the black stain community are also in line with previous results [Saba et al., 2006; Chen et al., 2019]. Microbial diversity is associated with the ecological shift, but other factors, such as age, diet, oral hygiene habits, and the biofilm sample size, can reflect the indices [Johansson et al., 2016]. Based on this result, we speculated that the mechanisms of the 2 kinds of oral diseases could not be explained only by the alpha diversity.

Considering that the perturbed microbial community of the dental plaque is the main reason behind the carious processes, the "core microbiome of caries" have been proposed in order to better understand the microbial community in the occurrence and development of caries [Kressirer et al., 2018; Kazemtabrizi et al., 2020]. Several highly acidogenic and acid-tolerant bacteria have been considered as "core microbiome" associated with caries, including *Streptococcus mutans*, *Streptococcus sobrinus*, and certain *Lactobacillus*, *Bifidobacterium*, and *Scardovia* [Saraithong et al., 2015; Neves et al., 2017; Achtman and Zhou, 2020]. When it comes to *Streptococcus mutans*, it was regarded as one of the major etiological agents of human dental caries with its capacity to orchestrate changes in the plaque microbiome via the extracellular polysaccharide matrix and acid production [Lemos et al., 2019]. However, certain studies show that individuals with high *Streptococcus mutans* levels do not necessarily develop caries lesions, while *Streptococcus mutans* is undetectable in 15% of the lesions and *Lactobacilli* is undetected in 19% of the caries lesions [Köhler and Bjarnason, 1992]. In our study, *Streptococcus mutans* was not a predominant bacterium in SECC, which is not in line with previous studies [Grier et al., 2020]. We explain this discrepancy with the different ethnic and eating habits that might alter the core SECC microbiome.

The aforementioned results could potentially help us gain a thorough and comprehensive understanding of the bacterial community associated with dental caries, black stain, and healthy teeth. However, certain aspects of our study need to be further improved. For example, fungal-bacterial ecological interactions might affect the microbial balance and promote disease development, which has been reported by several other research groups [Xiao et al., 2018b; de Jesus et al., 2020]. Environment conditions such as temperature, pH, salinity, oxygen, and nutrients might also impact the microbial community of dental biofilms, which could be meaningful and

taken into consideration in further studies. In addition, PICRUSt predictions only included 16S rRNA biomarker gene sequences, and the pattern detection possibility was dependent on the input data. So, further studies are needed to focus on the key bacteria and their functional features.

Our conclusions support the hypothesis that the complexity of microbial communities could decrease in the case of certain diseases, such as extrinsic black stain and caries. The relative changes in *Porphyromonas*, *Selenomonas_3*, *Fusobacterium*, *Leptotrichia*, and *Pseudopropionibacterium* might potentially predict the incidence of caries in preschool children.

Acknowledgement

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Statements of Ethics

The Ethics Committee of Peking University Health Science Center approved this study (PKUSSIRB-201839140). All the subjects agreed to study participation with an informed consent form.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

Author Contributions

Y.C. performed experiments, analysed the data, and wrote the first draft of the manuscript. G.D. and D.W. collected the clinical samples. J.Y. and Y.Z. interpreted and validated the 16S RNA sequencing data and participated in revising the current version of the manuscript. Y.C., Y.W., and B.X. designed the study and revised the manuscript. J.A.G. interpreted the data and polished the manuscript.

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