

Letter



Investigation of Associated Factors and Microbiota of Different Oral Niches in Early Childhood Caries

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Dental caries is a widespread oral disease and is particularly significant during childhood, as it can impact growth and development^[1]. Early childhood caries (ECC), defined as the presence of decayed, missing, or filled primary teeth in children under six years old, represents a major global health issue^[2]. Untreated ECC can lead to pain, impaired digestion, disruption of permanent tooth development, and can negatively affect both the physical and mental health of children.

The etiology of ECC involves complex interactions among bacteria, host factors, diet, and time. In addition, the condition is impacted by additional factors such as breastfeeding, oral hygiene practices, diet, family history, socioeconomic status, and geographic location. A growing number of studies have investigated the role of the oral microbiota in health and disease^[3]. Different oral niches, such as saliva, the tongue, and tooth surfaces, host distinct microbial communities. These communities exist in a dynamic balance, and external factors, including acidity, heat, and antibiotic use can disrupt this balance, increasing the risk of caries and other oral and systemic diseases^[4].

However, limited research has been conducted on the microbiology of dental caries in children. This study aims to identify factors associated with caries and employs 16S rDNA sequencing to evaluate the differences in oral microbiota between healthy children and those with caries across different oral niches, such as the dorsal tongue mucosa, buccal mucosa, and tooth surfaces. Additionally, the study aims to screen for microbial species with diagnostic value and explore their gene functions. This research is expected to deepen our understanding of the etiology of ECC, improve diagnostic methods, and develop targeted prevention strategies.

This study was approved by the Ethics Committee of Shandong Second Medical University (Approval No.: 2020YX072). The study subjects were

children aged 3 to 5 years. Informed consent was obtained from all legal guardians before sampling, and all the study procedures were conducted in adherence to the relevant provisions of the Declaration of Helsinki. The sample size was determined using the Fleiss correction formula $N_{corrected} = \frac{P(1-P) \times Z^2}{E^2} \times \frac{P_{max}(1-P_{max})}{P(1-P)}$, with a Z value corresponding to the standard normal distribution at a 95% confidence level (1.96). P_{max} was set at 0.5, and the dental caries rate among 5-year-old children was reported as 70.9% based on the results of the Fourth National Oral Health Survey in China^[5]. The allowable sampling error was set to less than 6%, leading to a theoretical sample size of 267. In anticipation of invalid samples, the sample size was increased to 315. The study subjects were randomly selected from three kindergartens in Weifang, China. The inclusion criteria were children who had lived in the area for more than six months and who were able to cooperate with oral examinations. The exclusion criteria were children who had used antibiotics, probiotics, or other medications in the past month, as well as those with systemic diseases who were unable to cooperate with the examination.

The oral examinations were conducted by two dentists. Additionally, a questionnaire was designed based on local economic conditions and lifestyle habits, collecting basic information from the participants, including oral hygiene practices, dietary habits, parental educational background, and oral health knowledge. The dentists provided detailed explanations of the questionnaire to the parents to ensure accurate information collection. To capture representative samples across different stages of caries severity and to ensure the feasibility of high-throughput sequencing and detailed microbial analysis, 35 children were selected for sampling in this study. These children were categorized into a

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healthy group (dmft = 0, Group H), a mild early childhood caries group ($1 \leq \text{dmft} \leq 4$, Group M), and a severe early childhood caries group ($\text{dmft} \geq 5$, Group S). Samples were collected from the 35 children (including 9 healthy children, 15 with mild ECC, and 11 with severe ECC) by oral health professionals using sterile cotton swabs; samples were collected from the dorsal tongue mucosa, teeth, and buccal mucosa. Samples contaminated with blood or other impurities were excluded. The samples were labeled, placed in sterile EP tubes containing buffer solution, and stored accordingly. A total of 105 samples were collected, including dorsal tongue mucosa plaques (T niche, T01-T35), buccal mucosa plaques (C niche, C01-C35), and dental plaque samples (D niche, D01-D35). Following DNA extraction from the oral microbiota samples, 16S rDNA genes were amplified using specific primers. Libraries were prepared according to sequencing requirements and sequenced on the Illumina MiSeq PE300 platform. Finally, functional predictions were conducted using PICRUST2 and STAMP software.

The data were organized into a database using Excel, and accuracy was ensured by double-entry verification. The dataset was then imported into R software (v4.2.1) and SPSS 27.0 (SPSS Inc, Chicago, IL, USA) for analysis. Count data were expressed as the number of cases, and univariate analysis was conducted using the χ^2 test. Subsequently, factors with $P < 0.05$ were included in a multivariate logistic regression analysis, with a significance level set at $\alpha = 0.05$. The differences in oral microbiota sequencing data between groups were assessed using the Kruskal-Wallis test and one-way ANOVA. Furthermore, differences at various bacterial community classification levels were evaluated using the LEfSe method. In this study, LDA score > 2.0 , $P < 0.05$ was considered statistically significant.

A total of 315 children aged 3–5 years were surveyed, including nearly equal proportions of boys and girls. The analysis revealed a caries prevalence of 67.93%. The children were divided into healthy, mild ECC, and severe ECC groups. Univariate analysis (Supplementary Table S1, available in www.besjournal.com) identified 19 factors significantly associated with dental caries, including oral hygiene and dietary habits. Multivariate analysis (Figure 1) revealed that frequent consumption of sweets and the use of parental utensils were major factors associated with a 6.043 and 4.445 times increase in the risk of caries, respectively. Conversely, regular tooth brushing, oral cleaning before the age of one, and frequent fruit consumption were identified as

protective factors. The study also indicated that parental education level influenced the severity of caries, with higher education levels associated with more severe caries. Interestingly, despite the lack of statistically significant difference in parental literacy between the healthy and ECC groups, literacy emerged as a determining factor in caries severity. These findings suggest that higher education levels do not always correlate with better oral hygiene practices. This difference highlights the need for targeted education on specific oral health behaviors, regardless of the parents' overall educational attainment.

Additionally, frequent intake of high-sugar foods was strongly associated with an increased risk of caries, as these foods provide nutrients for bacterial growth and acid production, leading to enamel demineralization. On the other hand, moderate fruit intake, particularly fiber-rich fruits, protects against caries by increasing saliva production, which neutralizes acids and removes food debris. Regular oral hygiene practices, including brushing and tongue cleaning, as well as routine dental checkups, play an essential role in reducing the occurrence of caries. Using separate utensils to feed children was also emphasized as an important preventive measure, reducing the risk of transmitting harmful oral bacteria from parents to children.

This study identified 18 phyla, 30 classes, 70 orders, 128 families, and 253 genera from the oral microbiota samples collected from children. The six most abundant phyla were Firmicutes, Proteobacteria, Fusobacteria, Bacteroidota, Actinobacteria, and Patescibacteria, accounting for more than 98% of the total sequences. In addition, significant differences in microbial composition were observed across the niches of the dorsal tongue mucosa, buccal mucosa, and tooth surfaces (Supplementary Figure S1, available in www.besjournal.com). Firmicutes were predominant on the dorsal tongue and buccal mucosa, whereas Actinobacteria were more prevalent on tooth surfaces. Notably, children with severe caries (Group S) showed a significant reduction in Firmicutes within the dental plaque niche, suggesting that the shift in microbial balance may be associated with caries progression. At the genus level, *Streptococcus*, *Actinomyces*, and *Neisseria* were the most common genera, with *Veillonella* being particularly prevalent in the T niche and *Streptococcus* in the C niche. Alpha and beta diversity analyses (Supplementary Figure S2, available in www.besjournal.com) revealed no statistical significance in overall

microbial diversity across the different niches; however, significant differences in community composition were observed, especially at the genus level. For example, *Rothia* was identified as a biomarker in the T niche of healthy children, whereas *Streptococcus cristatus* (*S. cristatus*) was prominent in the mild ECC group. In the C niche, *Prevotella scopos* and *Neisseria pharynges* (*N. pharyngis*) were identified as key species associated with varying degrees of ECC severity. *Streptococcus*, *Veillonella*, and *Actinomyces* were dominant genera associated with caries, with *Streptococcus* playing a crucial role in plaque formation and acid production,

leading to enamel demineralization. In addition, *Veillonella* interacts with other cariogenic bacteria, exacerbating acid production, and further promoting caries. LEfSe analysis (Supplementary Figure S2C–E) identified biomarkers in different niches, such as *Rothia* sp. in healthy children and *S. cristatus* in those with mild ECC, emphasizing the niche-specific nature of these microbial communities.

This study clarifies the relationship between dental caries and the oral microbiota across different niches. Notably, in children with severe ECC, the oral microbiota exhibited reduced diversity and shifts in dominant species. *Streptococcus* and *Veillonella*,

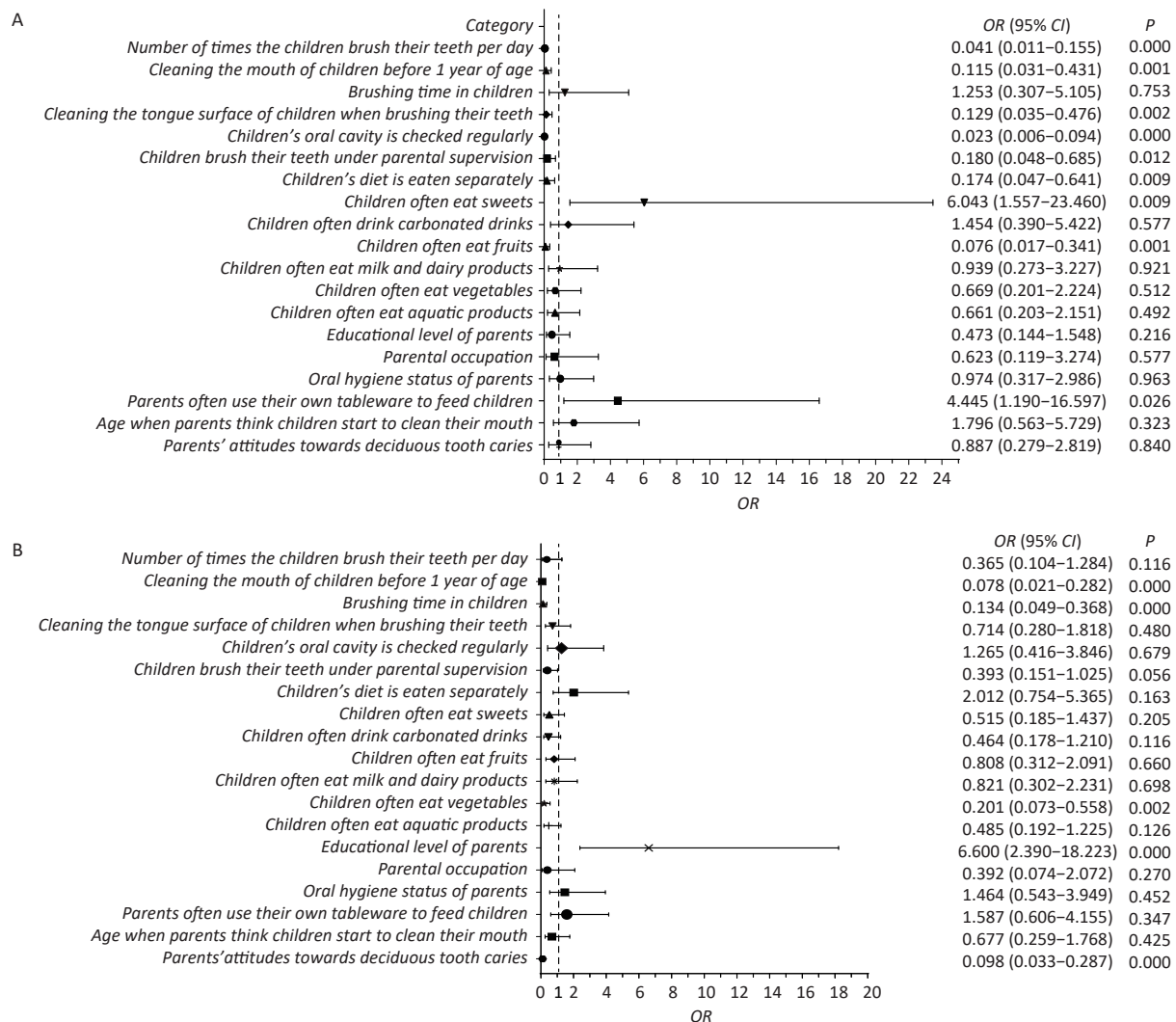


Figure 1. Forest plot. (A) Multifactorial regression analysis of health and ECC groups. (B) Multifactorial regression analysis of mild ECC and severe ECC groups. In the left planar rectangular coordinate system diagram, the horizontal scale with 1 as the center of the vertical null line indicates the protective factors on the left side of the line; the factors on the right side are associated factors, and the factors crossing the line are null factors. On the right side are the odds ratios with 95% confidence intervals and the P-values. ECC, early childhood caries.

known contributors to caries, were particularly prevalent in caries-affected niches, highlighting their role in acid production and enamel demineralization. Additionally, biomarkers such as *Prevotella intermedia* and *N. pharyngis* were identified in caries-affected niches, suggesting their potential role

in the pathogenesis of ECC. These findings highlight the complex interactions between the oral microbiota and dental caries, indicating the close association between microbial species and the development and progression of caries in specific oral niches^[6].

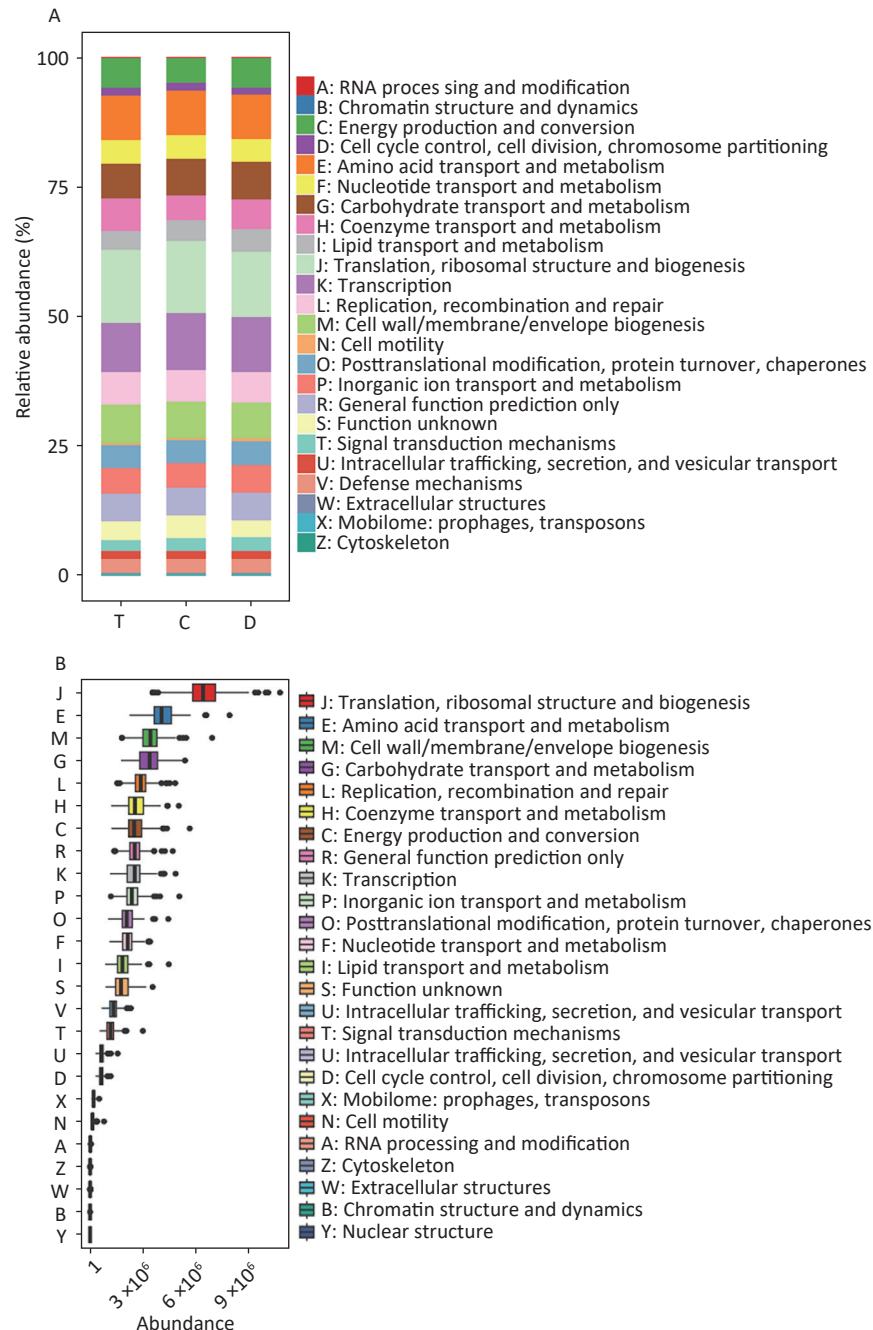


Figure 2. PICRUSt2 functional prediction and phylogenetic tree at the genus level. (A) Composition of COG functions in the three groups. (B) Abundance of COG functions in all samples. T: T niche, samples were collected from the dorsal tongue mucosa. D: D niche, samples from the teeth. C niche, samples were collected from the buccal mucosa. COG, Clusters of Orthologous Groups.

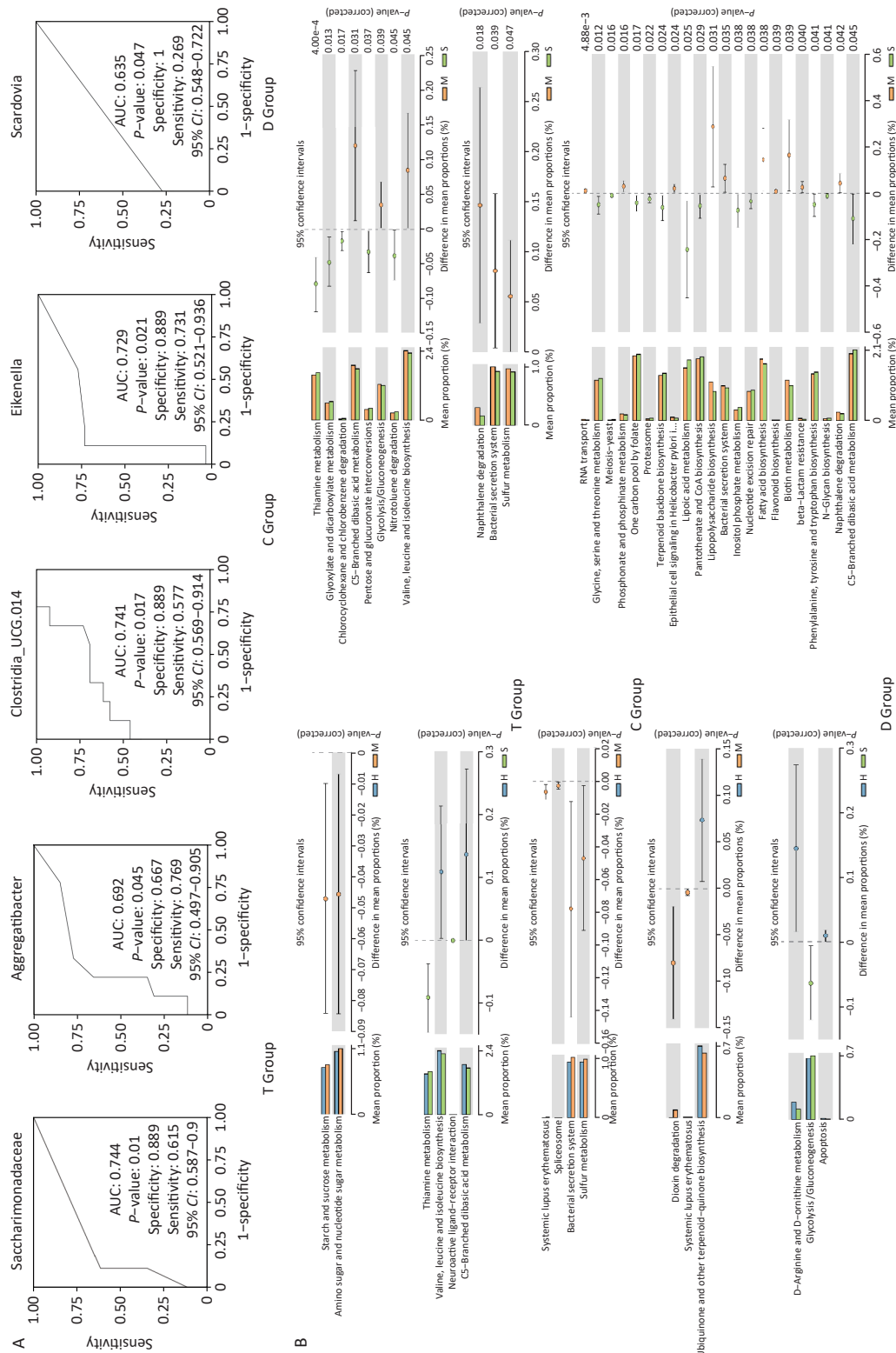


Figure 3. Predictive power of microbiota markers and metabolic pathway prediction. (A) ROC curve analysis. From left to right, *Saccharimonadaceae*, *Aggregatibacter*, *Clostridia_UCG.014*, *Eikenella*, and *Scardovia*; (B) KEGG pathway analysis. The bars on the left represent the abundance of a metabolic pathway as a percentage of all metabolic pathways in each of the two groups of samples, with the corrected P-values on the right. KEGG, Kyoto Encyclopedia of Genes and Genomes.

Functional predictions using PICRUSt2 (Figure 2) indicated variations in the metabolic potential of microbiota across niches, particularly in metabolism-related pathways, which could influence caries development. Notably, diagnostic analysis (Figure 3A) revealed that certain bacterial families, such as *Saccharimonadaceae* and *Aggregatibacter* in the T niche, had high predictive performance for caries, as evidenced by their AUC values. *Clostridia_UCG.014* is a known gut microbial strain^[7], and previous studies have explored the relationship between nutrition, oral microbiota, and dental caries. Nonetheless, its association with dental caries remains unclear. With ongoing research on the “oral-intestinal microbiota axis”, some important physiological functions and potential pathogenic roles may be revealed.

This study underscores the role of specific oral niches in shaping microbial communities and their potential impact on caries. Despite the similar overall diversity across groups, the presence of specific genera related to ECC severity, such as *Streptococcus* and *Veillonella*, highlights the importance of niche-specific microbial dynamics. The presence of specific bacteria such as *Streptococcus* and *Veillonella* in caries-affected niches indicates their role in disease progression^[8]. These findings suggest that individualizing interventions based on the specific microbial profiles of different oral niches could be more effective. However, the unexpected increase in the abundance of Bacteroidota in caries-affected niches suggests complex interactions, which require further investigation. Functionally, the differences in metabolic pathways between healthy and caries-affected children indicate that targeting microbial metabolism could be a potential strategy for preventing caries. Furthermore, identifying specific microbial markers also presents avenues for developing more accurate diagnostic tools. Functional predictions (Figure 3B) further support this, indicating that microbial metabolic activity may be a key factor in caries progression. Future research should focus on exploring these niche-specific interactions to develop more precise caries prevention strategies. Functional predictions revealed significant differences in metabolic pathways between the groups, which were primarily related to glucose, amino acid, and lipid metabolism; these findings highlight the importance of microbial metabolic activity in caries progression. Nevertheless, the present study relied on questionnaire data, which carries potential biases such as recall bias and the accuracy of parental

reporting^[9,10]. Still, the questionnaire data were combined with microbiological analysis to mitigate these limitations, thereby providing a more comprehensive understanding of the factors contributing to dental caries. Future research should focus on refining these methods and further exploring the metabolic functions of the oral microbiota in caries prevention.

Our findings depict the distinct microbial characteristics and biomarkers across the three oral niches in preschool children, demonstrating differences between healthy children and those with varying degrees of caries. The study emphasizes the importance of good dietary habits, reducing high-sugar food intake, and using personal tableware to maintain a balanced oral microbiota. Additionally, promoting healthy oral hygiene practices, such as regular brushing, flossing, and early detection of oral issues through routine checkups, is crucial. The results underscore the role of family and community environments in shaping children’s oral health and reducing the incidence of dental caries in young children.

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Competing Interests The authors declare that they have no conflict of interest.

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