Changes in the Upper Airway Microbiota in Pediatric Obstructive Sleep Apnea

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Abstract

Background: A few clinical studies have demonstrated that obstructive sleep apnea (OSA) is associated with dysbiosis of the oral and nasal microbiota. However, how upper airway microbial diversity, composition, and structure are altered in pediatric OSA has not been systemically explored.

Methods: In total, 30 polysomnography (PSG)-confirmed OSA patients with adenoid hypertrophy, and 30 controls who did not snore or adenoid hypertrophy, were enrolled. Swabs from four surface oral tissue sites (tongue base, soft palate, both palatine tonsils, and the adenoids) and one nasal swab from both anterior nares were collected. The 16S ribosomal RNA (rRNA) V3–V4 region was sequenced to identify the microbial communities.

Results: Alpha and beta diversity were not significantly different between the pediatric OSA patients and controls at the five upper airway sites. However, the microbial profiles were significantly different among the five upper airway sites. The abundances of *Haemophilus*, *Fusobacterium*, and *Porphyromonas* were higher in the adenoids and tonsils of pediatric patients with OSA. Functional analysis revealed that the differential pathway between the pediatric OSA patients and controls involved amino acid metabolism and signal transduction.

Conclusions: In this study, the upper airway microbiome of pediatric OSA patients only exhibited minor differences in composition compared with the controls. However, the microbiota data could be useful as a reference for studies on the upper airway microbiome or other relevant clinical phenomena.

Background

Obstructive sleep apnea (OSA) is a highly prevalent sleep disorder (affecting about 1–4% of children worldwide that can cause cardiovascular and metabolic problems in later life (1). Pathogenic adenoid hypertrophy and enlarged tonsils are the causes of airway obstruction, which can cause OSA in children. Hyperplasia of the tonsils and adenoids is correlated with the infectious diseases that can accompany OSA, such as acute otitis media (AOM) and secretory otitis media (SOM).

Many clinical studies have suggested that the adenoids and tonsils are pathogenic reservoirs, where 16S rRNA gene amplicon sequencing has revealed that the bacterial communities are complex, diverse, and highly variable. For example, *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Moraxella catarrhalis* have been detected in the adenoids, but not the palatine tonsils, of AOM and SOM patients (2). The abundances of *Haemophilus*, *Streptococcus*, *Neisseria*, *Capnocytophaga*, *Kingella*, *Moraxella*, and *Lachnospiraceae* are higher in cases of tonsillar hypertrophy, while the abundances of *Parvimonas*, *Bacteroidales* [G-2], *Aggregatibacter*, and *Atopobium* are higher in patients with chronic tonsillitis (3). *S. pneumoniae* significantly affects the composition and diversity of the adenoid microbiota, and *Acidobacteria* is related to chronic tonsillitis in non-healthy-weight patients with OSA (4). However, the findings are inconsistent. The upper airway cavity (i.e., oral and nasal cavity) is not a uniform ecosystem, instead comprising several fundamentally different niches with a high degree of microbial diversity. Thus,

microbial communities in the mouth and nasal cavity may be differentially affected by OSA via intermittent hypoxia or other mechanisms. Considering the limited data on the diversity and abundance of the upper airway microbiota in pediatric OSA patients, we characterized the microbiota at four oral sites by nasal swab sampling, and compared them between pediatric OSA patients and controls.

Materials And Methods

Study design and population

We performed a cross-sectional study on patients with polysomnography (PSG)-confirmed pediatric OSA and controls who did not snore and were recruited from Shanghai Jiaotong University Affiliated Sixth People's Hospital and Shanghai Children's Hospital. The research protocols were independently approved by the ethics committees of the two aforementioned tertiary hospitals (approval nos. 2018-073 and 2019 R061-F01, respectively). Informed consent was obtained from participants aged ≥ 7 years, and from the legal guardians of those aged < 7 years. All pediatric OSA patients, but no controls, had adenoid hypertrophy. Weight and height were carefully measured using an electronic balance and plastic tape measure, while the participants were wearing light clothes and no shoes. Body mass index (BMI) was calculated as weight/height^2 (kg/m^2). The inclusion criteria were as follows: aged 4–12 years; obstructive apnea hypopnea index (OAHI) > 1 event/h; no obvious dietary preferences; agreed to participate in the study; and willingness of parents to complete the questionnaire. The controls were children with congenital diseases who underwent general anesthesia in the hospital (e.g., for ear reconstruction, accessory ear, a cervical mass, or preauricular fistula without infection). The exclusion criteria included a special diet (gluten-free, casein diet, or specific carbohydrate diet); systemic disease (pulmonary, hepatic, renal, cardiovascular, gastrointestinal, or neurological disease), oral disease (dental caries or periodontal disease), or treatment for adenoid hypertrophy (tonsillectomy, adenoidectomy, corticosteroids, leukotriene antagonists); genetic/craniofacial syndromes, parents with obvious snoring or diagnosed with pediatric OSA; use of any medications, antibiotics or drugs to regulate the intestinal flora (prebiotics, synbiotics, or probiotics) during the previous month, or active infections (bacteria, fungi, or viruses); and pets in the home (a known source of bacteria). Ultimately, 30 pediatric OSA patients and 30 normal controls were enrolled.

Definitions of pediatric OSA

All patients with pediatric OSA were monitored by overnight standard PSG (Alice 5; Respiration, Murrysville, PA, USA) at our sleep center. In detail, during sleep from 10 pm to 6 am, electroencephalogram, electrooculogram, genioglossus electromyogram, thoracic/abdominal movement, leg movement, and percutaneous oxygen saturation (at the fingertip) were recorded by sensors. A well-trained technician manually scored the polygraphic data (OAHI, rapid eye movement, non-rapid eye movement, oxygen desaturation index, average and minimum SpO_2 , and microarousal index) according to the 2012 guidelines of the American Academy of Sleep Medicine (5). OSA severity was categorized as mild (OAHI 1–5 events/h), moderate (OAHI 5–10 events/h), or severe (OAHI ≥ 10 events/h)(6).

Biospecimen collection and DNA extraction

We collected four swabs from the surfaces of oral tissue sites [tongue base, soft palate, both palatine tonsils, and the adenoids (nasopharynx site)], and one nasal swab from each anterior nare (Figure 1a). The biospecimen collection procedure followed the Human Microbiome Project (HMP) (http://hmpdacc.org/doc/HMP_MOP_Version12_0_072910.pdf), as described previously (7). Bacterial genomic DNA was extracted using a DNA HDQ 96 Kit (M6399-01; Omega, Inc., Norcross, GA, USA) according to the manufacturer's instructions.

16S rRNA gene sequence analysis

The V3–V4 regions of the 16S ribosomal RNA (rRNA) gene were amplified from the microbial genomic DNA by polymerase chain reaction (PCR). The barcoded forward primer was 341F (5'-CCTACGGGNGGCWGCAG-3') and the reverse primer was 805R (5'-GACTACHVGGGTATCTAATCC-3'). The amplicons were purified using a QIAquick PCR Purification Kit (Qiagen, Hilden, Germany). PCR amplicons were sequenced to generate 250 bp paired-end reads using the Illumina NovaSeq platform according to the manufacturer's recommendations (LC-Bio Technology Co., Ltd., Hangzhou, China).

Bioinformatics and statistical analyses

The raw 16S rRNA gene amplicon sequences were processed and analyzed using QIIME (8). First, the sequences were demultiplexed based on the barcodes assigned to each sample. Then, the demultiplexed pair-end sequences from each sample were quality-controlled (stitched, filtered, trimmed, and de-noised, with ambiguous/chimeric sequences being removed) using DADA2 in QIIME2 and clustered to generate an amplicon sequence variant table (9). Alpha diversity analysis of all samples was carried out using the Chao1 and Shannon diversity indices. Beta diversity was investigated through nonmetric multidimensional scaling (NMDS) analysis according to Bray-Curtis distance matrices. The bacterial composition (unweighted UniFrac distance) in the microbiome community was examined using the Mantel test. Differences in the relative abundance of taxa were identified based on the linear discriminant analysis effect size (LEfSe) (10). Regarding potential functional implications, Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) was used to predict microbial metabolic pathways (11).

It was estimated that 20 subjects per group would be necessary to detect differences in unweighted pairwise distances with 90% power (12). Therefore, our study (30 subjects/group) had sufficient power to detect differences in taxa between pediatric OSA patients and control subjects.

Statistical analyses were performed using the SPSS (version 22.0; IBM Corp., Armonk, NY, USA) and R studio (R Foundation for Statistical Computing, Vienna, Austria) software packages. Categorical variables are presented as numbers and percentages, and continuous variables as medians with interquartile range. The pediatric OSA and controls groups were compared using the Mann-Whitney *U* test or chi-square test, as appropriate. A two-sided *p*-value < 0.05 was considered significant.

Results

Patient demographics

In total, 30 pediatric OSA patients and 30 control subjects were included in our study. No significant differences in age, gender, or BMI were observed between the two groups (all $p > 0.05$, Table 1). The detailed clinical and PSG data of the pediatric OSA patients are presented in Table 1. In total, 298 swab samples (60 from the tongue base, 60 from the soft palate, 60 from the palatine tonsils, 58 from the adenoids, and 60 from the anterior nares) were eligible for 16s RNA analysis.

Table 1
Basic characteristics of the overall population

Variable	Case (n = 30)	Control (n = 30)	P value
Demographics			
Age, years	7(5–8)	6(3–10)	0.395
Male (%)	16(53.3%)	16(53.3%)	0.999
Height, m	1.27(1.15–1.36)	1.18(1.05–1.44)	0.544
Weight, Kg	22.8(18.4–31.3)	22.6(17.8–36.1)	0.923
BMI, Kg/m ²	15.21(14.00–17.03)	16.58(14.66–17.95)	0.102
Sleep apnea			
OAHI	5.8(3–10.1)	-	-
OAHI _{REM}	7.3(3.4–15.9)	-	-
OAHI _{NREM}	4.1(2.4–10.1)	-	-
Average SaO ₂	97(94.8–98)	-	-
Minimum SaO ₂	87.5(84–91)	-	-
ODI	3.0(1.9–5.8)	-	-
MAI	11.8(7.9–28.6)	-	-
The data are presented as means and standard deviation; skewed data are presented as the median (IQR), and categorical data as the number (percentage). Differences in the baseline characteristics among the two groups were examined using Mann-Whitney <i>U</i> or Chi-square test as appropriate.			
Abbreviations: BMI, body mass index; OAHI, obstructive apnea-hypopnea index; OAHI _{REM} , rapid eye movement obstructive apnea-hypopnea index; OAHI _{NREM} , non-rapid eye movement obstructive apnea-hypopnea index; SaO ₂ , oxygen saturation; ODI, oxygen desaturation index; MAI, micro-arousal index.			

Differential upper airway microbiota diversity was observed between the pediatric OSA patients and controls

A total of 24,912,788 raw sequences were acquired. After sequence processing, 21,100,111 high-quality 16S rRNA gene sequences (accounting for 84.7% of the raw sequences) were identified, with a median read count of 71,381 (range: 48,649–84,926) per sample. A total of 57,438 feature tables were generated from these samples (13–437 tables per sample). The Wilcoxon rank-sum test detected no differences in feature tables between the pediatric OSA and control groups at the various upper airway sites ($p = 0.48$ for the adenoids; $p = 0.07$ for nares; $p = 0.25$ for the palate; $p = 0.93$ for the tongue; $p = 0.11$ for the tonsils) (Fig. 1b).

The Chao1 and Shannon diversity indices were not significantly different between the pediatric OSA and control groups at the various upper airway sites (Chao1 index: $p = 0.50$ for the adenoids; $p = 0.06$ for nares; $p = 0.27$ for the palate; $p = 0.99$ for the tongue, $p = 0.12$ for the tonsils; Shannon diversity index: $p = 0.68$ for the adenoids; $p = 0.21$ for nares; $p = 0.72$ for the palate; $p = 0.49$ for the tongue, $p = 0.34$ for the tonsils) (Fig. 1c and 1d). Overall, the microbiota alpha-diversity values were similar, indicating comparable alpha diversity of the microbiomes at these upper airway sites between the pediatric OSA and control groups.

Microbiota beta-diversity was compared between the pediatric OSA and control groups at the various upper airway sites by principal coordinate analysis (PCoA) and NMDS analysis. The latter analysis did not indicate significant dissimilarity ($P_{\text{Stress}} = 0.11$) in the microbial communities of the different upper airway sites (Fig. 1e). On the PCoA plot, the microbial communities of the different upper airway sites did not clearly differ between the groups (Fig. 1f). However, the pathogenic bacteria from the five upper airway sites were different between the groups (Fig. 1f).

Bacterial composition identified from the different upper airway sites between pediatric OSA patients and controls

In Fig. 2a-d, the average composition of bacterial communities at the phylum, family, genus, and species levels were presented. At phylum level, *Firmicutes*, *Proteobacteria*, *Bacteroidetes*, *Actinobacteria*, and *Fusobacteria* were significantly different in groups of different upper airway sites (Fig. 2a). At family level, *Streptococcaceae*, *Prevotellaceae*, *Pasteurellaceae*, *Neisseriaceae*, and *Moraxellaceae* were significantly different in these groups (Fig. 2b). At genus level, *Streptococcus*, *Haemophilus*, *Neisseria*, *Prevotella_7*, and *Moraxella* were significantly different in these groups (Fig. 2c). At species level, *Streptococcus_sp.*, *Dolosigranulum_sp.*, *Haemophilus_sp.* and *Moraxella_sp.* were significantly different in these groups (Fig. 2d).

Differences in taxa between the pediatric OSA and control groups at the various upper airway sites

We utilized LEfSe analysis to compare the microbiota between the pediatric OSA and control groups at the various upper airway sites (Fig. 3a–e). We identified important taxonomic differences between the

pediatric OSA and control groups based on a logarithmic linear discriminant analysis score ($\log_{10}$) > 2.0. The LEfSe results suggested remarkable differences in upper airway microbiota between the pediatric OSA and control groups. We were particularly interested in differences in taxa at the genus level. In adenoid, the relative abundances of the genera *Haemophilus*, *Fusobacterium*, *Porphyromonas*, *Prevotella*, *Treponema*, *Agathobacter*, *Parvimonas*, *Campylobacter*, and *Faecalibacterium* were higher in the pediatric OSA than control group, whereas the relative abundances of genera *Actinobacillus*, *Burkholderiales*, *Ruminococcaceae_UCG_005*, *Eikenella*, and *Romboutsia* were higher in the controls than pediatric OSA (Fig. 3a). In nares, only the relative abundances of the genera *Haemophilus*, *Porphyromonas*, and *Capnocytophaga* were higher in the pediatric OSA than control group (Fig. 3b). In palate, the relative abundances of the genera *Haemophilus*, *Actinobacillus*, *Porphyromonas*, *Fusobacterium*, *Prevotella*, *Streptobacillus*, and *Campylobacter* were higher in the pediatric OSA than control group, whereas the relative abundances of genera *Lachnoanaerobaculum*, *Abiotrophia*, *Trichococcus*, *Rothia*, and *Actinomyces* were higher in the controls than pediatric OSA (Fig. 3c). In tongue, the relative abundances of the genera *Porphyromonas*, *Fusobacterium*, *Haemophilus*, *Capnocytophaga* and *Prevotella* were higher in the pediatric OSA than control group, whereas the relative abundances of genera *Abiotrophia*, *Trichococcus*, *Lautropia*, *Alloprevotella*, and *Streptococcus* were higher in the controls than pediatric OSA (Fig. 3d). In tonsils, the relative abundances of the genera *Fusobacterium*, *Haemophilus*, *Porphyromonas*, *Moraxella*, *Neisseria*, *Aggregatibacter*, *Treponema*, *Prevotella*, *Parvimonas*, *Streptobacillus*, *Campylobacter*, and *Collinsella* were higher in the pediatric OSA than control group, whereas only the relative abundance of genera *Trichococcus* was higher in the controls than pediatric OSA (Fig. 3e).

Functional prediction of microbiota at the different upper airway sites in the pediatric OSA and control groups

PICRUSt analyses were performed to explore functional and metabolic variations in the upper airway microbial communities, and the results are presented in Fig. 4a–e. The PICRUSt analysis identified 20 Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways with significant differential abundance between pediatric OSA and controls in adenoid (Fig. 4a), 10 KEGG categories in nares (Fig. 4b), 20 KEGG categories in palate (Fig. 4c), 20 KEGG categories in tongue (Fig. 4d), and 20 KEGG categories in tonsils (Fig. 4e), respectively. Similar patterns of differentially enriched genes functioning in amino acid biosynthesis, metabolism, and signal transduction were detected at the upper airway sites in both groups. The enriched pathways were related to human pathogenesis, as expected.

Discussion

This is the first study to provide an overview of the differences in upper airway microbiota between pediatric OSA patients and controls. We observed minor microbial differences between pediatric OSA patients and controls at the various upper airway sites using microbiome 16S rRNA gene sequencing. We also investigated the bacterial functions. Although our sample size provided sufficient power, we found no associations between the microbial features and OSA at any upper airway site. Only the microbial

profiles differed significantly among the five oral and nasal sites. These results suggest that the microbial communities in the upper airway might be resistant or resilient to disturbances caused by OSA.

Many studies have focused on the effects of environmental factors on the oral and nasal microbiota, such as cigarette smoking and alcohol consumption (7, 13, 14). Exposure to these factors could lead to a higher abundance of opportunistic pathogens in the upper respiratory tract (7, 13, 14). Sleep can also affect microbial abundance in the pediatric oral cavity (15). OSA is a common chronic sleep disorder characterized by intermittent hypoxia during sleep. The abundance of oral microbiota changes during the anoxia and reoxygenation cycle. Microbiome profiling has played an integral role in understanding the development and exacerbation of OSA (16). In our previous study, we reported that the microbiota (*Firmicutes*, *Proteobacteria*, *Bacteroidetes*, *Fusobacteria*, and *Actinobacteria*) on the buccal mucosa in pediatric OSA patients was altered (17). However, in this study, no associations between microbial features and OSA were observed at any of the oral sites. This is similar to cigarette smoking, which only affects the oral microbiota in the buccal mucosa (7). One study showed that the salivary microbial composition was stable after antibiotic use at various follow-up timepoints (18). Thus, oral microbial organisms may be tolerant to many adverse conditions (19). This could partially explain why the microbial communities in the upper airway are resistant or resilient to disturbances such as intermittent hypoxia.

Data on the effect of OSA on nasal or nasopharynx microbiota are sparse, and the results are inconsistent. One study found that the nasal microbiome of adult patients with severe OSA was enriched with *Streptococcus*, *Prevotella*, and *Veillonella* (20). Another study reported no difference in the composition of the nasopharynx microbiome between mild-to-moderate adult OSA patients and those without OSA (21). No previous study has explored the composition of the nasal microbiome in pediatric OSA patients, while one study found no significant difference in nasopharynx or nasal cavity composition or diversity between patients with chronic rhinosinusitis and controls (22). The adenoids and tonsils are lymphoid tissues; hypertrophy thereof plays an important role in the onset and development of pediatric OSA, but few studies have explored the adenoid or tonsil microbiome in OSA to determine whether it participates in hypertrophy. Higher abundances of the genera *Haemophilus*, *Fusobacterium*, and *Porphyromonas* were found in the adenoids and tonsils of our pediatric OSA group. *Haemophilus* is the main pathogenic biofilm bacteria constituting the adenoid reservoir (23, 24). *Fusobacteria* were detected at a tonsillar site in pediatric OSA patients (25) and associated with the AHI (20). The abundance of *Porphyromonas* is higher in patients with OSA; it profoundly affects the likelihood of developing OSA-related cardiovascular diseases (26). The roles of these genera in the development of the adenoids and tonsils need to be further explored.

Several limitations of this study should be acknowledged. First, we used 16S rRNA sequencing (rather than deep-shotgun sequencing) to detect microbial diversity, which is not capable of in-depth analysis of the function of the upper airway microbiome. Second, although the sample size was sufficient to detect differences in microbes between the case and control groups, our cohort was relatively small. Thus, caution is required when interpreting the data. Third, the normal controls were classified as such based

on a questionnaire rather than objective sleep parameters (i.e., standard PSG). Fourth, various confounding factors, such as food intake, eating habits, and mouth breathing during sleep were not analyzed. Finally, our study used a case-control design, so causality could not be inferred. The role of the upper airway microbiota in pediatric OSA merits further exploration. Despite these limitations, our results constitute useful information for future studies exploring the role of upper airway floral disturbances in pediatric OSA.

Conclusions

This study revealed minor differences in the upper airway microbiota between pediatric OSA patients and controls. Large-scale metagenomic studies are warranted for in-depth examination of the upper airway microbiota in pediatric OSA patients.

Abbreviations

OSA, obstructive sleep apnea; PSG, polysomnography; BMI, body mass index; OAH, obstructive apnoea-hypopnea index; ODI, oxygen desaturation index; MAI, micro-arousal index; AOM, acute otitis media; SOM, secretory otitis media; PCR, polymerase chain reaction; NMDS, nonmetric multidimensional scaling; LEfSe, linear discriminant analysis effect size; PICRUSt, Phylogenetic Investigation of Communities by Reconstruction of Unobserved States; KEGG, Kyoto Encyclopedia of Genes and Genomes. 16S rRNA, 16S ribosomal RNA.

Declarations

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Authors' contributions

The authors take responsibility and vouch for the accuracy and completeness of the data and analyses. Prof. SY, JG and MG had full access to all of the data in the study and took responsibility for the integrity of the data and the accuracy of the data analysis. Study design: HX, JG, MG and SY; Statistical analysis: HX, XL; Manuscript draft: HX, JG, and SY. All the other authors have participated in data collection. All authors have seen and approved the manuscript.

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Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

This study was performed in accordance with the Declaration of Helsinki. The study protocol was approved by the Ethics Committee of Shanghai Jiao Tong University Affiliated Sixth People's Hospital and Shanghai Children's Hospital (Approved No. 2018-073 and No. 2019 R061-F01) and the registered number is ChiCTR1800018479. We obtained informed consent from all subjects or their legal guardian.

Consent for publication

Not applicable.

Competing interests

All the authors declare that there is no conflict of interest in the study.

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Figures

Figure 1

a Graphic representation of sampling from five upper airway sites in each subject. **b** Mean number of feature tables of pediatric OSA and controls in different upper airway sites. **c** The Shannon index between pediatric OSA and controls in different sample sites. **d** The Chao1 index between pediatric OSA and controls in different sample sites. **e** Nonmetric multidimensional scaling analysis showed that the microbial beta diversity was not significantly different. ANOSIM test was performed for comparing different groups. **f** Principal coordinates analysis plot based on the unweighted UniFrac distance depicting differences in the bacterial community between pediatric OSA and controls in different sample sites.

Figure 2

Average composition of upper airway bacterial composition at the phylum, family, genus, and species level between pediatric OSA and controls in different sample sites. **a** at the phylum level, **b** at the family level, **c** at the genus level, **d** at the species level.

Figure 3

Differentially abundant bacterial taxa identified by linear discriminant analysis (LDA) coupled with effect size measurements (LEfSe) in comparisons of pediatric OSA and controls. **a** in adenoid site. **b** in nares site. **c** in palate site. **d** in tongue site. **e** in tonsils site.

Figure 4

the bacterial functions of pediatric OSA and controls in different sample sites were predicted by PICRUSt2 analysis. **a** in adenoid site. **b** in nares site. **c** in palate site. **d** in tongue site. **e** in tonsils site.