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SCIENTIFIC INVESTIGATIONS

# Pediatric Obstructive Sleep Apnea is Associated With Changes in the Oral Microbiome and Urinary Metabolomics Profile: A Pilot Study

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**Study Objectives:** Several cross-sectional studies have reported associations between oral diseases and obstructive sleep apnea (OSA). However, there have been no reports regarding the structure and composition of the oral microbiota with simultaneous evaluation of potential associations with perturbed metabolic profiles in pediatric OSA.

**Methods:** An integrated approach, combining metagenomics based on high-throughput 16S rRNA gene sequencing, and metabolomics based on ultra-performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry and gas chromatography coupled with time-of-flight mass spectrometry, was used to evaluate the oral microbiome and the urinary metabolome.

**Results:** 16S rRNA gene sequencing indicated that the oral microbiome composition was significantly perturbed in pediatric OSA compared with normal controls, especially with regard to *Firmicutes*, *Proteobacteria*, *Bacteroidetes*, *Fusobacteria*, and *Actinobacteria*. Moreover, metabolomics profiling indicated that 57 metabolites, 5 of which were metabolites related to the microflora of the digestive tract, were differentially present in the urine of pediatric patients with OSA and controls. Co-inertia and correlation analyses revealed that several oral microbiome changes were correlated with urinary metabolite perturbations in pediatric OSA. However, this correlation relationship does not imply causality.

**Conclusions:** High-throughput sequencing revealed that the oral microbiome composition and function were significantly altered in pediatric OSA. Further studies are needed to confirm and determine the mechanisms underlying these findings.

**Keywords:** metabolomics, metagenomics, obstructive sleep apnea, oral microbiota

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**BRIEF SUMMARY**

**Current Knowledge/Study Rationale:** The oral microbiota has been suggested to be associated with several systemic diseases, but little is known about whether the structure and composition of the oral microbiota are perturbed in pediatric obstructive sleep apnea (OSA). To date, there have been no clinical studies using metabolomics approaches to evaluate metabolic status in pediatric OSA. This study was performed to investigate the oral microbiome and urinary metabolomics profile in OSA.

**Study Impact:** Five genera and 57 metabolites were different between OSA and controls. These alterations suggest perturbation of metabolic status and functional alterations in the oral microbiota in pediatric OSA, supporting the hypothesis that changes in the oral microbiome may represent a new mechanism leading to or exacerbating OSA-related metabolic disorders.

## INTRODUCTION

Obstructive sleep apnea (OSA) is one of the most common sleep disorders in children, with a high prevalence rate of 3% to 4%.[1] Pediatric patients with OSA experience repeated episodes of complete or partial obstruction of the upper airway at night, leading to increased intrathoracic pressure, sympathetic tone, microarousals, and decreased oxygen desaturation.[2] OSA can cause a series of clinical sequelae, such as cardiovascular and metabolic disturbances.[3]

Metabolomics is an innovative and highly sensitive profiling method that seeks to identify and quantify small molecules in biological systems in a specific physiological state.[4] Multiple metabolites and metabolic pathways associated with adult OSA have been identified using metabolomics approaches.[5,6] However, no reported metabolomics study had explored metabolic changes in children using a high-throughput method.

Several clinical studies have found positive correlations between periodontitis and OSA.[7,8] Because oral health is closely related to oral microbial status, we hypothesized that the oral health–OSA relationship may have an underlying microbial basis. In the oral cavity, the oral microbiome comprises more than 700 different bacterial species.[9] Increasing evidence shows that there is a close relationship among oral microbiota

inflammation, insulin resistance, and even common systemic diseases, such as diabetes and symptomatic atherosclerosis.[10,11] However, no reported study has directly evaluated such associations between the oral microbiome and OSA.

The adenoids are now considered to be a pathogen reservoir, as evidenced by the presence of group A streptococcus, *Haemophilus influenzae*, *Streptococcus pneumoniae*, *Staphylococcus aureus*, *Moraxella catarrhalis*, and *Neisseria meningitidis*.[12–14] Because the oral cavity and adenoids are structurally adjacent, the oral microbiota in pediatric OSA may be altered.

Recently, a pilot study revealed that the urine metabolomic profiles of children fluctuated with their tract infection status.[15] Thus, we suggest that such profiles might also be perturbed by changes in the oral microbiota of children. To explore the potential characteristic metabolite signatures associated with pediatric OSA, a non-targeted metabolomics technique was applied to discover potential urinary metabolites, and metagenomics technology was used to compare the metagenomics data of pediatric patients with OSA with those of healthy subjects to assess whether the metabolites might have originated from the oral cavity. Such an integrated analysis may provide a new way to determine interactions between host and oral microbes.

## METHODS

This study was approved by Shanghai Jiao Tong University Affiliated Sixth People's Hospital and Shanghai Jiao Tong University Affiliated Shanghai Children's Hospital ethics committee and was performed in accordance with the Declaration of Helsinki. Informed written consent was obtained from the legal guardian of each participant, and children older than 7 years old agreed to participate prior to their enrollment.

### Study Population

During the period from June 2014 to June 2015, children with habitual snoring and suspected OSA aged between 3 and 11 years (without specific diet preferences) were recruited from the Department of Otolaryngology, Head and Neck Surgery and the Center of Sleep Medicine, Shanghai Jiao Tong University Affiliated Sixth People's Hospital and from the Department of Otolaryngology, Head & Neck Surgery, Shanghai Jiao Tong University Affiliated Shanghai Children's Hospital. The normal control subjects, who had no medical history of any acute/chronic disorders and showed no clinical features of OSA (eg, intermittent sleep breathing pauses, snoring, and/or daytime sleepiness) according to their parents, were enrolled from schools or kindergartens at the same time in Jingan District, Shanghai. A detailed clinical questionnaire was completed by all parents. Height and weight were measured for each child, and body mass index (BMI) z-score was calculated according to the Centers for Disease Control and Prevention (CDC) 2000 growth standards (www.cdc.gov/growthcharts) using the Epi Info suite of software tools (www.cdc.gov/epiinfo). The following exclusion criteria were applied in both pediatric OSA and control subjects: systemic disease (ie, pulmonary, hepatic, renal, cardiovascular, gastrointestinal, or neurological disease), oral disease (ie, dental caries or periodontal disease), any treatment for adenoid hypertrophy (ie, tonsillectomy and adenoidectomy, corticosteroids, or leukotriene antagonists), genetic/craniofacial syndromes, parents with obvious snoring or diagnosed OSA, receipt of any medications—antibiotics or drugs used to regulate intestinal flora (ie, prebiotics, synbiotics, or probiotics)—during the previous month, and any active infections (ie, bacteria, fungi, or viruses). Subjects who had pets at home (a known source of bacteria) were also excluded.

### Overnight Polysomnography Monitoring

All suspected OSA subjects recruited from the two hospitals underwent standard nocturnal polysomnography (PSG) evaluations (Alice 5; Respironics, Murrysville, Pennsylvania, United States). Electrocardiography, electroencephalography, bilateral electrooculography, chin electromyography, oral/nasal airflow, nasal pressure, chest/abdominal movement, and pulse oximetry were recorded simultaneously while patients slept. The definitions of sleep variables followed the 2007 American Academy of Sleep Medicine guidelines. The apnea-hypopnea index (AHI) was calculated as the number of obstructive apnea events (defined as a > 90% decrease in signal amplitude for > 90% of the entire event) and hypopnea events (defined as a ≥ 50% fall in the amplitude and associated with arousal/awakening or ≥ 3% desaturation) per hour during sleep. The definition of OSA was as follows: the presence of AHI ≥ 1 events/h of total sleep time.[16]

### Urine Collection

Midstream urine samples were collected from all participants in the morning (7:00 AM). Urine samples (1 mL) were immediately stored in a centrifuge tube at 80°C until ultra-performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry (UPLC-Q-TOF-MS) and gas chromatography coupled with time-of-flight mass spectrometry (GC-TOF-MS) analyses.[6]

### Swab Sample Collection and Bacterial Genomic DNA Extraction

The oral cavity is not a uniform ecosystem but represents several fundamentally different niches with a high degree of microbial diversity. Therefore, one sampling site is not sufficient to gauge the diversity of the oral ecosystem. However, taking samples from all sites in the oral cavity is time consuming, laborious, and expensive. Previous studies in rodents showed that taking buccal swabs allows quick and noninvasive sampling for metagenomics analysis.[17] Therefore, we collected samples derived from the buccal mucosa of each participant before they had anything to eat or drink in the morning. Before sample collection, participants rinsed their mouths twice with water. Then, we firmly rubbed the buccal mucosa with disposable medical sterile swabs. Finally, the swab was clipped off and immersed in 1 mL of saline solution. These samples were immediately stored at −80°C until further use. The samples derived from the buccal mucosa were suspended and then pelleted by centrifugation (15,000×*g*, 10 minutes, 4°C). Bacterial genomic DNA was extracted using a QIAamp DNA Kit (QIAGEN, Hilden, Germany). The microbial genomic DNA was stored at 20°C before further analysis.

### Polymerase Chain Reaction Amplification of the 16S rRNA and Amplicon Sequencing

The V1–V3 regions of 16S rRNA were amplified by polymerase chain reaction from microbial genomic DNA using the following forward and reverse primers: 5′-AGAGTTTGATCCTGGCTCAG-3′ and 5′-TTACCGCGGCTGCTGGCAC-3′, respectively. After extraction and quantification of the polymerase chain reaction products, they were sequenced using a 454 Life Sciences Genome Sequencer FLX+ system (Roche, Basel, Switzerland) following the vendor's standard protocols.

### Bioinformatics and Statistical Analyses

Bioinformatics and statistical analyses were performed as described previously.[18] Quantitative Insights into Microbial Ecology (QIIME) software (version 1.8.0; http://qiime.org) was used to analyze all 454 FLX+ pyrosequencing data sets. All samples were rarefied to the same number of reads.

Briefly, raw sequences that showed exact matches to the barcode sequences were assigned to each sample and identified as valid sequences, the primers and barcodes of which were trimmed for further quality control. The low-quality sequences were filtered according to the following criteria: (1) sequence of length ≤ 150 bp; (2) sequences with average Phred scores ≤ 20; and (3) sequences with ambiguous bases and those containing mononucleotide repeats of > 8 bp.[19,20] Paired-end reads were assembled using fast length adjustment of short reads, or FLASH.[21]

After chimera detection, the remaining high-quality sequences were clustered into operational taxonomic units (OTUs) at 97% sequence identity by UCLUST.[22] A representative sequence was selected from each OTU using the default parameters. OTU taxonomic classification was performed by BLAST searching the representative sequence against the Greengenes Database (Release 13.8, http://greengenes.secondgenome.com/)[23] using the best hit.[24] An OTU table was further generated to record the abundance of each OTU in each sample, and the taxonomy of these OTUs. OTUs containing < 0.001% of total sequences across all samples were discarded. To minimize the differences in sequencing depth across samples, an averaged rarefied OTU table was generated by averaging 100 evenly resampled OTU subsets under the 90% minimum sequencing depth for further analysis.

The richness and evenness of species were assessed by a ranked abundance curve. Alpha diversity indexes, including Good coverage, Chao1, ACE, Shannon, Simpson, inverse Simpson, and Simpson evenness, were calculated at 97% identity.[25] β diversity was used to investigate the similarity between the bacterial community structures of the pediatric patients with OSA and the normal controls using unweighted UniFrac distances; the results were visualized using principal coordinate analysis (PCoA) and the unweighted pair–group method with arithmetic means (UPGMA) hierarchical clustering analysis.

Principal component analysis (PCA) was performed to evaluate similarities among various bacterial communities.[26] PCA is a method used to summarize the variance in a multivariate scatter of points in a low-dimensional space, and provides an overview of linear relationships between objects and variables. This can often act as a good starting point in multivariate data analysis highlighting trends, groupings, key variables, and potential outliers. Further, for data sets with many variables and relatively few objects, PCA can help collapse these variables into a few principal components that can then be used in further analyses.

A similarity (ANOSIM) test was performed to assess group-related sample aggregation.

Based on the occurrence (versus the relative abundance) of OTUs in a sample group, a Venn diagram was generated to show the shared and unique OTUs in the two groups. The taxa abundances at the phylum, class, order, family, genus, and species levels were analyzed between pediatric patients with OSA and controls. Furthermore, LEfSe (version 1.0) was used to detect different abundant genera in the two groups; the threshold on the linear discriminant analysis score was set to 3.0.

We performed partial least-squares discriminant analysis (PLS-DA) to identify key genera that could differentiate the oral microbiota in pediatric patients with OSA from that in normal control patients. Genera with variable importance in projection (VIP) > 1 and $P < .05$ on Student $t$ test in the PLS-DA model were selected. The correlation between the 50 most abundant genera was visualized in a heat map. Genera with rho > .6 and $P < .01$ were visualized in the network using Cytoscape software (version 3.4.0, Cytoscape Consortium, New York, New York, United States).

The Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) software (version 1.0.0) and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases were used to predict oral microbial functions.

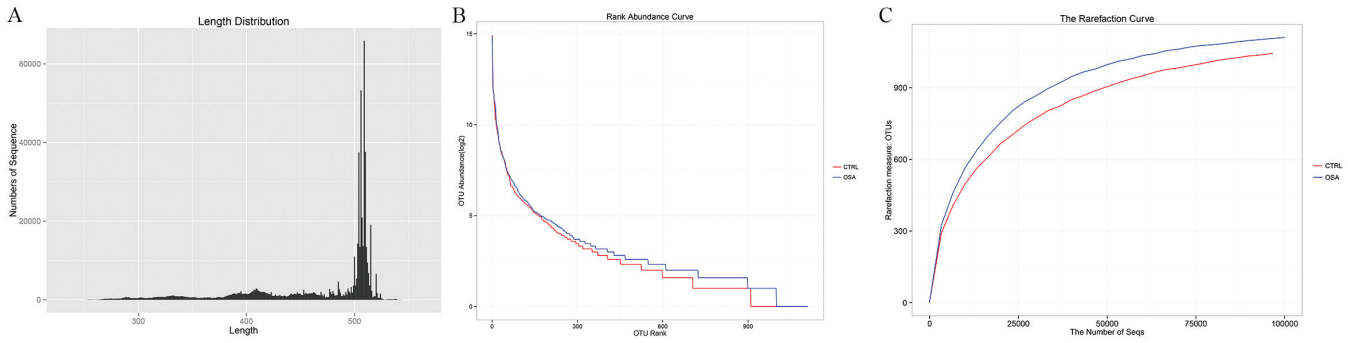
### Statistical Analyses of Metabolites

Urinary UPLC-Q-TOF-MS and GC-TOF-MS analyses were performed as described in our previous report.[6] Orthogonal partial least-squares discriminant analysis (OPLS-DA) was performed to visualize the metabolic differences between pediatric patients with OSA and normal controls.[27] Metabolites with VIP > 1 and $P < .05$ on Student $t$ test in the OPLS-DA model were selected. The fold change was the intensity mean value ratio of the two groups. All statistical analyses on metabolomics data were performed using SPSS (version 20.0; IBM Corp., Armonk, New York, United States) and SIMCA-P software (version 13.0; Umetrics, Umea, Sweden).

Co-inertia analysis (CIA) was performed to assess the consistency of the two sets of data (the 100 most abundant OTUs and 57 differential metabolites).[28] CIA is a multivariate method that identifies trends or co-relationships in multiple datasets containing the same samples; that is, the rows or columns of the matrix have to be weighted similarly and must therefore be "matchable." CIA simultaneously identifies ordinations (dimension reduction diagrams) from the datasets that are most similar by finding successive axes from the two datasets with maximum covariance. CIA can be applied to datasets where the number of variables (genes) far exceeds the number of samples (arrays), as is the case with microarray analyses.

We also used a heat map to visualize the oral microbiome differences between patients with OSA and

**Figure 1**




**(A)** Length distribution of sequences determined by 454 pyrosequencing. **(B)** Rank abundance curve of the bacterial OTUs derived from the two groups. **(C)** Rarefaction curve of the bacterial OTUs derived from the two groups. OTU = operational taxonomic unit.

**Table 1**—Demographic data of pediatric subjects stratified by OSA and matched normal control subjects.

| Characteristics | OSA | Control | *P* |
|---|---|---|---|
| No. of participants | 30 | 30 | – |
| Age (years) | 6 (5–8) | 6 (6–8) | .55 |
| Males, n (%) | 22 (73.3) | 23 (76.7) | .50 |
| BMI z-score | 1.7 ± 0.8 | 1.6 ± 0.3 | .43 |
| AHI (events/h) | 4.1 (1.9–7.3) | n.d. | – |
| Mean $SaO_2$ (%) | 96 (95–97) | n.d. | – |
| $LSpO_2$ (%) | 79.0 (74.8–86.3) | n.d. | – |
| MAI (events/h) | 4.2 (1.1–7.7) | n.d. | – |

Data are presented as mean ± standard deviation, median (interquartile range), or n (%). AHI = apnea-hypopnea index, $LSpO_2$ = lowest pulse oxygen saturation, MAI = microarousal index, n.d. = not determined, OSA = obstructive sleep apnea, $SaO_2$ = oxygen saturation.

control patients. The correlation matrix between metabolites and oral bacterial species was generated using Pearson correlation coefficient.

Currently, there are no general recommendations for sample size calculation in microbiome studies; it has been estimated that 20 participants in each group could detect differences in unweighted (effect size = 0.008) and weighted (effect size = 0.04) pairwise distances with 90% power.[29] Thus, our sample size (n = 30 each group) was appropriate for unweighted and weighted UniFrac analysis. For correlation analysis, using G*power software 3.1.9.2,[30] our sample of 60 participants was powered to detect an *r* of approximately .32 with an α error of 5% and statistical power of 80%. Thus, though our sample size is small, it is large enough to detect variability in microbiome of controls versus OSA.

## RESULTS

### Basic Characteristics

In total, data from 60 subjects (30 pediatric patients with OSA and 30 normal control subjects) were finally analyzed by an integrated approach that combined metagenomics and metabolomics methods. No differences in age, sex, or BMI-z score were observed between the groups. PSG monitoring data are also presented (**Table 1**).

### Decreased Bacterial Diversity of Oral Microbiota Associated With Pediatric OSA

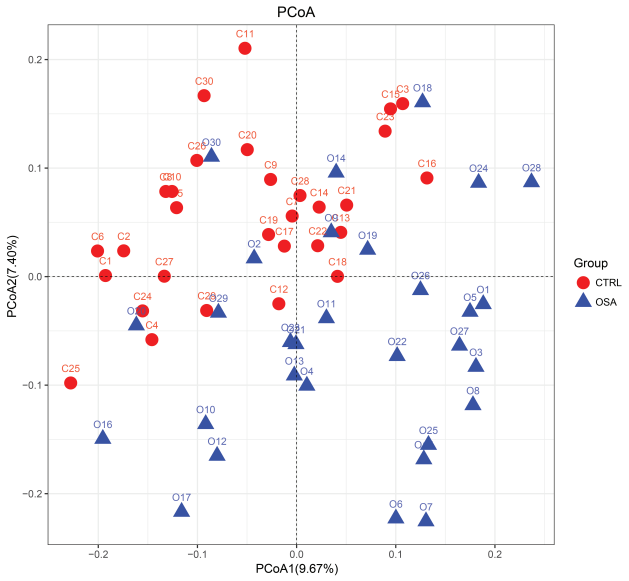
A total of 814,085 preliminary raw sequences, with a mean of 13,568 sequences per sample (range, 10,065 to 22,915), were obtained. After quality trimming, filtering, denoising, and chimera checking, a total of 603,756 high-quality reads finally remained, accounting for 74.16% of the valid reads; an average of 10,063 reads (range, 6,951 to 18,368) per sample were recovered for downstream analyses. The sequence lengths, which ranged from 232 to 563 bp, were distributed around 500–515 bp (**Figure 1A**). Clustering of all high-quality sequences at 97% identity resulted in 3,156 OTUs. After removing the low-credibility OTUs (which contributed only 0.7% of all sequences), 1,297 OTUs with an average of 260 OTUs per sample (range, 51 to 413) were identified.

The Shannon diversity index was slightly higher in patients with OSA than in controls (4.45 ± 0.54 versus 4.14 ± 0.58; $P = .04$); however, there were no significant differences between the groups in ACE (205.7 ± 46.57 versus 211.6 ± 48.11; $P = .63$), Chao1 richness index (166.3 ± 35.88 versus 179.0 ± 36.66; $P = .18$), Simpson diversity index (0.84 ± 0.06 versus 0.86 ± 0.06; $P = .34$), inverse Simpson diversity index (7.29 ± 2.54 versus 8.59 ± 3.86; $P = .13$), or Simpson evenness index (0.04 ± 0.02 versus 0.05 ± 0.02; $P = .43$). Based on the results of the OTU analysis, the rank-abundance curves for the bacterial communities of the pediatric OSA and normal control groups showed similar patterns (**Figure 1B**). The Good coverage values were high for all sequences in the two groups (> 97%), indicating that the sequencing depth was sufficient to explore the oral microbiota of pediatric OSA. Rarefaction analysis estimates showed that the species richness of the oral microbiota of pediatric OSA was slightly higher than that of the normal controls (**Figure 1C**).

### Bacterial Community Structure Analysis

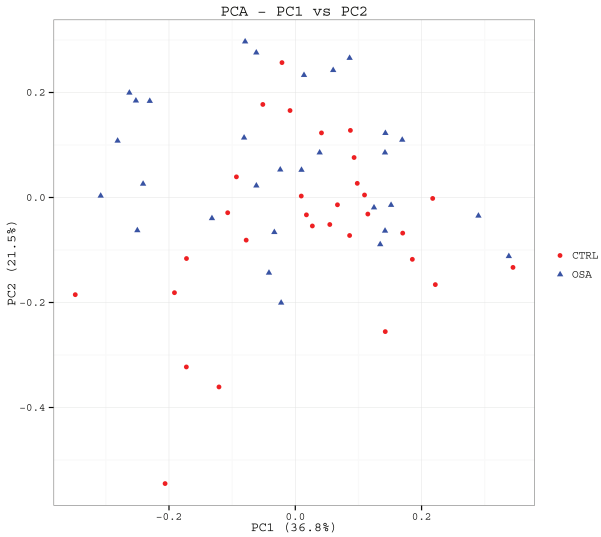
To view the similarities in oral bacterial community structures between patients with OSA and controls, we performed PCoA of β-diversity according to the unweighted UniFrac distances.

**Figure 2**—Principal coordinate analysis plot of the oral microbiota based on the results of the unweighted UniFrac metric.




The blue triangles represent subjects with obstructive sleep apnea and the red dots represent control subjects. Smaller distances between two points indicate greater similarity in microbial community structure between the two samples. CTRL = control, PCoA = principal coordinate analysis.

**Figure 3**—Principal component analysis at the genus level.




One dot represents one sample. The blue triangles represent subjects with obstructive sleep apnea (OSA) and the red dots represent control subjects. Smaller distances between two points indicate greater similarity in microbial community structure between the two samples. CTRL = control, PCA = principal component analysis.

The results revealed differences in the bacterial community structures between patients with OSA and controls (**Figure 2**). The results of PCA at the genus level showed slight segregation of the bacterial community structures of the two groups, with the first two principal components representing 36.8% and 21.5% of the total variation, respectively (**Figure 3**). ANOSIM test also revealed significant separation between patients with OSA and controls ($r$ = .2034, $P$ = .001). UPGMA hierarchical clustering analysis showed that the samples formed separated clusters that corresponded to the OSA and control groups (**Figure S1A** in the supplemental material).

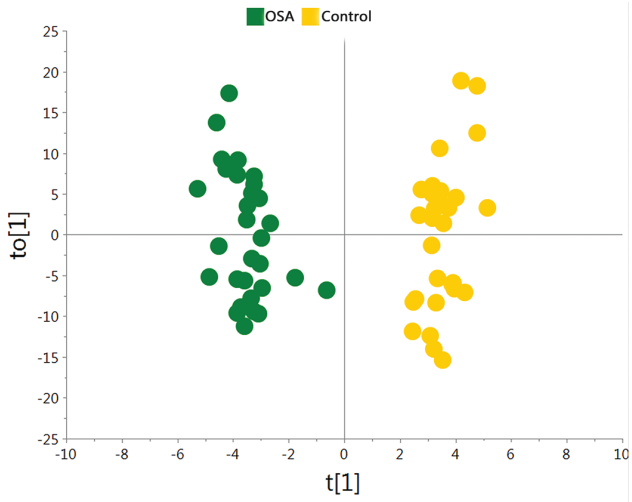
## Bacterial Abundance and Distribution

In total, 20 phyla, 40 classes, 73 orders, 125 families, 188 genera, and 228 species were found in the oral buccal mucosa samples. The taxonomic distributions of the predominant bacteria at the aforementioned six levels are presented in **Figure S2** in the supplemental material. The five most abundant phyla were *Firmicutes* (48.7%), *Proteobacteria* (26.9%), *Bacteroidetes* (17.2%), *Fusobacteria* (3.0%), and *Actinobacteria* (2.8%), which together accounted for 98.6% of the total sequences. The five rarest phyla were *Verrucomicrobia*, *Gemmatimonadetes*, *Chlorobi*, *Nitrospirae*, and *one candidate division* (*SR1*). The most prevalent genera were *Streptococcus* (36.3%), *Neisseria* (12.5%), *Haemophilus* (8.2%), *Porphyromonas* (5.6%), *Prevotella* (4.2%), *Veillonella* (3.7%), *Granulicatella* (3.6%), *Lautropia* (2.2%), *Leptotrichia* (1.4%), *Actinobacillus* (1.3%), *Capnocytophaga* (1.3%), and *Fusobacterium* (1.3%), which together comprised 81.8% of all sequences. The compositions of the entire microbial communities in terms of taxa are provided in **Figure S1B**. Four orders, *Lactobacillales*, *Bacteroidales*, *Neisseriales*, and *Pasteurellales*, were relatively abundant, and a higher abundance of *Bacteroidales* was observed in the OSA group; the other three were similarly abundant in the two groups. The significant differences were then assessed by LEfSe and PLS-DA analyses. A taxonomy tree could rapidly identify the dominant taxa from within the complex microbial data. The colored nodes represent the 20 most abundant taxa, including *Firmicutes* (*Streptococcus*), *Gammaproteobacteria* (*Haemophilus*), *Betaproteobacteria* (*Neisseria*), and *Bacteroidetes* (*Porphyromonas*) (**Figure S1C**). The heat map shows correlations between the participants and the 50 most abundant genera represented in the microbiota samples (**Figure S3A** in the supplemental material).

## Differing Microbiota Compositions

The community compositions differed between patients with OSA and normal control patients. A representation of the taxa that differed across groups was prepared using LEfSe (**Figure S3B** and **Figure S3C**). There were 12 different families, with enrichment of *Veillonellaceae*, *Campylobacteraceae*, *C111, and Paraprevotellaceae* in patients with OSA, and of *Thermaceae*, *Pseudomonadaceae*, *Nocardioidaceae*, *Gemellaceae*, *Comamonadaceae*, *Cardiobacteriaceae*, *Burkholderiaceae*, and *Alcaligenaceae* in controls. We also found significantly different microbial compositions at the genus level, with nine genera that differed significantly between the groups. *Thermus*, *Pseudomonas*, *Lautropia*, and *Achromobacter* showed higher abundances in the normal control group, whereas *Veillonella*, *Prevotella*, *Mogibacterium*, *Campylobacter*, and *Butyrivibrio* were more abundant

**Figure 4**—Score plots of the orthogonal partial least-squares discriminant analysis model for obstructive sleep apnea and normal control groups.




Green represents obstructive sleep apnea (OSA), yellow represents normal control groups. The model parameters were: $R^2X$ = 0.433, $R^2Y$ = 0.954, $Q^2Y$ = 0.665.

in the patients with OSA (linear discriminant analysis > 3, $P < .05$). A PLS-DA model was also developed to identify genera that were differentially distributed between OSA and normal control groups (**Figure S3D**). In total, 29 genera (VIP > 1) were identified as key genera. Of these, 13 genera were enriched in the control group: *Thermus*, *Lautropia*, *Eikenella*, *Flavobacterium*, *Cardiobacterium*, *Ochrobactrum*, *Kingella*, *Microbacterium*, *Pseudomonas*, *Sphingobium*, *Prosthecobacter*, *Sphingomonas*, and *Allobaculum*. Another 16 genera were more abundant in patients with OSA. Furthermore, three genera were identified with VIP > 2: *Prevotella*, *Thermus*, and *Campylobacter*. We identified 1,160 and 1,112 OTUs in patients with OSA and control patients, respectively. As shown in **Figure S4A** in the supplemental material, 975 OTUs, representing 75.2% of all OTUs (1,297 OTUs) and 99.3% of all OTU abundance, were shared between the two groups. The Venn diagram revealed OTUs that were unique to each group; specifically, 185 and 137 unique OTUs were found in patients with OSA and control patients, respectively. These unique OTUs were low in abundance, containing 7 to 78 sequences. The 185 unique OTUs in the OSA group belonged to 8 species: *debontii*, *dispar*, *firmus*, *melaninogenica*, *parainfluenzae*, *boronitolerans*, *mizutaii*, and *cinerea*.

### Co-Occurrence Network Analysis

We used co-occurrence network analysis to determine the interrelationships of oral microbiota at the genus level. Seven genera showed positive associations (*Thermus-Acinetobacter* (rho = .66), *Acinetobacter-Flavobacterium* (rho = .70), *Flavobacterium-Ochrobactrum* (rho = .70), *Bulleidia-Prevotella* (rho = .61), and *Prevotella-Campylobacter* (rho = .72) (rho > .6 and $P < .01$). The network diagram is presented in **Figure S4B**.

### Functional Predictions

We performed PICRUSt analysis to predict the bacterial functions of the oral microbiota community in pediatric OSA based on 16S rRNA sequencing data. In total, we predicted 39 metabolic functions based on all samples with the most enrichment: membrane transport (12.4%), replication and repair (9.9%), carbohydrate metabolism (9.6%), amino acid metabolism (9.1%), translation (6.8%), and energy metabolism (5.3%). Some other metabolic functions (nucleotide metabolism, metabolism of cofactors and vitamins, glycan biosynthesis and metabolism, folding/sorting and degradation, genetic information processing, and cellular processes and signaling; **Figure S4C**) were also identified.

### Metabolic Profiling of Pediatric Patients With OSA Versus Controls

An untargeted metabolomics analysis was performed on urine samples from 30 pediatric patients with OSA and paired normal controls. The OPLS-DA model demonstrated a clear separation between pediatric patients with OSA and normal controls (**Figure 4**). In total, 57 metabolites that distinguished pediatric patients with OSA from controls were identified (**Table S1** in the supplemental material, VIP > 1 and $P < .05$). Of these, 17, 15, 5, 3, 2, 3, 3, 3, and 1 metabolites were classified as amino acid metabolism, carbohydrate metabolism, microbial metabolism, vitamin metabolism, ornithine cycle, nucleic acid metabolism, fatty acid metabolism, butanoate metabolism, and bilirubin metabolism, respectively. The other five metabolites were intermediate in other metabolic pathways (**Table S1**).

### Correlation Between the Oral Microbiome and Urinary Metabolites

We first performed a CIA to assess the consistency of the data from the oral microbiome and urinary metabolomic profiling; the results showed that the similarity between the two datasets was low although significant (RV = 0.24, $P = .004$; **Figure 5**). Then, the relationship between oral microbiome (the top 50 abundance levels of genera) changes and urinary metabolite (the 57 differential metabolites) perturbations in pediatric OSA was explored by correlation analysis. It was found that several specific metabolites identified by the metabolomics approach were correlated with the typical oral bacteria identified by 16S rRNA diversity analysis, which indicated that a functional correlation between the oral microbiome and associated metabolites might exist (**Figure S4D**).

## DISCUSSION

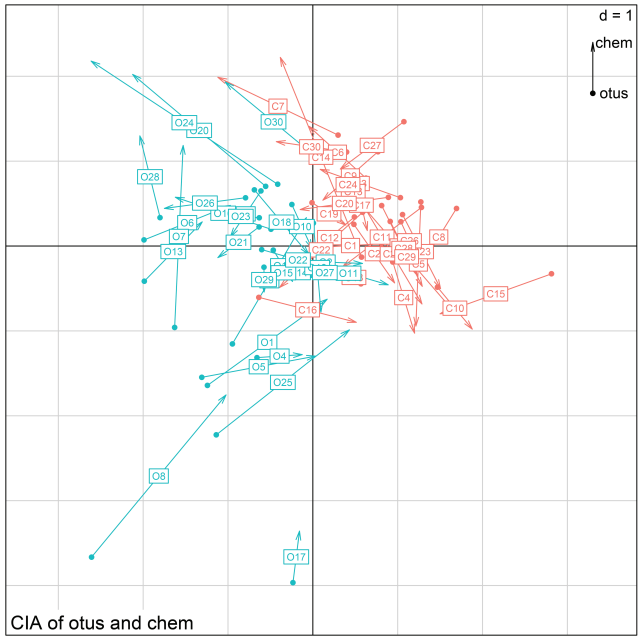
This is the first report regarding the use of metagenomics and metabolomics approaches to identify perturbations in the oral microbiota and altered urinary metabolites in pediatric OSA. Our study showed that oral microbiome compositions were different between pediatric OSA and controls, but our observations could not reveal causal relations. In addition, these perturbed oral microbiota were associated with changes in several metabolomic profiles, indicating that OSA not only disturbed the oral microbiota at the abundance level but also

substantially altered the metabolomic profile related to the oral microbiome, resulting in disturbances in host metabolite homeostasis. These results might provide mechanistic insights regarding perturbations of the oral microbiome as a new mechanism of OSA-induced metabolic disorders.

Intermittent hypoxia and sleep fragmentation, two important characteristics of OSA, can alter the microbial community structure in mice.[31,32] Moreover, altered gut dysbiosis has been correlated with OSA-related hypertension, systemic/adipose tissue inflammation, and insulin resistance.[32,33] Given that the oral cavity and intestine share the same digestive tract, we suggest that OSA could markedly alter the gut microbiome and lead to changes in the composition of the oral microbiota. Although several studies have shown intestinal flora disturbances in OSA, our clinical study is the first to reveal that the oral microbiome is also perturbed in pediatric OSA, as evidenced by five predominant phyla (*Firmicutes*, *Proteobacteria*, *Bacteroidetes*, *Fusobacteria*, and *Actinobacteria*), constituting 98.6% of the total oral microbiota. The dominant phyla in our study were highly similar to those described previously in pediatric dental caries.[34] Previous rodent studies identified a higher amount of *Firmicutes* and lower amounts of *Bacteroidetes* and *Proteobacteria* phyla under intermittent hypoxia conditions.[31] In humans, an increased intestinal oxygen level could affect the composition of fecal and mucosal adherent microbiota (eg, *Proteobacteria* and *Actinobacteria*, *phyla*).[35] All of these studies indicated that changing oxygen concentrations can affect the microbiota. However, it is still unclear whether changes in the oral bacterial profile are a cause or a consequence of pediatric OSA or vice versa. During OSA episodes, air often travels through different airway channels, so the exposure of oral microbiota to oxygen may be markedly different in OSA than in healthy children. Because oxygen is a major parameter shaping the bacterial community, even in otherwise stable environments,[36] oxygen in the air and not in the blood may be more important for microbes on the buccal mucosa.

To date, only three reported studies have applied metabolomics methods to adult OSA,[6,37,38] and two of them were aimed at exploring differences in metabolomic profiles.[6,37] Ferrarini et al. first identified 14 statistically significant metabolites (platelet-activating factor, lysophospholipids, bile pigments, and pipecolic acid) in severe OSA versus nonsevere OSA.[37] Our previous metabolomics study with more detailed demographic data and a larger sample size revealed 24 metabolites that were consistently higher or lower in normal controls, simple snorers, and subjects with OSA.[6] In this study, the results of the metabolomic profiling in pediatric patients with OSA were somewhat different from those in adult patients with OSA. In addition to the metabolites associated with amino acid metabolism, carbohydrate metabolism, microbial metabolism, nucleic acid metabolism, and fatty acid metabolism, which were also elevated in adult OSA, metabolites associated with vitamin metabolism, ornithine cycle, butanoate metabolism, and bilirubin metabolism were uniquely elevated in pediatric patients with OSA. A potential explanation is that the pathophysiological process differs in pediatric and adult OSA. The major etiology of adult OSA has been described as decreased upper airway muscle relaxation, and, because there is typically a long time between

**Figure 5**—Co-inertia analysis to assess the consistency of oral microbiome and urinary metabolomic profile data.




One dot and one line with an arrow represent one sample. The blue dots and blue lines with arrows represent subjects with obstructive sleep apnea and the red dots and red lines with arrows represent control subjects. The dots represent oral microbiota compositions, while the arrows indicate metabolomic data. Shorter lengths of the lines with arrows indicate greater consistency of the two datasets. CIA = co-inertia analysis, OTUs = operational taxonomic units.

the occurrence and the diagnosis of this condition, these three major nutrients are commonly altered. Adenoid hypertrophy is an important issue in pediatric OSA, and many metabolic pathways will also be involved.[39] Generally, metabolomics is a relatively new analytical method and is still at a descriptive stage in human diseases. Large-scale multicenter studies with both adult and pediatric patients with OSA are needed to further address these differences.

Overall, the two sets of data (metabolomics and microbiome) were highly correlated in the CIA analysis. CIA was reported to be a useful method for identification of the relationships between large datasets.[28] Indeed, the relationship between oral dysbiosis and systemic diseases has been well documented. Several clinical studies have shown that decreased diversity of oral microbiota exists in various diseases, such as esophageal squamous cell carcinoma,[40] pancreatic cancer,[41] and rheumatoid arthritis.[42] Moreover, metabolomic analyses showed metabolic disturbances in these diseases.[43–45] Similarly, we suggest that oral dysbiosis and metabolic disorders coexist in OSA. However, the relationship between urinary metabolites and oral microbiota is purely correlative without further controlled experiments. It is very likely that other effects of OSA beyond the oral microbiota are likely to influence the urinary metabolites; large-sample, prospective studies should be performed to address any causal relationships.

Several limitations of this study should be acknowledged. First, this was a pilot study and characterized the microbiota

in only a particular oral site in the enrolled subjects. Given that the oral cavity is a miniature microbial community that comprises more than 700 species or phylotypes and given that the compositions of the oral microbiome vary at different oral sites, analysis of the microbiome from only one site cannot represent the entire oral flora. Therefore, samples (including saliva samples) should be collected from multiple oral sites in further studies. Second, the gut microbiota in these subjects were not investigated simultaneously. Although previous studies showed that gut microbiota were disturbed by swallowing bacteria, a direct linkage between compositions of oral and gut microbiota was not established in this study. Third, because it is difficult to persuade children and their legal caregivers to undergo PSG monitoring in the absence of any sleep disturbances, we defined the "normal" control subjects via questionnaires rather than with standard PSG. Fourth, we preliminarily demonstrated that the altered oral microbiome was weakly associated with urinary metabolomic profiles in CIA; however, the observed correlation does not necessarily indicate a causal link. In addition, the cross-talk between the microbes and the host is complex. The underlying mechanisms of these perturbations should be examined in further clinical or rodent studies. Fifth, the OSA symptoms may also alter eating habits[46] and thus change the microbiota composition and metabolomic profile. However, it is difficult to quantify the amounts and species of food in the diet; this is also a common question in omics studies. Sixth, genetic predisposition may also be important and should not be ignored. Seventh, a direct link between levels of metabolites from the oral cavity and oral microbiota was not established in our study and should be examined in further studies. Eighth, there exists compositional and functional oscillation of the microbiota during the 24-hour light-dark cycle.[47,48] Though we have taken the factor into consideration during sample collection, we did not check the circadian variation in our study. Finally, the sample size of this study was relative small; multicenter studies with larger samples are needed to confirm our conclusions.

In conclusion, in this study, we combined 16S rRNA sequencing and metabolomics analyses to assess the oral microbiome and metabolic profiles in pediatric OSA. The sequencing revealed that OSA might be associated with oral dysbiosis and various metabolites involved in diverse metabolic pathways. In addition, a correlation analysis showed that some oral bacteria families were strongly correlated with the altered urinary metabolite profiles. These alterations might suggest a perturbed metabolic status and functional alterations in the oral microbiota in pediatric OSA, supporting the hypothesis that changes in the oral microbiome may be a new mechanism that leads to or exacerbates OSA-related metabolic disorders.

## ABBREVIATIONS

AHI, apnea-hypopnea index
CIA, co-inertia
GC-TOF-MS, gas chromatography coupled with time-of-flight mass spectrometry
KEGG, Kyoto Encyclopedia of Genes and Genomes
OPLS-DA, orthogonal partial least-squares discriminant analysis
OSA, obstructive sleep apnea
OTU, operational taxonomic unit
PCA, principal component analysis
PCoA, principal coordinate analysis
PICRUSt, Phylogenetic Investigation of Communities by Reconstruction of Unobserved States
PLS-DA, partial least-squares discriminant analysis
PSG, polysomnography
QIIME, quantitative insights into microbial ecology
UPLC-Q-TOF-MS, quadrupole time-of-flight mass spectrometry
UPGMA, unweighted pair-group method with arithmetic means

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## DISCLOSURE STATEMENT

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