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# Alterations of oral microbiota in young children with autism: Unraveling potential biomarkers for early detection

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ABSTRACT

**Objectives:** This study investigated the oral microbiota in young children with autism spectrum disorder (ASD) to determine possible alterations in microbial composition and identify potential biomarkers for early detection.

**Methods:** Dental plaque samples from 25 children with ASD (aged 3–6 years; $M = 4.79$, $SD = 0.83$) and 30 age- and sex-matched typically developing (TD) children were analyzed using 16S rRNA sequencing.

**Results:** The results showed lower bacterial diversity in children with ASD compared to controls, with distinct microbial compositions in the ASD and TD groups. Six discriminatory species (*Microbacterium flavescens, Leptotrichia* sp. HMT-212*, Prevotella jejuni, Capnocytophaga leadbetteri, Leptotrichia* sp. HMT-392*,* and *Porphyromonas* sp. HMT-278) were identified in the oral microbiota of ASD children, while five discriminatory species (*Fusobacterium nucleatum* subsp. *polymorphum, Schaalia* sp. HMT-180, *Leptotrichia* sp. HMT-498, *Actinomyces gerencseriae*, and *Campylobacter concisus*) were identified in TD controls. A model generated by random forest and leave-one-out cross-validation achieved an accuracy of 0.813. Receiver operating characteristic analysis yielded a sensitivity of 0.778, a specificity of 0.857, and an AUC (area under curve) of 0.937 (95 % CI: 0.82 – 1.00) for differentiating children with and without ASD.

**Conclusion:** The present study has unveiled significant disparities in the oral microbial composition between ASD and TD children.

**Significance:** These findings contribute to understanding the microbiome-brain connection in ASD and its implications for early detection and management. Further research is needed to validate these oral bacterial biomarkers and explore their mechanistic association with ASD pathophysiology.

## 1. Introduction

Characterized by persistent deficits in social communication and interaction, as well as restricted and repetitive patterns of behavior, children with autism spectrum disorder (ASD) often have difficulties getting along with peers, show resistance to changes, and face challenges in their academic, social, and daily functioning. The latest prevalence rate in the United States estimates that ASD affects approximately 1 in 36 children [1], highlighting its increasing significance as a global public health concern. Early identification and timely support for children with ASD are crucial, as they significantly enhance the chances of independent living and improved social functioning [2,3].

A meta-analysis of studies from 2012 to 2019 found that the average age at ASD diagnosis was 60 months, varying from 31 to 235 months across countries based on ASD type, comorbidity, and gender [4]. Severe cases may be diagnosed by ages 2 to 3, while milder ones often go unnoticed until school's social demands highlight their impairments [5]. Limited ASD screening tools, primarily reliant on subjective teacher and parent assessments, underscores the necessity for more robust objective measures. Exploring biomarkers that can provide a more robust foundation for early ASD detection is imperative for improving diagnostic accuracy and intervention outcomes.

Prior studies have suggested that gut and oral microbiome play important roles in the pathogenesis of inflammation, immune dysfunction, and disruption of the gut–brain axis, which may contribute to ASD pathophysiology [6–9]. For instance, some researchers propose that oral

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bacteria may impact neuroimmune activity and inflammation by crossing the blood-brain barrier (BBB), thereby potentially affecting the central nervous system through inflammation or changes in metabolic activity [10]. Besides, it has been suggested that the Gram-negative bacterium *Haemophilus parainfluenza* can generate metabolites capable of traversing a compromised BBB, leading to impaired brain functions [11]. Other studies have indicated immune dysregulation in individuals with ASD and their families, suggesting a genetic link between the immune system and ASD, as well as correlations between ASD and various immune phenotypes such as allergic diseases, lymphocyte count, and autoimmune disorders [12]. The interplay between maternal immune activation during pregnancy and epigenetic dysregulation in the fetal brain may contribute to ASD development [13]. These factors could potentially influence oral and gut dysbiosis. Moreover, animal research has shown that transferring oral microbiota from autistic individuals to mice can induce behaviors resembling those seen in ASD and lead to alterations in brain gene expression, particularly affecting genes related to serotonin [14]. These findings suggest a plausible oral-gut-brain axis that may underlie the manifestation of ASD in individuals.

Some researchers have investigated the relationship between gut microbiota and ASD [9,15,16]. Wan et al. [9] examined stool samples from children aged 3–6, and identified five bacterial markers that could differentiate children with ASD from typically developing children. Their findings suggested a persistent under-development of gut microbiome in children with ASD relative to TD children. Given that the gastrointestinal tract begins with the oral cavity, which hosts the second most complex microbiota in humans [17,18], variations in the oral microbiota of children with ASD compared to TD children may be detectable.

While the reliability and validity of ASD identification through gut microbiome is increasing, research on oral biomarkers for screening ASD in children is limited. To the best of our knowledge, only a few studies have examined this aspect and indicated differences in oral microbiota between children with and without ASD [16,19–21]. Qiao et al. [21] observed lower bacterial diversity in saliva and dental plaque samples among children with ASD compared to TD controls. They found higher levels of *Haemophilus* in saliva and *Streptococcus* in dental plaque in the ASD group, alongside lower levels of *Prevotella, Selenomonas, Actinomyces, Porphyromonas*, and *Fusobacterium*. In another study, Hicks et al. [18] noted five oral microbial ratio variances between ASD and TD subjects, and three ratio differences between ASD and non-autistic individuals with developmental delay, suggesting that oral microbiome profiling might aid in ASD identification.

This study aimed to analyze oral microbiota variations between children with ASD and typically developing (TD) children, exploring if specific bacterial species or the overall composition of dental plaque biofilms could act as biomarkers to differentiate ASD children from their TD counterparts while addressing potential confounding variables (e.g. the presence of dental caries). This would test the null hypothesis of no differences in oral microbial profiles between children with and without ASD. The study pioneers the exploration of oral microbial profiles in ASD children compared to matched peers in Hong Kong.

## 2. Materials and methods

This is an initial comparative cross-sectional study to examine putative differences in the oral microbiota in young ASD children compared to age and sex-matched TD controls using high-throughput 16S rRNA gene amplicon sequencing. This study was approved by the Human Research Ethics Committee of the authors' university (EA220235) and conducted in compliance with regulations and STROBE guidelines. Written informed consent was obtained from parents of all participants prior to the study. An *a priori* power analysis was conducted using MedCalc for Windows, version 19.4 (MedCalc Software, Ostend, Belgium) to determine the minimum sample size required to test the study hypothesis. The required sample size to achieve 80 % power for detecting a medium effect, at a significance criterion of $\alpha = 0.05$, was $n = 62$ for ROC analysis of AUC = 0.7. Thus, the obtained sample size of $n = 62$ is adequate to test the study hypothesis.

### 2.1. Participants

A total of 64 preschoolers, including 32 diagnosed with ASD and 32 TD children, matched for age and sex, were recruited from the community through local preschools and rehabilitation centers operated by non-governmental organizations. Inclusion criteria for the ASD group included the following: 1) aged 3–6, currently studying in local kindergartens or special childcare centers; 2) received a clinical diagnosis of ASD from qualified healthcare professionals (e.g. paediatricians, clinical psychologists), based on the criteria specified in the Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (DSM-5). Preschoolers were included in the TD group based on the following criteria: 1) aged 3–6, currently studying in mainstream kindergartens; 2) screened negative for ASD on the Social Responsiveness Scale (SRS)−2 short form (see below) [22]. At the time of enrollment, parents of all participants completed a written informed consent, child medical/-demographic questionnaire and the SRS-2.

### 2.2. Parent-Report measures

#### 2.2.1. Demographic information, daily brushing and feeding habits, and medical history

Demographic data, toothbrushing, and feeding habits were gathered using a parent-report questionnaire, along with developmental history, medical records, and dietary information such as birth details, probiotic use, gastrointestinal issues, allergies, and dietary restrictions, etc. (see Appendix Questionnaire).

#### 2.2.2. Social Responsiveness Scale-2 short form

The presence of social impairments was assessed using the SRS-2 short form [22], a 16-item rating scale known for its strong psychometric properties, including high internal reliability (Cronbach's alpha = 0.96). A higher score on the SRS-2 indicates greater severity of ASD symptoms.

### 2.3. Oral examination and sample collection

All children underwent a clinical examination performed by an experienced dentist who was blinded to the participants' ASD status. Clinical examinations took place at preschools or rehabilitation centers. Participants refrained from eating, drinking, and oral hygiene for at least 3 h before their appointment. The time of their last meal and toothbrushing were recorded. Dental caries was assessed at tooth level according to the diagnostic criteria published by the World Health Organization in 2010 [23]. The presence of decayed, missing, or filled teeth was recorded. Other oral health conditions, including the stage of dentition and dental restorations, were also documented. Plaque samples were collected from each participant following a standardized protocol [21]. Supragingival plaque samples were sequentially obtained from the buccal surfaces of all maxillary and mandibular primary molars, canines, and incisors using sterile cotton swabs, and pooled in 1.5 mL Eppendorf tubes containing phosphate-buffered saline. All samples were immediately placed on ice, transported to the laboratory within 3 h, and stored at −80 °C until DNA extraction.

### 2.4. Laboratory analysis

Genomic DNAs were extracted from the freshly thawed plaque samples, following the standard protocol for gram-positive bacteria of the QIAamp DNA Mini Kit (Qiagen, Germany). The DNA extraction procedure followed the protocol outlined in Appendix D: Protocols for Bacteria of the manufacturer's QIAamp DNA Mini and Blood Mini

Handbook (Ref: 10,663,018 04/2010). The resulting DNA concentration and purity were assessed optically using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, USA).

### 2.5. 16S rRNA gene sequencing

16S rRNA gene amplification (V3-V4 hypervariable region) and Illumina Novaseq PE250 sequencing were performed by Novagene Co., Ltd. (China). Sequence data were trimmed, quality filtered and subjected to data processing and taxonomic assignment using the DADA2 pipeline [24]. Taxa were assigned to amplicon sequence variants (ASVs) for downstream computational and statistical analysis [25].

### 2.6. Statistical and data analyses

Demographics and clinical parameters of subjects in ASD and TD groups were compared using *t*-test for continuous variables and Fisher's exact test for categorical variables. Rarefaction curves were generated with "rarecurve" function in R package "vegan" to assess the sequencing depth. Each unique amplicon sequence variant (ASV) was aligned with the expanded Human Oral Microbiome Database (eHOMD) [26,27] using blast+. The "estimate richness" function in R package "phyloseq" was used to evaluate α-diversity indices. For phylogenetic $\beta$-diversity measures, non-metric multidimensional scaling (NMDS) was conducted based on weighted UniFrac distances. The statistical differences were tested by permutational multivariate ANOVA (PERMANOVA) between groups.

Linear discriminant analysis (LDA) effect size (LEfSe) was used to identify taxa that differentiated the microbial communities specific to different groups. Caries and without caries groups were also analyzed using LEfSe to account for any oral microbiome shifts due to dental caries. Random Forest algorithm and leave-one-out cross-validation (LOOCV) were employed to select microbial markers and their combinations and to validate the selection of microbial markers. The performance of the obtained combinations was evaluated using receiver operator characteristics (ROC) analysis. All statistical analyses were conducted using R software package (version 4.2.0.; The R Project for Statistical Computing).

## 3. Results

### 3.1. Demographic characteristics, daily brushing and feeding habits

Of the 64 pooled supragingival plaque samples collected from the cohort, 55 samples (ASD: 25; TD: 30) yielded genomic DNA of sufficient concentration and purity that enabled successful 16 rRNA amplicon sequencing using Illunina Novaseq platform. The demographic and dental characteristics of these participants are depicted in Table 1.

The mean ages of ASD group and TD controls were 4.79 and 4.90 years, respectively. All participants were in the primary dentition stage as none of them had experienced the eruption of the first permanent teeth. There were no significant differences in terms of gender, age, birth weight, dietary frequency and milk intake between ASD and TD children.

### 3.2. Group comparison of the structure and composition of the oral microbiota

A total of 11,143 ASVs were identified using the DADA2 pipeline [24]. Rarefaction analysis indicated sufficient sequencing depth (Appendix Fig. 1). The dataset was taxonomically agglomerated to the species level and 127 species remained after filtering with a relative abundance cutoff rate of 0.1 % and prevalence cutoff of 10 % (Appendix Fig. 2).

Alpha diversity measures comparing dental plaque between ASD and TD groups are presented in Fig. 1**A**. The Shannon and Gini-Simpson indices, which measure species evenness and abundance, did not show significant differences between groups. However, more contrasting results were found for the Observed and Chao1 indices, which assess species richness. Although the Observed index did not reach statistical significance ($p = 0.082$), its lower value in the ASD group suggested a reduced variety of species types compared to the TD group. Moreover, the Chao1 index, which considers rare species in its calculation, was significantly lower in the ASD group ($p = 0.035$), indicating a decreased complexity of species communities. Collectively, these findings

**Table 1**
Demographic and dental characteristics of the participants.

| | TD ($n = 30$) | ASD ($n = 25$) | $p$[a] |
|---|---|---|---|
| **Gender** | | | |
| Male | 24 (80.0 %) | 19 (76.0 %) | 0.753 |
| Female | 6 (20.0 %) | 6 (24.0 %) | |
| **Age (years)** | | | |
| Mean (SD) | 4.90 (0.85) | 4.79 (0.83) | 0.621 |
| **Preschool grade level** | | | |
| 1 | 6 (20.0 %) | 6 (24.0 %) | 1 |
| 2 | 7 (23.3 %) | 6 (24.0 %) | |
| 3 | 15 (50.0 %) | 13 (52.0 %) | |
| Missing | 2 (6.7 %) | 0 (0 %) | |
| **Full term to give birth** | | | |
| No | 3 (10.0 %) | 4 (16.0 %) | 0.689 |
| Yes | 27 (90.0 %) | 21 (84.0 %) | |
| **Birth weight (kg)** | | | |
| Mean (SD) | 3.09 (0.41) | 2.87 (0.56) | 0.109 |
| **Frequency of dentist visit** | | | |
| Periodic visit | 3 (10.0 %) | 2 (8.0 %) | 0.630 |
| Unscheduled visit | 8 (26.7 %) | 4 (16.0 %) | |
| Never | 19 (63.3 %) | 19 (76.0 %) | |
| **Caries** | | | |
| Mean (SD) | 2.63 (3.61) | 0.44 (1.61) | 0.005 |
| **Missing teeth** | | | |
| Mean (SD) | 0.10 (0.31) | 0.08 (0.40) | 0.838 |
| **Filled teeth** | | | |
| Mean (SD) | 0.30 (0.88) | 0 (0) | 0.071 |
| **Number of primary teeth** | | | |
| Mean (SD) | 19.9 (0.25) | 19.9 (0.40) | 0.886 |
| **Tooth brushing frequency** | | | |
| Two times or more per day | 29 (96.7 %) | 14 (56.0 %) | 0.001 |
| Once per day | 1 (3.3 %) | 7 (28.0 %) | |
| Sometimes | 0 (0 %) | 2 (8.0 %) | |
| Never | 0 (0 %) | 2 (8.0 %) | |
| **Use of fluoridated toothpaste** | | | |
| Yes | 19 (63.3 %) | 16 (64.0 %) | 0.104 |
| No | 3 (10.0 %) | 7 (28.0 %) | |
| Don't know | 8 (26.7 %) | 2 (8.0 %) | |
| **Feeding method in infancy** | | | |
| Breastfeeding | 9 (30.0 %) | 15 (60.0 %) | 0.062 |
| Mixed | 10 (33.3 %) | 3 (12.0 %) | |
| Formula milk | 11 (36.7 %) | 7 (28.0 %) | |
| **Sleep with milk bottle** | | | |
| Yes | 3 (10.0 %) | 2 (8.0 %) | 1 |
| No | 27 (90.0 %) | 23 (92.0 %) | |
| **Frequency of diet** | | | |
| 1–2 | 13 (43.3 %) | 11 (44.0 %) | 0.253 |
| 3–4 | 6 (20.0 %) | 9 (36.0 %) | |
| 5–6 | 9 (30.0 %) | 4 (16.0 %) | |
| 7–8 | 0 (0 %) | 1 (4.0 %) | |
| 9–10 | 0 (0 %) | 0 (0 %) | |
| Over 10 | 2 (6.7 %) | 0 (0 %) | |
| **Social Responsiveness Scale-2** | 11.3 | 22.7 | $< 0.001$ |

[a] $p$-value of Student's $t$-test for continuous data and Fisher's exact test for categorical data.

All participants were in the primary dentition state as there were no first eruption of the first permanent tooth. There were no significant differences in terms of gender, age, birth weight, dietary frequency and milk intake between the ASD and TD children. As expected, children with ASD displayed substantially higher scores (i.e. more severe ASD symptoms) on the SRS-2 in contrast to the TD children ($p < 0.01$). The ASD children were reported to brush less frequently compared to their TD peers ($p = 0.001$), yet they were found to have fewer instances of dental caries ($p = 0.005$). Further information for caries and non-caries classification can be found in Appendix Table 1.

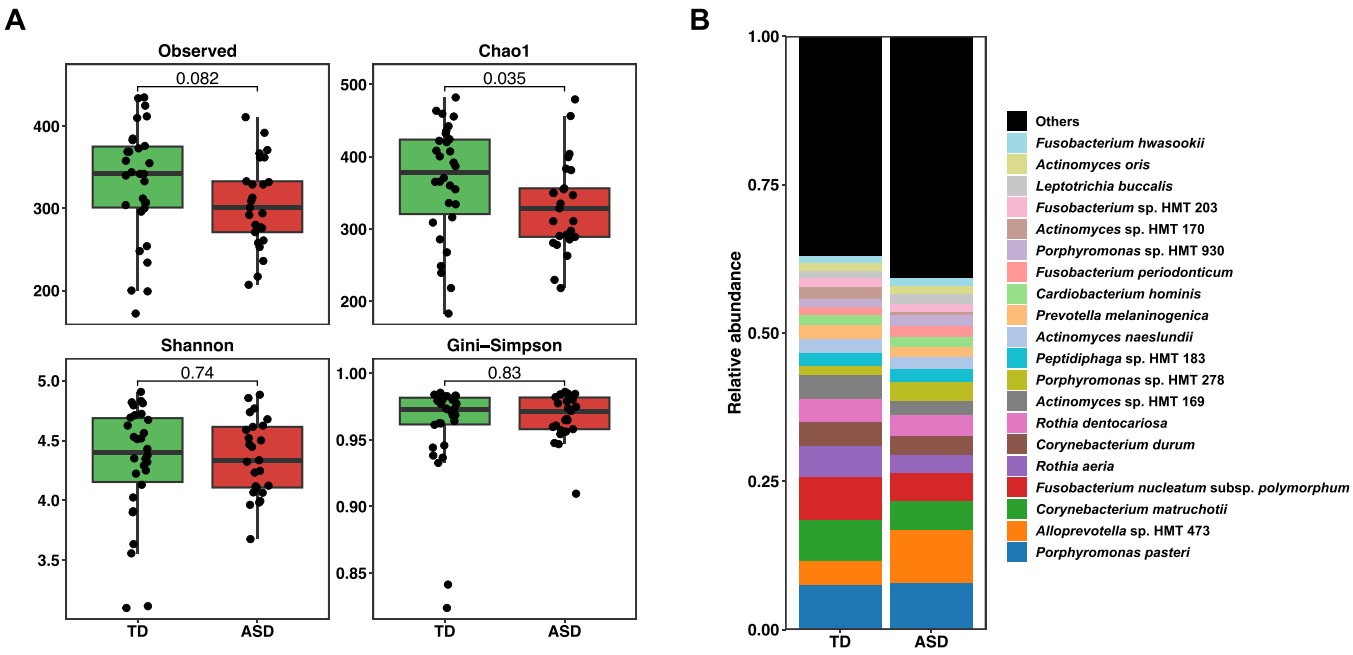


**Fig. 1.** Alpha diversities and relative abundances of 20 most abundant species within dental plaque from ASD and TD groups. (A) Four different Alpha diversity estimations: Observed species (Observed), Chao1, Shannon, and Gini-Simpson indices; of dental plaque composition within ASD (red) and TD (green) groups. Only the Chao1 index indicated a statistically significant difference between the ASD and TD groups ($p = 0.035$; Wilcoxon Rank Sum test). (B) Relative abundance of the top 20 most abundant species within ASD and TD groups.

highlight a discrepancy in species diversity between the ASD and TD groups. Fig. 1**B** depicts the top 20 abundant species for both the ASD and TD groups. The top 20 species were consistent between the two groups, but their relative abundances differed.

For $\beta$-diversity, non-metric multidimensional scaling (NMDS) based on weighted UniFrac metrics was performed to examine differences in the community structure of the oral microbiota (Fig. 2**A**). Subjects clustered differently according to their respective caries experience and clinical diagnosis. A significant difference was observed between the ASD and TD groups in weighted UniFrac distances ($p = 0.016$; Fig. 2**B**), signifying a notable variation in oral microbial composition between the two groups. To address the potential confounding effects of dental caries on bacterial composition, similar analyses were conducted for all participants with and without dental caries. Results showed no significant difference between two groups in weighted UniFrac distances ($p = 0.051$; Fig. 2**B**), indicating that dental caries did not significantly contribute to the observed differences in microbial community composition between ASD and TD children in this study.

The LDA score plot (Fig. 3**A**) shows the respective bacterial taxa (at the phylum, class, order, genus and species level) that could be used to differentiate the ASD and TD groups. A cladogram showing the taxonomic relationships between these taxa is shown in Fig. 3**B**. Notably, 11 species could be used to respectively discriminate ASD and TD groups, which are referred to as 'discriminatory' species or 'bacterial species biomarkers'. ASD subjects could be identified using six species: *Prevotella jejuni, Porphyromonas* sp. HMT-278, *Capnocytophaga leadbetteri, Microbacterium flavescens, Leptotrichia* sp. HMT-212 and HMT-392. Analogously, TD subjects could be identified using 5 species: *Fusobacterium nucleatum* subsp. *polymorphum, Schaalia* sp. HMT-180, *Leptotrichia* sp. HMT-498, *Actinomyces gerencseriae*, and *Campylobacter concisus*. Box plots showing the respective relative abundances of the 11 bacterial species biomarkers within ASD and TD groups (as well as results from Wilcoxon rank-sum test comparisons) are shown in Fig. 3**C**.

### *3.3. Diagnostic capabilities of bacterial composition in dental plaque for discriminating ASD and TD children*

Random forest analysis was performed to further examine whether the 11 bacterial species biomarkers identified by LEfSe could discriminate between ASD and TD children. The data was divided into training and testing sets, with 70 % of the samples included in the training set. The LOOCV method evaluated the model by repeatedly training the data with all the sequenced samples except one, and calculating the average accuracy. The best model, with 100 trees and a mtry parameter of 2, was then used for further analysis. The testing dataset was used for validation, resulting in an accuracy of 0.813 (95 % CI: 0.544 – 0.960), a sensitivity of 0.778, specificity of 0.857, and an AUC (area under curve) of 0.937 (95 % CI: 0.82 – 1.00) for the ROC analysis (Fig. 4**A**).

The feature importance plot (Fig. 4**B**) illustrates the mean decrease in the Gini coefficient, which assesses the significance of individual species in contributing to the model's homogeneity in the resulting random forest. A higher mean decrease in the Gini score indicates greater importance of the respective biomarker species within the model. In the current model, *Porphyromonas* sp. HMT-278 holds the highest importance among species in the ASD group.

## 4. Discussion

Previous research has shown a strong association between gut microbiota and ASD [10,11]. The oral microbiota is a resilient microbial population residing within the human oral cavity, most notably in dental plaque biofilms, constituting the second most complex microbial community in humans [17,18]. This investigation was conducted to examine differences in the composition of bacterial species within the dental plaque of young children with ASD versus age- and sex-matched TD controls. The present study considered multiple factors during the sampling process, including stage of dentition, caries experience, and diet, along with age and sex, due to their potential influence on the composition of dental plaque. Results from the current bioinformatic analyses revealed significant differences in oral microbiota composition in children with ASD compared to TD children. Notably, ASD children

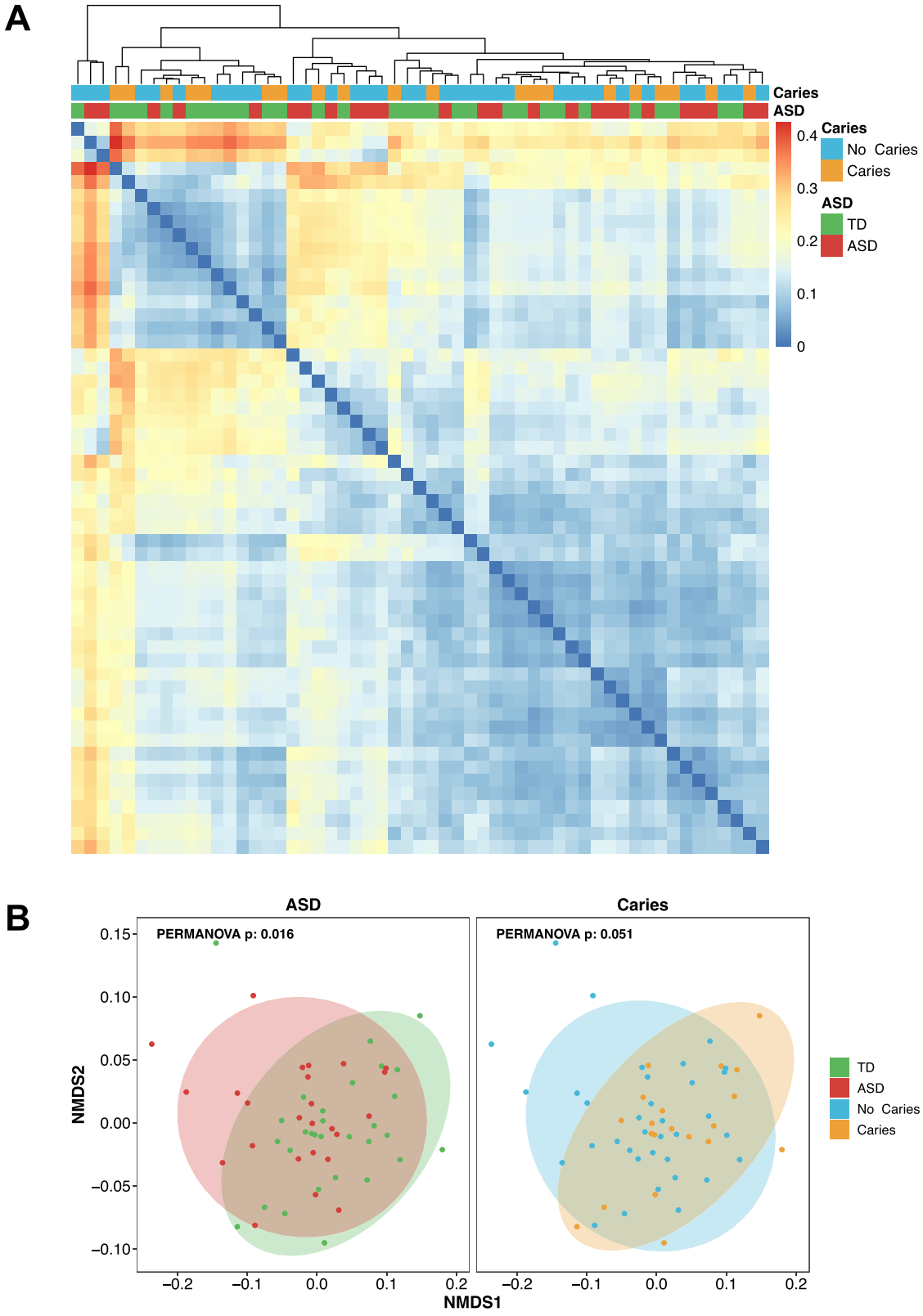


**Fig. 2.** Differences in bacterial community composition (beta diversity) within ASD and TD subjects; subjects with and without caries. (A) Heatmap showing the magnitude of differences between individual species. A phylogram showing the clustering of community composition in each sample according to caries experience (caries, no caries) and clinical diagnosis (ASD, TD). (B) Non-metric multidimensional scaling (NMDS) plots based on weighted UniFrac distances according to clinical diagnosis (ASD) and caries experience (caries). There was a statistically significant distinction between the ASD and TD groups (PERMANOVA, $p = 0.016$), whereas no significant difference was observed between the groups based on caries experience.

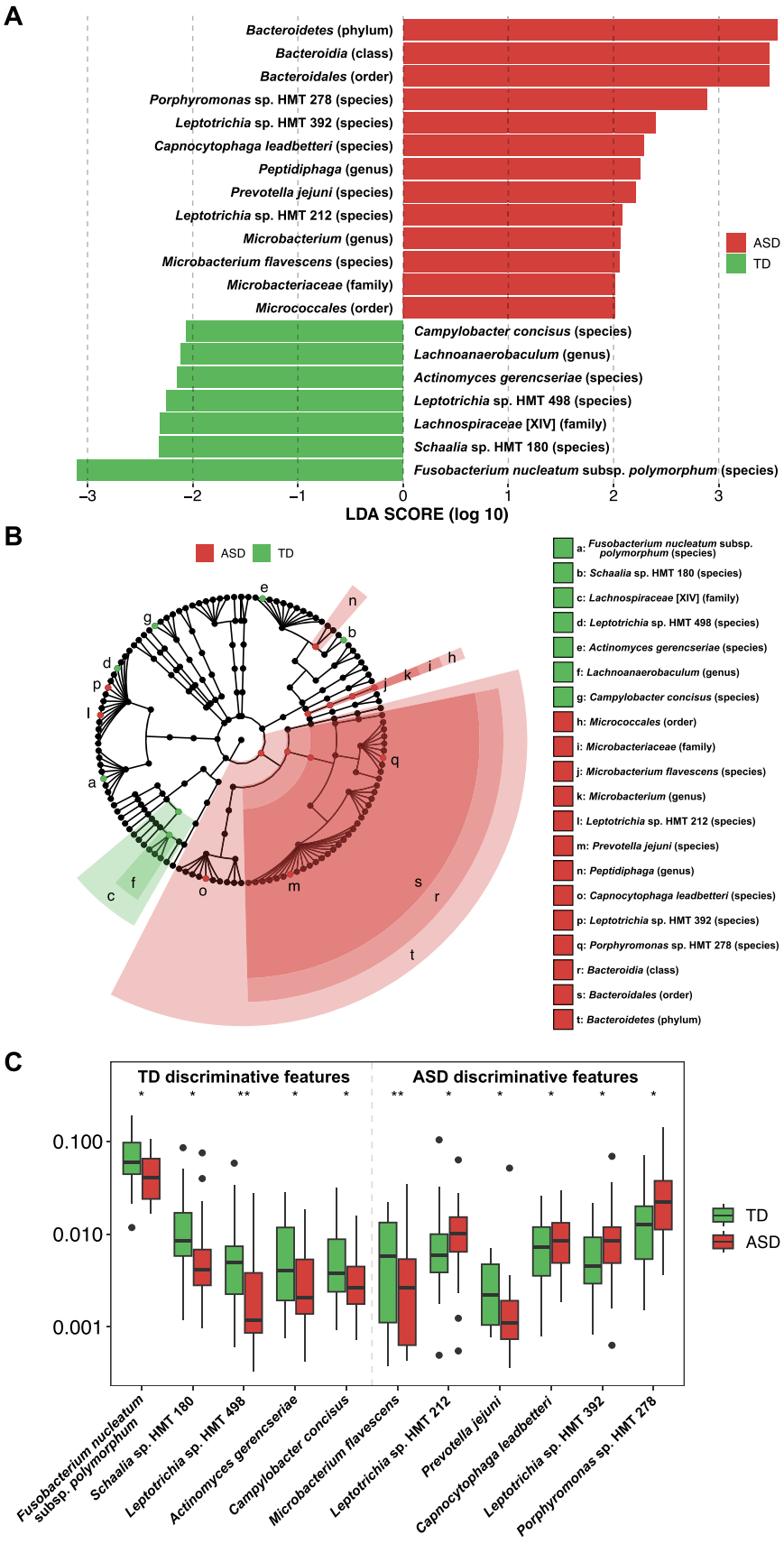


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**Fig. 3.** Differential distributions of oral taxa within ASD and TD children. (A) LDA score plot of the discriminatory taxa for the ASD and TD groups. (B) Cladogram of the relationship of the discriminatory taxa in the ASD and TD groups (showing taxonomic levels from phylum to species). (C) Box plot of the 6 discriminatory species for the ASD group, and 5 discriminatory species for the TD group. Children with ASD exhibited an increased relative abundance of *Leptotrichia* sp. HMT-212, *Capnocytophaga leadbetteri, Leptotrichia* sp. HMT-392, and *Porphyromonas* sp. HMT-278, while showing a decreased relative abundance of *Microbacterium flavescens* and *Prevotella jejuni*.

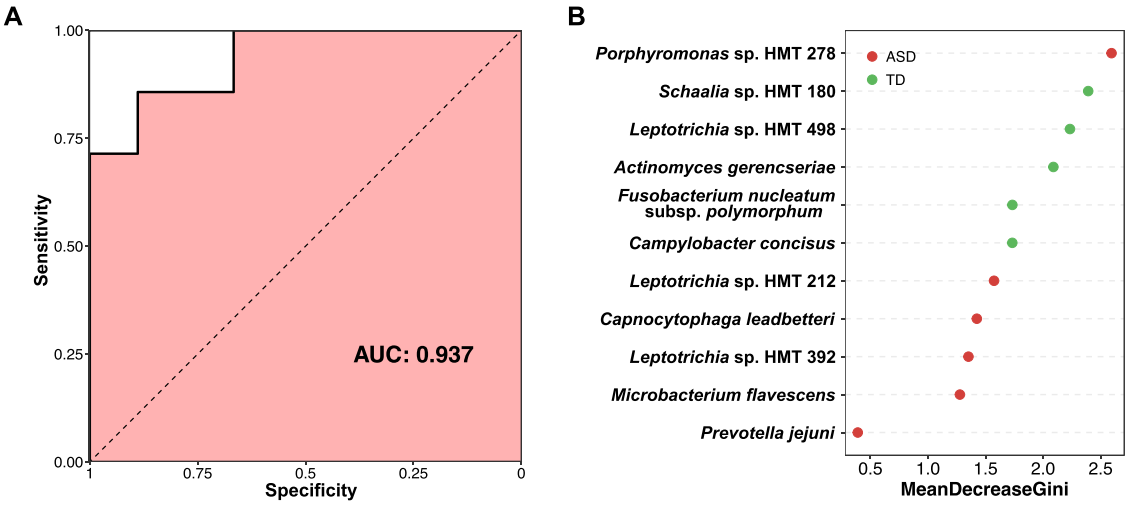


**Fig. 4.** Receiver operating characteristic (ROC) curve analysis and feature importance plot of bacterial species biomarkers. (A) ROC curve analysis shows the diagnostic performance of the bacterial species biomarkers ($n = 11$) for the prediction of children with ASD. (B) Feature importance plot showing the mean decrease in the Gini coefficient for the 11 respective bacterial species between ASD (red) and TD (green) children.

exhibited lower microbial community complexity (lower beta-diversity) than TD children (Fig. 2).

A balanced and diverse microbiome, characterized by ecological harmony (eubiosis) within the complex biofilm communities and physiological compatibility with the host, is essential for resilient oral health [17,28–30]. Oral microbial dysbiosis (unhealthy ecological imbalance), especially within subgingival microbiomes, has been associated with several systematic diseases and conditions [31,32]. The consensus is that reduced bacterial diversity can compromise the overall resilience of the microbial community, weakening its ability to withstand environmental stressors, such as the introduction or proliferation of disease-promoting microbes [33]. Prior studies have suggested that alterations in oral microbial profiles are potentially associated with ASD [16,34]. However, prior studies have not included a detailed clinical examination of the subjects' oral health status. In contrast, this study applied strict inclusion criteria targeting primary dentition, age control, and conducted a standardized oral examination recording abscesses, sinuses, and DMFT status.

The current findings suggest that whilst there were differences in alpha diversity, community composition (beta-diversity), and the distributions of certain taxa between ASD and TD children, both groups shared notable similarities in the dominant species present (Fig. 1**B**). This indicates that there were relatively subtle differences in overall taxonomic composition between ASD and TD children. Subjectively, both groups had a healthy oral microbiome, evidenced by the fact that there was no overabundance of taxa previously associated with caries or periodontal disease. Caries did not appear to greatly affect the differences in oral microbiota within TD and ASD children (Fig. 2**B**).

These findings were consistent with previous studies [19,21], which also reported lower beta diversity in the ASD group compared to the TD group. However, there were some differences in the respective identities and levels of bacterial taxa that could be used to differentiate the clinical groups. In previous studies [19,21], bacterial genera including *Streptococcus* and *Haemophilus* were more abundant in the oral microbiome of children with ASD, whilst others, including *Prevotella, Actinomyces, Porphromonas,* and *Fusobacterium* were reduced. In the current study, after adjusting for caries, the prevalence of *Streptococcus* and *Haemophilus* did not stand out significantly in either group.

In this study, eleven bacterial biomarker species could be used to discriminate ASD and TD groups. Of note, certain species within the *Bacteroidetes* phylum, including *Porphyromonas* sp. HMT-278, *Prevotella jejuni*, and *Capnocytophaga leadbetteri*, were found to be biomarkers for the ASD group. Taxa from the *Bacteroidetes* phylum have been associated with ASD in previous studies looking at the oral microbiota [16] and the gut microbiota [32]. For instance, supplementation with *Bacteroidetes* species has been shown to ameliorate ASD-related behaviors in mouse models, suggesting their potential as a probiotic therapy for behavioral symptoms [14,35].

The current findings revealed a lower abundance of *Prevotella jejuni* in children with ASD compared to controls. *Prevotella* spp. have been linked to gastrointestinal and respiratory health [34]. Originally isolated from the gut of a juvenile with coeliac disease, *P. jejuni* is obligately anaerobic, saccharolytic, proteolytic and hemolytic [36]. It predominantly inhabits the oral cavity, and phylogenetically, is most closely related to *Prevotella melaninogenica* and *Prevotella histicola* [37,38]. Most notably, its salivary levels were found to be elevated in subjects with Crohn's disease [39]. A recent large-scale oral metagenome-human genome associate study linked *P. jejuni* with rs1196764 at the APPL2 locus [40]. APPL2 encodes a multifunctional regulatory protein that is involved in the regulation of glucose-stimulated insulin secretion, and may play a role in negatively modulating inflammation [41,42]. These intriguing pathophysiological associations merit further detailed investigations.

The 11 bacterial biomarker species identified are known inhabitants of the human oral cavity, and are listed in the extended Human Oral Microbiome Database (eHOMD, v3.1) [26,43,44]. According to the eHOMD (v3.1) [44], *Porphyromonas* sp. HMT-278 strain W7784, *Schaalia* sp. HMT-180 strain F0310 and *Leptotrichia* sp. HMT-212 strain W10393 have been genome sequenced, but to the best of our knowledge, there are no published reports into their respective activities, clinical distributions or associations with disease. *Leptotrichia* sp. HMT-392 and HMT-498 are as-yet uncultivated, making it difficult to propose

pathophysiological roles for these taxa. In addition, *Microbacterium flavescens* (NCBI:txid69366), previously known as *Arthrobacter flavescens*, is one of the six distinctive species indicated in this study. It should not be confused with *Mycolicibacterium flavescens* (NCBI:txid1776), formerly referred to as *Mycobacterium flavescens*. As far as is known, no published reports are linking either species to ASD.

Future research should explore the potential contributions of these bacteria to ASD, including their transcriptional and metabolic activities, interactions with other oral microbiota, and impact on neurodevelopment and ASD-related behavior. Understanding these connections can enhance the existing knowledge of the oral microbiome-brain axis and their potential as early screening markers for ASD in young children.

Apart from early screening markers, potential probiotics therapy may also be a future direction for investigation. Multiple studies have documented enhancements in gastrointestinal symptoms and ASD manifestations following probiotic supplementation [45,46,47]. The oral cavity serves as an entry point to the digestive system, and maintaining a healthy balance of oral bacteria can have downstream effects on the gastrointestinal tract. Probiotic treatment may potentially alter the oral microbiota composition of children with ASD and influence the gut microbiota through the ingestion of saliva and other oral secretions. It is noteworthy that the results of probiotic therapy are not consistent across studies, emphasizing the need for further research to explore the optimal strains, doses, and durations of probiotic treatment for children with ASD. Studies should also investigate the long-term effects of probiotic supplementation and its potential to serve as a complementary therapy alongside other treatments for ASD.

Moreover, recognizing the limitations posed by the small sample size in this study highlights the importance of incorporating a larger and more ethnically diverse cohort of children in future research to ensure more robust and generalizable results. Subsequent studies should incorporate test-retest reliability measures to enhance the consistency of the oral microbial profile as an early ASD screening marker.

## 5. Conclusion and implications

In conclusion, the present study has unveiled significant disparities in the oral microbial composition between ASD and TD children aged 3–6 years. The overall community composition as well as 11 specific bacterial species (taxa) demonstrated high accuracy in differentiating between children with and without ASD. Our results underscore the potential clinical relevance of using oral microbiome analysis for the early detection of ASD. Further research is imperative to elucidate the implications and underlying mechanisms of these microbial differences in relation to ASD.

All authors gave their final approval and agreed to be accountable for all aspects of the work.

## CRediT authorship contribution statement

**Jacqueline Wai-yan Tang:** Writing – review & editing, Writing – original draft, Project administration, Methodology, Investigation, Conceptualization. **Charles Cheuk-fung Hau:** Writing – review & editing, Methodology, Investigation, Conceptualization. **Wai-man Tong:** Writing – review & editing, Methodology, Formal analysis, Data curation. **Rory Munro Watt:** Writing – review & editing, Methodology, Conceptualization. **Cynthia Kar Yung Yiu:** Writing – review & editing, Resources, Methodology, Conceptualization. **Kathy Kar-man Shum:** Writing – review & editing, Resources, Methodology, Investigation, Funding acquisition, Conceptualization.

## Declaration of competing interest

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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*Ethics approval and consent to participate*

The research project has been approved by the Human Research Ethics Committee of the University of Hong Kong (EA220235). Written consent was obtained from parents of all participants prior to data collection.

*Data Availability*

The de-identified data in the current study will be available from the corresponding author upon reasonable request.

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## Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.jdent.2024.105486.

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