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Correlations of Nasal Microbiome with Allergic Rhinitis and Its Symptoms Severity in Children Progression

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Objective: Human microbiome is involved in the pathogenesis of allergic diseases, but the impact of nasal microbiota on allergic rhinitis (AR) symptoms severity has not been evaluated. This study aimed to characterize nasal microbiome in AR children and its correlations with AR symptoms.

Methods: According to diagnostic guidelines for AR, 45 AR children and 40 healthy subjects were recruited from July to August in 2023. Based on the total score of nasal symptoms (TNSS), the 45 AR patients were divided into a mild AR group (MAR) (n = 16) and a moderate or severe AR group (MSAR) (n = 29). Nasal swabs were collected for microbiome analysis using 16S-rDNA sequencing.

Results: The Simpson and Shannon indices were significantly higher in the AR group compared to the health control group, indicating an increase of nasal microbiota at the species evenness level in AR children. Moreover, the species evenness was significantly increased in the MSAR group compared to the MAR group. *Staphylococcus* (member of the *Firmicutes* phylum) was significantly dominant in the AR group, but *Moraxella* (member of the *Proteobacteria* phylum) was significantly dominant in the CG group. The LEfSe analysis showed that the mean relative abundances of *Ralstonia* in the MSAR group was higher than that in the MAR group. Meanwhile, the abundance divided by *Ralstonia* of Spearman correlation coefficients was positively correlated with the TNSS of AR symptoms (r = 0.4, P = 0.009).

Conclusion: The elevation of species evenness in nasal microbiome was likely related to the aggravation of AR symptoms. The *Ralstonia* may play a pro-inflammatory role in AR.

Plain Language Summary: In this study, 45 AR children and 40 healthy subjects were recruited and the AR patients were divided into a mild AR group (MAR) (n = 16) and a moderate or severe AR group (MSAR) (n = 29) based on the total score of nasal symptoms (TNSS). High throughput 16S rDNA analysis was employed to evaluate the nasal microbiome in AR children and its correlations with AR symptoms. We found that the Simpson and Shannon indices were significantly higher in the AR group than in the CG group, and in the MSAR group than in the MAR group, indicating that the rise of species evenness in nasal microbiome may be related to the occurrence and aggravation of AR symptoms. *Staphylococcus* (member of the *Firmicutes* phylum) was significantly dominant in the AR group, and the abundance of *Ralstonia* was positively correlated with the TNSS of AR symptoms.

Keywords: nasal microbes, allergic rhinitis, symptoms severity, children

Introduction

Human microbiome was coined in 2001 to describe a collection of commensal, symbiotic, and pathogenic microorganisms living in the human body.[1] New DNA sequencing technologies and data-mining tools have transformed microbiome analyses.[2] High throughput 16S rDNA analysis can be employed to evaluate the overall alpha and beta diversities in microbiome.

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In recent years, research has rapidly expanded into the role of human microbiome in health and disease pathogenesis.[3] Previous studies have focused on microbiota in the gastrointestinal tract[4] and lower airways (lung)[5] but rarely in the upper airways (nasal and oral passages). The human nasal mucosa harbors abundant microorganisms, including commensal microbes that maintain a symbiotic relationship.[6] Culture-independent 16S-rDNA sequencing revealed that at the phylum level, the nasal microbiota of healthy subjects consists primarily of *Actinobacteria, Firmicutes*, and *Proteobacteria*.[7,8] At the genus level, the most prevalent are *Moraxella, Haemophilus, Streptococcus*, and *Flavobacterium*.[9] Knowledge about microbiota dysbiosis in the allergic nasal mucosa may help explain an individual's susceptibility to allergens and allergic inflammation.[10]

Allergic rhinitis (AR), an immunoglobulin E (IgE) and T helper (Th)2-mediated inflammatory nasal disease, is caused by sensitized immune responses to inhaled allergens, such as pollens, environmental fungi, dust mites, bacteria, and animal dander.[11] Accumulating evidence suggests that the microbiome in the nasal mucosa may exert an important function in the immune responses and development of AR.[12] Jeon YJ et al, revealed that colonization by *Staphylococcus* species is more dominant in allergic nasal mucosa, and nasal commensal *S. aureus* from subjects with AR mediates anti-allergic effects by modulating IL-33-dependent Th2 inflammation.[13]

Most studies observed no significant change or difference in microbial diversity between AR patients and healthy controls, while others reported a greater species diversity in the AR group compared to controls.[14] We used high throughput 16S rDNA and metagenomic sequencing to compare the nasal microbiota characteristics of AR and healthy controls. Meanwhile, we divided the AR patients were into a mild AR group (MAR) (n = 16) and a moderate or severe AR group (MSAR) based on the total score of nasal symptoms (TNSS), and compared the nasal microbiota characteristics between the two group. The purpose of this study is to determine the nasal microbiome characteristics in AR children and its correlations with the AR severity of clinic symptoms.

Materials and Methods

Patients and Samples

This study was approved by the Ethics Committee of Children's Hospital of Nanjing Medical University and complied with the Declaration of Helsinki. Informed consents were obtained from the guardians of participants. From July to August 2023, 45 children initially diagnosed as perennial AR due to dust mite allergy were consulted in the Department of Otorhinolaryngology, Children's Hospital of Nanjing Medical University. Meanwhile, 40 healthy subjects underwent physical examination during the same period.

AR was confirmed according to Guideline for Diagnosis and Treatments of Pediatric Allergic Rhinitis (2022, revision): the occurrence of two or more symptoms (sneezing, clear watery nasal discharge, itchy nose and nasal congestion) which persist or accumulate for more than 1 hour per day, 4 days per week and four consecutive weeks, accompanied by ocular symptoms such as itchy eyes, tearing and red eyes; nasal endoscopy of the nasal mucosa showing pallor and edema; allergen test result positive for serum-specific IgE.[15] Meanwhile, all the AR children showed perennial dust sensitization to avoid the influence of season and allergen type on TNSS score of AR patients. Data about the age, gender, body mass index (BMI) of all children were recorded.

AR symptoms included nasal obstruction, itching, sneezing, and clear nasal discharge. The INSS values of nasal obstruction, nasal leakage, nasal itching and sneezing were used to evaluate the severity of AR symptoms in children. INSS: 0 to 3 (0 = asymptomatic; 1 = mild; 2 = moderate; 3 = severe). Mild: no symptoms that cause obvious discomfort; moderate: symptoms cause discomfort but do not affect daily life or interfere with sleep; severe: symptoms interfere with daily activities and sleep status. The INSSs of all symptoms were added to get a TNSS.[16]

Exclusion criteria: (1) patients on systemic or topical antibiotics, immune agents, glucocorticoids, or antihistamines within 2 months; (2) other related diseases in the nasal cavity, including sinusitis, nasal polyps, benign and malignant tumors, nasal boils, carbuncles, intranasal infections, or nose bleeding within 1 month; (3) other respiratory system diseases; (4) patients with nasal irrigation within 2 weeks. AR children were divided into an MAR group (TNSS score: 0–4) and an MSAR group (TNSS score: 5–12).

Nasal microbiome of each child was sampled with nasal swab from the common meatus. The nasal swab was inserted into each nostril under anterior rhinoscopy, rotated for three times, constantly pressured, and placed immediately into a sterile tube. Samples were immediately transported to the microbiology laboratory and kept at −80°C for further analysis.

DNA was extracted from samples by CTAB according to the manufacturer's instructions. Nuclear-free water was used for blank. Total DNA was eluted in 50 μL of elution buffer and stored at −80°C until measurement in the PCR by LC-Bio Technology Co., Ltd. (Hang Zhou, China).

16S rDNA is located on the small subunit of the ribosome of prokaryotic cells, has a total length of 1542bp and contains of 9 variable regions and 10 conserved regions (variable regions from V1 to V9), which is considered as the most suitable index for bacterial phylogeny and classification identification. Among them, V3, V4 and V5 regions have a good specificity and complete database information. Thus, we selected the V3–V4 region of the bacterial 16S rRNA gene sequences to amplify using the primers: 341F(5'-CCTACGGGNGGCWGCAG-3') and 805R(5'-ACTACHVGGGTATCTAATCC-3').[17] PCR amplification was performed in a total volume of 25 μL reaction mixture containing 25 ng of template DNA, 12.5 μL of PCR Premix, 2.5 μL of each primer, and PCR-grade water to adjust the volume. PCR conditions to amplify the prokaryotic 16S fragments consisted of an initial denaturation at 98°C for 30 seconds, 32 cycles of denaturation at 98°C for 10 seconds, annealing at 54°C for 30 seconds, extension at 72°C for 45 seconds, and then a final extension at 72°C for 10 minutes. The PCR products were confirmed with 2% agarose gel electrophoresis. Throughout the DNA extraction process, ultrapure water, instead of sample solution, was used to exclude the possibility of false-positive PCR results as a negative control. PCR products were purified by AMPure XT beads (Beckman Coulter Genomics, Danvers, MA, USA) and quantified by Qubit (Invitrogen, USA). The amplicon pools were prepared for sequencing the size, and quantity of the amplicon library was assessed on Agilent 2100 Bioanalyzer (Agilent, USA) and with the Library Quantification Kit for Illumina (Kapa Biosciences, Woburn, MA, USA), respectively. The libraries were sequenced on NovaSeq PE250 platform.

Sequencing and Data Processing

Samples were sequenced on an Illumina NovaSeq platform (LC-Bio, Hangzhou, China), according to the manufacturer's recommendations. Paired-end reads were assigned to samples based on their unique barcode, and truncated by cutting off the barcode and primer sequence. Paired-end reads were merged using FLASH. Quality filtering on the raw reads was performed under specific conditions to obtain high-quality clean tags according to the Fqtrim (v0.94). Chimeric sequences were filtered using Vsearch software (v2.3.4). After dereplication using DADA2, we obtained Amplicon Sequence Variants (ASV) feature table and ASV feature sequence.

Statistical Analysis

Statistic Package for Social Science 25.0 (SPSS 25.0) software was used for statistical analysis of age, general and BMI data. Differences in age and BMI among groups were tested by *t*-test, and gender among groups by chi-square test. $P < 0.05$ was considered as statistically significant.

Bioinformation sequences were mainly analyzed using the QIIME2 software, and the diagrams were plotted using the R package (v3.5.2). QIME2 software was preferred to draw dilution curves for saturation analysis to evaluate whether the current sequencing depth was sufficient to include microbial diversity. Alpha diversity was evaluated with five indices, including Chao1, Observed-otus, Shannon, Simpson and Goods-coverage. The Wilcox test was used to detect the difference in indices between the two groups, and $P < 0.05$ was considered as statistically significant. The Principal Coordinates Analysis based on weighted UniFrac distances of the samples was used to evaluate the beta diversity by QIIME2 and R package. PcoA simplifies the data structure by decomposing the sample distance matrix to maximize the distribution of the relationship between samples at a certain distance scale, the more similar the species composition of the two samples.

Then, according to SILVA (release 138) classifier,[18] the relative abundance of each sample was quantified. Blast was used for sequence alignment, and sequences were annotated with SILVA database. The Wilcox test was used to analyze the differences between the two groups of samples with biological replicates, $P < 0.05$ was considered as statistically

significant. LDA Effect Size (LEfSe) analysis was used to find species with significantly different abundances among groups (biomarkers). The LEfSe analysis was mainly divided into three steps: First, Kruskal–Wallis rank sum test was used to detect abundance differences in all characteristic species among groups, and significantly different species were obtained. Then, the Wilcoxon rank sum was used to detect whether all subspecies of the significantly different species obtained in the previous step could converge into one taxon. Finally, the linear discriminant analysis (LDA) was performed to obtain the final differential species (ie, biomarker). Spearman correlation coefficients of species with AR symptoms were calculated.

Results

Clinical Characteristics of the Subjects

A total of 95 children were enrolled, including 45 AR patients (AR group) and 40 health controls (CG group). The clinical characteristics of the two groups are shown in Table 1. Gender, mean age and BMI showed no significant differences between the two groups (all $P > 0.05$).

Alpha and Beta Diversity in AR and CG Groups

Alpha diversity, a diversity within one sample, was interpreted by Chao1 index for species richness, and Shannon and Simpson indices for richness and evenness. As shown in Table 1, there were no significant differences in Chao1 or Observed-otus indices between AR and CG groups, but the Shannon (CG3.55 ± 1.28, AR: 4.20 ± 1.35) (Figure 1B) and Simpson (CG0.73 ± 0.18, AR: 0.80 ± 0.14) (Figure 1A) indices of the AR group were significantly higher than those in the CG group, demonstrating a higher evenness of the nasal microbiome in AR patients compared to the health controls. Beta diversity, expressed as a distance matrix, was used to describe dissimilarity between one pair of samples. The Principal Coordinates Analysis (PCoA) determined that there was no obvious clustering between the two groups (Figure 1C).

Nasal Microbial Compositions in AR and CG Groups

A total of 44 bacteria phyla and 11268 genera were detected in the two groups. The top 30 most abundant species at phylum (Figure 2A) and genus (Figure 2B) levels in each sample were identified. At the phylum level, *Firmicutes, Proteobacteria* and *Actinobacteria* were the most dominant in both groups (Figure 2C). Specifically, *Proteobacteria* (CG42.31%, AR24.19%, $P = 0.0037$) was more dominant in the CG group, while *Firmicutes* (CG33.69%, AR49.61%, $P = 0.002$) was more dominant in the AR group.

At the genus level, the 10 dominant members are shown in Figure 2C. Of them, *Staphylococcus* (member of the *Firmicutes* phylum) was the most abundant in the AR group (AR: 23.84%, CG14.16%, $P = 0.0045$), while *Moraxella* (member of the *Proteobacteria* phylum) was the most abundant in the CG group (AR: 4.90%, CG22.99%, $P = 0.0002$). The LEfSe analysis further confirmed these findings (LDA score > 4.0 and $P < 0.05$) (Figure 2D). Taken together, the composition of symbiotic

Table 1 Clinical Characteristics and Alpha Diversity in AR and CG Groups

Group	AR(45)	CG(40)	P value
Gender			0.82
Male	28	25	
Female	17	15	
Age	8.60±2.36	7.38±2.73	0.29
BMI	16.82±3.81	15.67±3.16	0.13
Chao 1	289.23±125.36	279.88±117.74	0.82
Observed_otus	280.64±121.58	271.18±114.50	0.85
Shannon	4.20±1.35	3.55±1.28	0.03
Simpson	0.80±0.14	0.73±0.18	0.04

Abbreviations: AR, patients with allergic rhinitis; CG, control group.

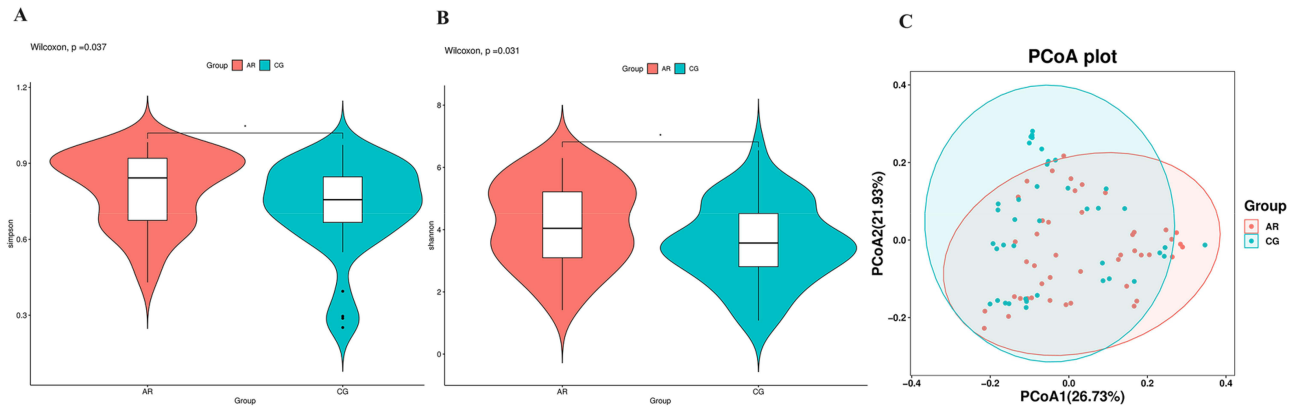

Figure 1 Comparison of alpha and beta diversities between AR and CG group.
Notes: Analysis of Simpson (**A**) and Shannon (**B**) indices for alpha diversity, the x-coordinate represents the group name, and the ordinate represents the size of Simpson (**A**) or Shannon (**B**) indices of each group. The Principal Coordinates Analysis (PCoA) for beta diversity (**C**), the points in the figure represent samples, and samples of different colors belong to different groups. The distance between points represents the degree of difference between samples. There are significant differences between the groups. * $P < 0.05$.
Abbreviations: AR, patients with allergic rhinitis; CG, control group.

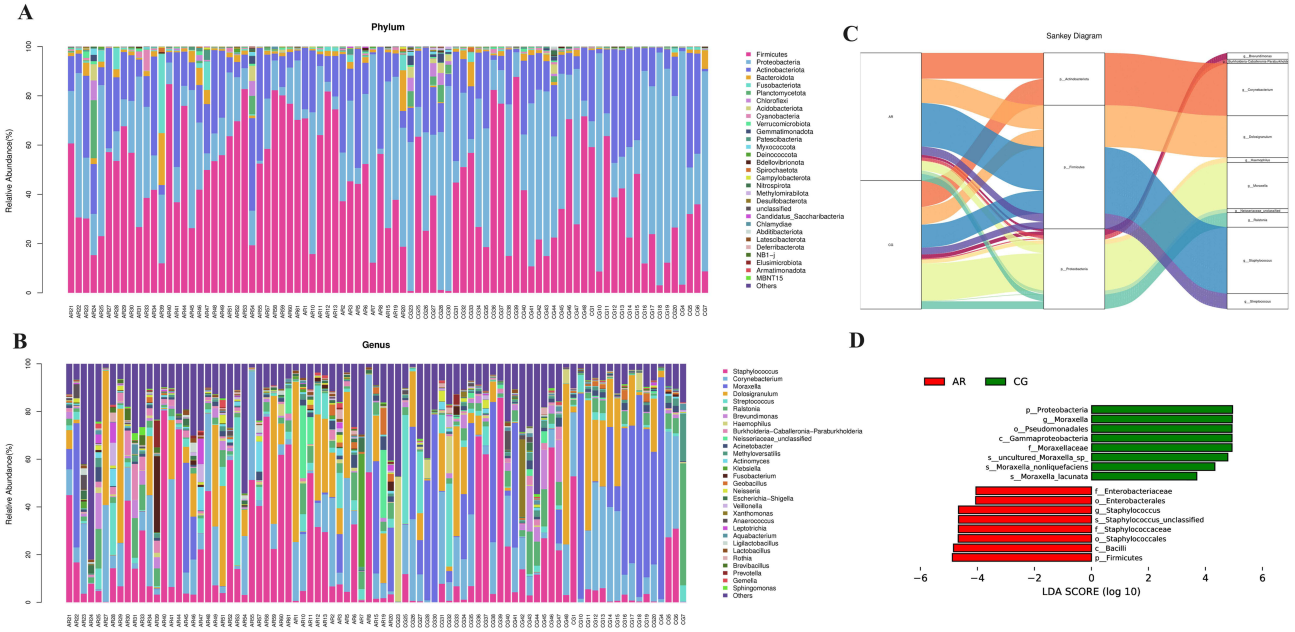

Figure 2 Comparison of nasal microbiome compositions between AR and CG groups.
Notes: Bacterial structure comparisons between AR and CG groups at phylum (**A**) and genus (**B**) levels, including the top 30 genera, the horizontal axis represents the sample name, and the vertical axis represents the relative abundance of a certain classification. Different colors correspond to different species at the same level. Sankey plot of the dominant species in AR and CG groups at phylum and genus levels (**C**), different color branches show the relative abundances of species at phylum (middle) and genus levels (right) in two groups (left), the width of the branch indicates the size of the abundance. The LEfSe analysis screening differential species with LDA > 4 (**D**) between AR and CG groups, the color of the bar graph represents the group in which each species is more abundant, while the length represents the LDA score, which is the degree of influence of biomarker between different groups.
Abbreviations: AR, patients with allergic rhinitis; CG, control group.

microbiome in the nasal mucus was distinctively different at both phylum and genus levels between healthy participants and AR patients and the *Staphylococcus* (member of the *Firmicutes* phylum) might be a biomarker in nasal cavity for AR patients.

Correlations of Nasal Microbiome with AR Symptoms

Spearman rank correlation coefficients were calculated to correlate age, gender, BMI, INSS and TNSS with the abundances of bacterial gene. The results showed that *Brevundimons* (r = 0.3, $P = 0.034$), *Ralstonia* (r = 0.5, $P =$

0.002) and *Methyloversat*ilis (r = 0.3, *P* = 0.004) were positively correlated with age; *Haemophilus* (r = 0.3, *P* = 0.02) positively with gender, and *Ralstonia* positively with TNSS (r = 0.4, *P* = 0.009). There was no significant correlation between BMI and bacterial gena. The abundances of *Brevundimons, Ralstonia, Actinomycetales-unclassifi*ed, *Moraxell*a and *Methyloversatilis* were positively correlated with the occurrence of snot in the INSS, and *Brevundimons* positively correlated with nasal obstruction, but negatively correlated with sneezing (Figure 3).

Nasal Microbial Compositions in MAR and MSAR Patients

Based on the TNSS, we divided 45 AR patients into an MAR group (n = 16, TNSS 0–4) and an MSAR group (n = 29, TNSS 5–12). As shown in Table 2, gender, mean age and BMI between the two groups had no statistical difference (all *P* > 0.05). In terms of alpha diversity, the Simpson (Figure 4A: MAR3.38±1.13, MSAR: 4.38 ± 1.54) and Shannon (Figure 4B: MAR0.71±0.13, MSAR: 0.82 ± 0.15) indices were significantly increased in the MSAR group, compared to the MAR group, which demonstrated a higher evenness of the nasal microbiome in MSAR patients. The LEfSe analysis showed that the mean relative abundances of *Ralstonia, Acinetobacter* and *Proteobacteria* in the MSAR group were higher than those in the MAR group, suggesting the close association of nasal microbiome with the severity of AR symptoms (LDA score > 3.0 and *P* < 0.05) (Figure 4C).

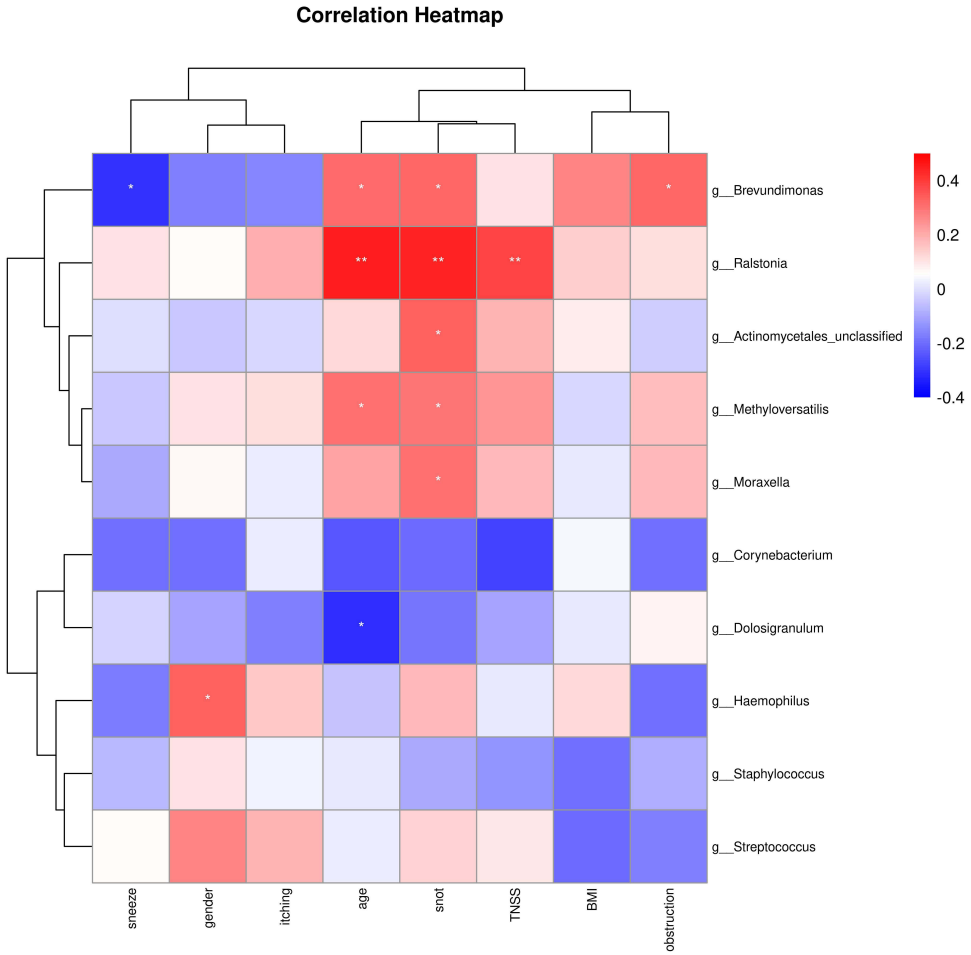

Figure 3 Spearman rank correlations of nasal microbiome with age, gender, BMI, INSS and TNSS in AR patients.
Notes: * denotes p < 0.05 and **p < 0.01.
Abbreviations: AR, patients with allergic rhinitis; CG, control group; BMI, body mass index; INSS, individual nasal symptom score; TNSS, total score of nasal symptoms; correlations significance.

Table 2 Clinical Characteristics and Alpha Diversity in MAR and MSAR Groups

Group	MAR(16)	MSAR(29)	*P* value
Gender			0.51
Male	10	18	
Female	6	11	
Age	7.56±1.71	9.17±2.19	0.03
BMI	16.84±3.34	16.81±4.11	0.02
Chao 1	240.42±102.11	324.88±285.48	0.35
Observed_otus	232.44±97.75	318±218.86	0.35
Shannon	3.38±1.13	4.38±1.54	0.02
Simpson	0.71±0.13	0.82±0.15	0.01

Abbreviations: MAR, patients with mild allergic rhinitis (TNSS score <4); MSAR, patients with moderate or severe allergic rhinitis (TNSS score 5–12).

Discussion

AR, a common condition of the upper respiratory tract, affects up to 40% of the global population.[19] AR can be treated with environmental control, pharmacotherapy, immunotherapy and surgical intervention, but their outcomes are often challenged by low long-term compliance and a high financial burden.[20] Therefore, exploring new mechanisms of AR is beneficial for developing more satisfactory treatments.

In recent years, the importance of the nasal microbiome in immune responses, especially that Th2 cytokine-regulated, has been increasingly recognized.[21] Choi et al have found that *Lactobacillus plantarum* can increase the production of Th1-type cytokines (IFN-γ, specific serum IgG2a) and decrease that of Th2-type cytokines (IL-4, IL-5, IL-13) in a mouse AR model, thus reconstructing the of Th1/Th2 balance.[22] To our best knowledge, the present study is the first systematic analysis on the relationship between nasal microbiome and AR symptoms severity in children.

Our previous study has shown a significant difference in the evenness of nasal microbiome between the AR patients and health controls, but no difference in richness.[23] The present study also exhibited a significant increase in Simpson and Shannon indices in the AR group, compared to the CG group, indicating an expected increase in species evenness in AR patients. However, there were no significant differences in Chao1 or Observed-otus indices between two groups. Thus, although richness amongst samples was not significant, the distribution of species showed difference across groups was, which has also been reported by Bender before.[24] Meanwhile, we found that the species evenness was significantly increased in the MSAR group, compared to the MAR group. These demonstrate that AR symptoms might aggravate with

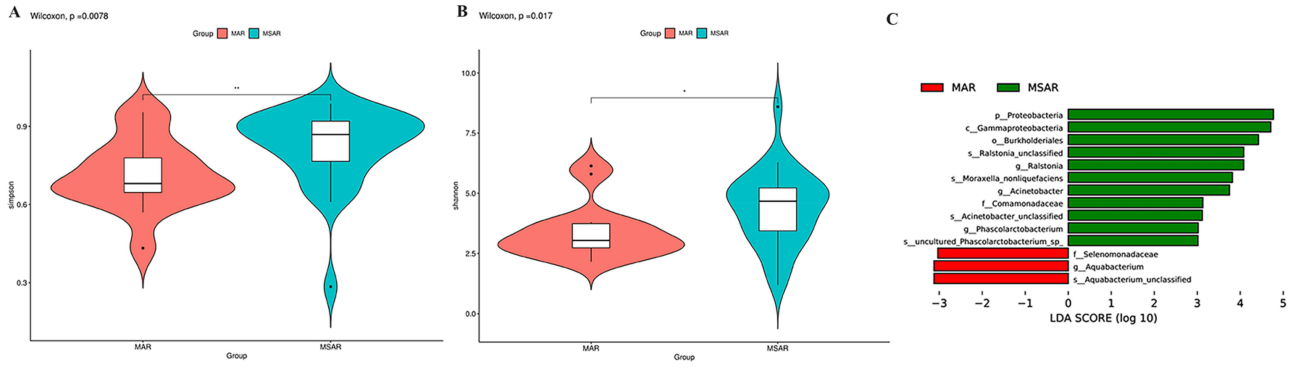

Figure 4 Comparison of nasal microbiome compositions between MAR and MSAR groups.
Notes: Simpson (**A**) and Shannon (**B**) indices for alpha diversity, the x-coordinate represents the group name, and the ordinate represents the size of Simpson (**A**) or Shannon (**B**) indices of each group. The LEfSe analysis screening differential species with LDA > 3 (**C**) between the MAR and MSAR groups, the color of the bar graph represents the group in which each species is more abundant, while the length represents the LDA score, which is the degree of influence of biomarker between different groups.
Abbreviations: MAR, patients with mild allergic rhinitis (TNSS score <4); MSAR, patients with moderate or severe allergic rhinitis (TNSS score 5–12).

the unevenness of nasal microbiome. But other studies have observed no significant difference in microbial diversity between AR patients and healthy controls.[25,26] These studies exhibit substantial variability in sample sizes and sources (such as nasal secretions, inferior turbinate mucosa, and aseptic swabs of both the vestibules and the lower nasal passages), potentially influencing the results.

Despite these variations, a consistent finding exists across all studies: AR patients exhibit a significantly different microbial abundance compared to the healthy control group.[27] Consistently, our data showed that *Proteobacteria, Actinobacteria*, and *Firmicutes* phyla were more abundant in the nasal mucus.[28] Interestingly, the abundance of *Moraxella* (member of *Proteobacteria* phylum) was reduced, while that of *Staphylococcus* (member of *Firmicut*es phylum) was elevated in the nasal cavity of AR patients. Similarly, *Staphylococcus* nasal colonization has been found more evident in asthmatic patients' respiratory extracts than in healthy controls, which induces human nasal epithelial cells to release inflammatory factors and aggravates Th2 cell-mediated inflammatory response.[29] Hyun et al have investigated the relationship between nasal microbiota and serum IgE sensitization, and reported higher relative abundance of *Firmicutes* (notably *Staphylococcus* aureus) in individuals with high total IgE compared to those with low IgE levels.[30] Therefore, we surmise that allergic inflammation in the nasal mucosa may provide an environment favoring *Staphylococcus* to colonize and become abundant in the nasal mucus of AR patients.

We further evaluated the correlation of nasal microbiome with the severity of AR symptoms. We discovered that the abundance of *Ralstonia* was positively correlated with snot and TNSS. We also observed a significant difference in the composition of nasal microbiota between the MAR and MSAR groups. The MSAR group had a higher relative abundance of *Ralstonia*, which could be related to its pro-inflammatory effect. Consistently, previous research has also highlighted the pro-inflammatory role of *Ralstonia* in patients with asthma[31] or food allergy,[32] implying the detrimental effect of *Ralstonia* on host immune system and immunity. Particularly, the abundance of *Ralstonia* was positively correlated with Th2/Treg ratio, indicating its inhibition on Treg differentiation and promotion on tropomyosin-induced allergy.[33] Therefore, we can conjecture that *Ralstonia* may play a pro-inflammatory role in AR patients by disturbing Th2/Treg balance.

In summary, the *Staphylococcus* and *Ralstonia* of the human microbiota might exert an influence on the pathophysiology of AR, necessitating future studies to elucidate underlying mechanisms.

However, our study focuses on perennial AR children and greatly reduced the effects of demographic variables on the alterations in the nasal microbiome and AR symptoms, such as smoking, season and age. There are still some limitations to this study. First, the nasal swabs cannot completely immune to the patient's recent environmental exposure impacts. Second, in this cross-sectional design, we cannot observe the dynamics changes of microbiome and their relation to symptom severity. Meanwhile, based on correlational data, we can only prove the correlation but not the causation between nasal microbiome composition and AR symptom severity. Therefore, further animal model studies and large-sample longitudinal clinical correlation studies are needed.

Conclusion

Nasal microbiome in AR patients differed significantly from that in healthy controls. The relative abundance of *Staphylococcus* increased significantly in AR patients. The species evenness in nasal microbiome was correlated with AR symptom severity, and the abundance of *Ralstonia* was positively correlated with the TNSS of AR symptoms. These findings provide a new idea for the diagnosis, treatment, or prevention of AR.

Data Sharing Statement

The data are available from the corresponding author: Xiao-fei Shen on reasonable request.

Ethics Statement

This study was approved by the Ethics Committee of Children's Hospital of Nanjing Medical University and complied with the Declaration of Helsinki. Informed consents were obtained from the guardians of participants.

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Disclosure

There is no potential conflict of interests of this manuscript.

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