

Dysbiosis of Fecal Microbiota in Allergic Rhinitis Patients

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Abstract

Background: The gut microbiota plays an important role in shaping the immune system and may be closely connected to the development of allergic diseases.

Objective: This study aimed to determine the gut microbiota composition in Chinese allergic rhinitis (AR) patients as compared with healthy controls (HCs).

Methods: We collected stool samples from 93 AR patients and 72 age- and sex-matched HCs. Gut microbiota composition was analyzed using QIIME targeting the 16S rRNA gene. Functional pathways were predicted using Phylogenetic Investigation of Communities by Reconstruction of Unobserved States. Statistical analysis was performed using the R program, linear discriminant analysis effect size (LefSe), analysis of QIIME, and statistical analysis of metagenomic profiles, among other tests.

Results: Compared with HCs, AR patients had significantly lower gut-microbiota α -diversity ($P < .001$). The gut microbiota composition significantly differed between the 2 study groups. At the phylum level, the relative abundance of *Bacteroidetes* was higher while those of *Actinobacteria* and *Proteobacteria* were lower in the AR group than in the HC group ($P < .001$, $q < 0.001$). At the genus level, *Escherichia-Shigella*, *Prevotella*, and *Parabacteroides* ($P < .001$, $q < 0.001$) had significantly higher relative abundances in the AR group than in the HC group. LefSe analysis indicated that *Escherichia-Shigella*, *Lachnospirillum*, *Parabacteroides*, and *Dialister* were potential biomarkers for AR. In addition, predictive metagenome functional analysis showed that pyruvate, porphyrin, chlorophyll, purine metabolism, and peptidoglycan biosynthesis significantly differed between the AR and HC groups.

Conclusion: A comparison of the gut microbiota of AR patients and HCs suggested that dysbiosis of the fecal microbiota is involved in the development of AR. The present results may reveal key differences and identify targets for preventive or therapeutic intervention.

Keywords

allergic rhinitis, allergy, gut microbiome, *Prevotella*, pyruvate, purine

Introduction

Allergic rhinitis (AR) is an extremely common condition. AR can occur at all ages, but its incidence peaks during the teenage years.¹ People with AR are at an increased risk for asthma, especially difficult-to-control asthma. In addition, AR impairs patients' quality of life and decreases their productivity at school or at work. The gut microbiota is now considered to be an important part of the body that is linked with health and disease including allergic diseases.

Characteristics of the gut microbiota, including, diversity, relative abundance, and metabolism, crucially

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affect the maintenance of human homeostasis and health.^{2,3} Exposure to pets, siblings, or many people during the early years of life has been demonstrated to be a protective factor against allergic diseases, while low richness and diversity of the gut microbiota have been linked to the development of allergic diseases.² The above findings are commonly referred to as the hygiene hypothesis.⁴ Enrichment of certain microbes in the gut microbiota is associated with an increase in the differentiation of T-cells into regulatory T-cells.⁵ In addition, the gut microbiota, and hence the risk of allergic diseases, can be affected by numerous factors such as environment (rural vs urban), antibiotic exposure, mode of delivery (vaginal vs cesarean section), and breastfeeding during infancy (present vs absent).⁶ Exposure to antibiotics during infancy alters the abundance of commensal bacteria in the gut and leads to an increased risk of food allergies, as compared with unexposed controls.⁷ Furthermore, the use of probiotics and/or prebiotics for the treatment of established allergic diseases has yielded some positive results; for example, the treatment of pregnant and nursing women with certain probiotics reduces eczema risk in their infants.⁸ Thus, the gut microbiota may play an important role in shaping the immune system, which is vitally important for the development of AR.

Although recent studies suggest a close connection between gut microbiota and allergic diseases,⁹ no study has yet performed a detailed comparative analysis of the composition of the gut microbiome in AR patients versus healthy control (HC) subjects. A deeper understanding of the differences in the gut microbiota of AR patients and HCs may help in the select of appropriate therapeutic strategies, which may then reduce the burden of AR and improve patients' quality of life. We hypothesized that (a) compared with HCs, AR patients have significant alterations in the composition of the gut microbiome (dysbiosis) and (b) the function of the gut microbiome significantly differs between AR patients and HCs. Therefore, the aim of this study was to comparatively analyze the gut microbiomes of patients with AR and HC subjects in order to find out specific differences in the composition and function of the gut microbiome in these 2 groups.

Methods

Subjects and Sample Collection

The protocol of this case-control study was approved by the ethics committee of the Affiliated Hangzhou First People's Hospital, Zhejiang University School of Medicine. Written informed consent was obtained from the participants or their legally authorized caregivers. We enrolled patients who visited our hospital

for AR treatment between April 2015 and December 2016. The inclusion criteria were as follows: patients who were diagnosed with AR based on their immunoglobulin E level and results of immediate hypersensitivity skin tests. Patients with a history of antibiotics, probiotics, prebiotics, or synbiotics use within 2 months before the start of the study, and patients with significant gastrointestinal abnormalities, neurodevelopmental disabilities, or any severe diseases, including those involving the brain, heart, liver, kidneys, and hematopoietic system, were excluded from the study. In addition, age- and sex-matched HC subjects without allergies were recruited from the community.

We collected the following demographic and clinical data via in-person interviews: age, gender, weight, height, body mass index (BMI), history of being breastfed, mode of delivery, and probiotic, prebiotic, and antibiotic use. Stool specimens were collected either by a researcher during a study visit or by the subjects themselves, at home, using sterile collection kits. The stool samples were immediately transferred on ice packs to the hospital, where the samples were placed in 2 mL Eppendorf tubes (250 mg/tube), and immediately frozen to -80°C until DNA extraction.

DNA Extraction and Sequencing

Total DNA was extracted from the fecal samples (200–250 mg, wet weight) by using a QIAamp Power Fecal DNA-extraction kit (QIAGEN, Hilden, Germany), with the following minor modification to the manufacturer's protocol. In brief, the fecal samples were incubated with lysis buffer and proteinase K. Glass beads (0.1 mm, Sigma, St. Louis, Missouri) were added to the lysed samples, which were then homogenized for 60 seconds in a FastPrep bead beater (MP Biomedicals, Carlsbad, California) at a speed of 4 m/s. The homogenized samples were purified using spin columns and then eluted with 200 μL buffer AE. Microbial DNA was quantified using spectrophotometry (NanoDrop ND-1000; Thermo Electron Corporation, Waltham, Massachusetts), and 1.0% agarose gel electrophoresis was performed for quality assessment.

Next, we performed DNA amplification and next-generation sequencing of the V3–V4 region of the 16S rRNA gene by using the NEXTflexTM 16S Amplicon-Seq Kit 2.0 (Bio Scientific, Austin, Texas) and the following primers: 5'-CAAGCAGAAGACGGCATACGAG ATGTGACTGGAGTTCAGACGTGTGCTCTT CCGATCTBARCODEACTCCTACGGGAGGCAGC AG-3' (forward) and 5'-AATGATACGGCGACCA CCGAGATCTACACTCTTCCCTACACGACGCT CTTCCGATCTBARCODEGGACTACHVGGGTWT CTAAT-3' (reverse). The resultant sequences were spectrophotometrically quantified (NanoDrop ND-1000)

and each sample's amplicon was mixed at the same concentration. The amplicons were then sequenced using high-throughput sequencing (Illumina HiSeq 2500; Illumina, San Diego, California) according to standard Illumina platform protocols.

The sequencing data were processed through the QIIME pipeline (v1.9.1)¹⁰ as previously described.¹¹ In brief, the raw paired-end reads were demultiplexed and merged. After quality filtering, operational taxonomic units (OTUs) were clustered using a modified UPARSE algorithm.¹² SILVA (v132)¹³ was used as the reference database for taxonomic assignment. Bacterial α -diversity was measured using the Chao1, observed species, Shannon, and Simpson indexes. Nonmetric multidimensional scaling (NMDS) and principal component analysis (PCA) were complemented using nonweighted and weighted UniFrac distance matrices and the Bray–Curtis dissimilarity matrix of OTUs or functional gene abundance. The downstream analysis was implemented using R v3.2.2 with the vegan package v2.4.0¹⁴ and ade4 package v1.7.4,¹⁵ and plotted with the ggplot2 package v2.1.0.¹⁶

Statistical Analysis

Adonis analysis and analysis of similarities (ANOSIM) were used to compare the α -diversity between the 2 study groups. The linear discriminant analysis (LDA) effect size (LEfSe) method was applied to identify potentially significant and biologically relevant bacterial biomarkers that were associated with the 2 study groups.¹⁷ The thresholds for the LEfSe analysis were an alpha value of .05 on the Kruskal–Wallis test and an LDA score >3 . The Kruskal–Wallis H test was used to perform pairwise comparisons.¹⁸

Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis and Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) analysis were used to predict microbial functions.¹⁹ Functional differences in orthologs were analyzed using the Statistical Analysis of Metagenomic Profiles and Kruskal–Wallis test, with the Benjamini–Hochberg correction to control the false-discovery rate (FDR) at 5%.²⁰ The Kruskal–Wallis H test was also used to determine statistical significance, and the P values were FDR-corrected to account for the performance of multiple tests (q value). Differences with FDR-corrected P values of $<.05$ were deemed to be statistically significant.¹⁸

Results

General Characteristics of Participants

In this study, we enrolled 165 subjects in total, including 93 AR patients and 72 HCs. No significant difference

Table 1. Demographics and Clinical Characteristics.

	Allergic Rhinitis (n = 93)	Healthy Controls (n = 72)	P
Age	29	24	.09
<10 years	18	27	
10–30 years	27	20	
31–60 years	46	22	
>60 years	2	3	
Sex			.863
Female	40	30	
Male	53	42	
Height, cm	157	153	.203
Weight, kg	52	47	.175
Probiotics			.862
Yes	1	1	
No	92	71	
Prebiotics			.418
Yes	1	2	
No	92	70	
Antibiotics			.811
Yes	6	4	
No	87	68	
Cesarean birth			.83
Yes	7	10	
No	86	62	
Breastfed			.465
Yes	87	61	
No	6	11	

was found in the mean ages of the AR patients and HCs (29 ± 15.59 vs 24.47 ± 19.46 years, respectively; $P = .079$). In addition, no statistical differences were found between the AR and HC groups in terms of the following parameters: gender, height, weight, BMI, and antibiotic, prebiotic, and probiotic use (Table 1). The proportion of Cesarean births was slightly higher in the HC group than in the AR group, but the difference was not statistically significant.

AR Patients Have Decreased Gut Microbiota Diversity

Bacterial α -diversity was significantly lower in the AR group than in the HC group ($P < .001$; Figure 1). The Chao1, observed species, Shannon, and Simpson indexes were all significantly lower in the AR patients than in the HCs (Adonis: $P < .001$; ANOSIM: $P < .001$). These results indicate that the diversity of the gut microbiota significantly differed between the AR patients and the HCs.

Alterations of Gut Microbiome in AR Patients

Differences were found in the overall composition of the gut microbiome profile between the 2 groups. At the phylum level, *Proteobacteria* and *Bacteroidetes*

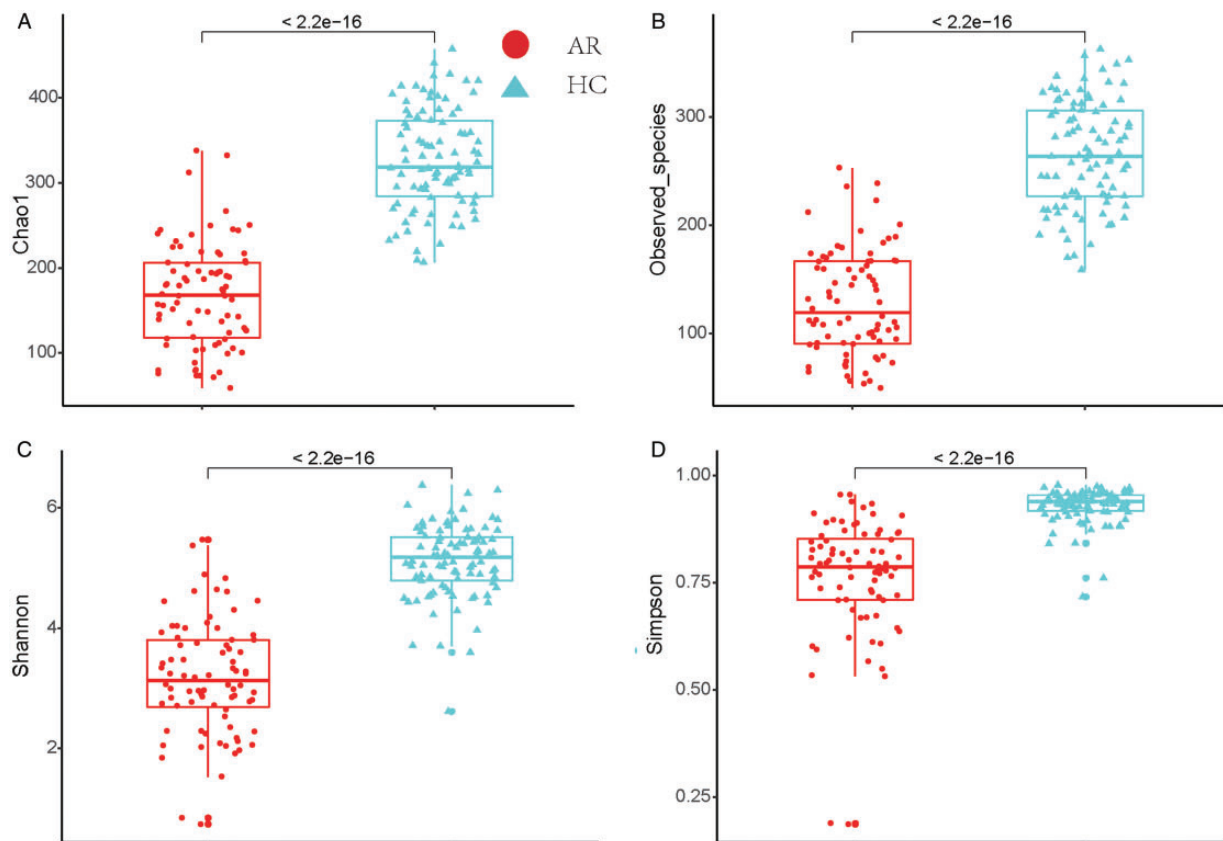


Figure 1. Alpha-diversity in AR patients and HCs. A and B, The Chao1 index and total number of observed species significantly differ between the AR and HC groups ($P < .001$). C and D, The Shannon and Simpson diversity indexes significantly differ between the AR and HC groups ($P < .001$). AR, allergic rhinitis; HC, healthy control

accounted for the main differences in the AR and HC groups (Supplemental Figure S1A and S1B). At the genus level, *Bacteroides* and *Prevotella9* accounted for much of the differentiation between the 2 groups (Supplemental Figure S1C and S1D).

Comparison of the gut microbiome composition between the 2 groups showed that at the phylum level, *Bacteroidetes* ($P = .000999$, $q = 0.004958$) was significantly more common and *Actinobacteria* and *Proteobacteria* ($P = .000999$, $q = 0.004958$) were significantly less common in the AR group than in the HC group (Figure 2A). At the family level, the relative abundances of *Prevotellaceae* ($P = .000999$, $q = 0.000805$), *Enterobacteriaceae* ($P = .000999$, $q = 0.000805$), and *Ruminococcaceae* ($P = .023976$, $q = 0.009656$) were significantly lower in the AR group than in the HC group. In contrast, the relative abundances of *Desulfovibrionaceae* ($P = .000999$, $q = 0.000805$), *Porphyromonadaceae* ($P = .000999$, $q = 0.000805$), and *Rikenellaceae* were significantly higher in the AR group ($P = .054945$, $q = 0.016305$; Figure 2B).

Statistically significant differences were also found at the genus level; several genera associated with the AR group were identified (Supplemental Figure S2).

Increases in the relative abundances of *Escherichia-Shigella* ($P = .000999$, $q = 0.004661$), *Lachnoclostridium* ($P = .000999$, $q = 0.004661$), and *Parabacteroides* ($P = .000999$, $q = 0.004661$) were noted in the AR patients, while *Faecalibacterium* ($P = .000999$, $q = 0.004661$), *Prevotella9* ($P = .000999$, $q = 0.004661$), *Akkermansia* ($P = .018981$, $q = 0.0382$), and *Roseburia* ($P = .005994$, $q = 0.016627$) were more abundant in the HC group (Figure 3).

We found evidence of a unique gut microbiome composition in AR patients. *Prevotella9*, which showed a decreased abundance in the AR group, was most prominent in the HC group. Several other bacteria with low abundances in the AR group were identified. The relative abundances of *Ruminococcaceae* UCG-004, *Allisonella*, *Clostridium innocuum* group, *Bilophila*, and *Flavonifractor* were significantly higher in the AR group than in the HC group ($P = .000999$, $q = 0.004661$). OTUs identified as *Escherichia-Shigella*, *Prevotella*, and *Parabacteroides* were predominantly detected in the AR group. Decreased relative abundances of *Ruminococcaceae* UCG-003 and *Prevotellaceae* NK3B31 group were observed in the AR group.

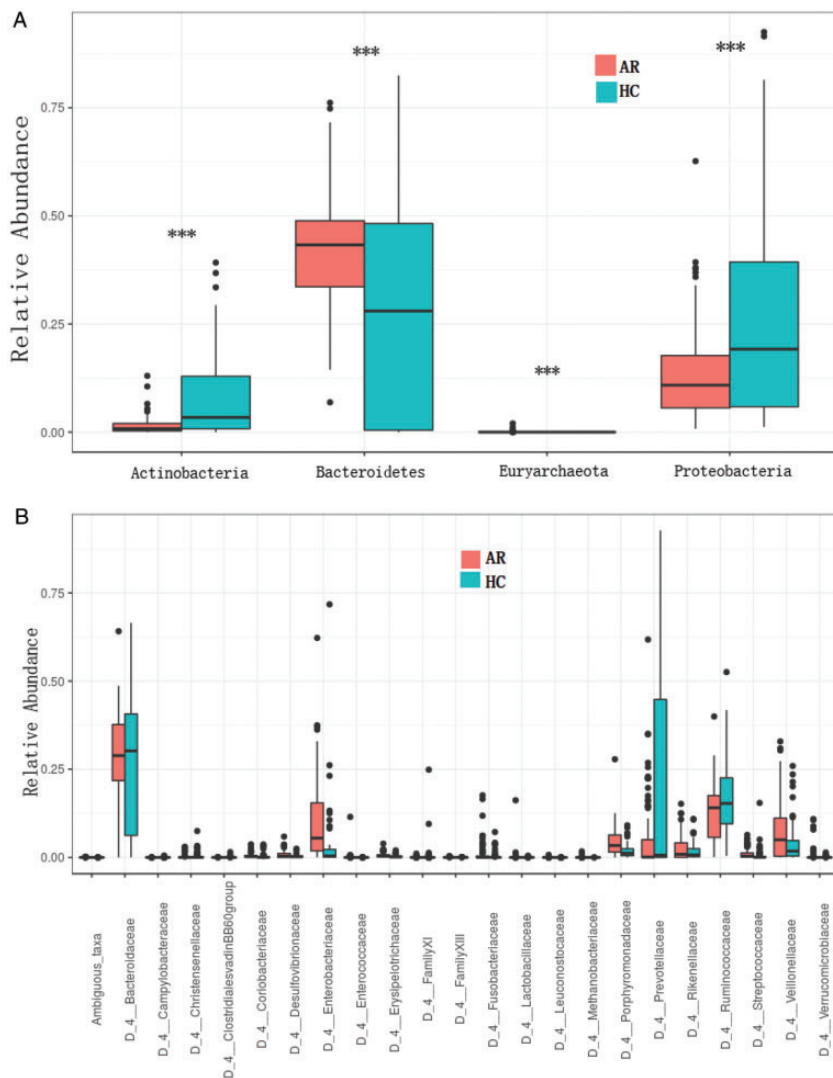


Figure 2. Gut microbiota composition and relative abundances of microbes in the AR and HC groups. A, Differences at the phylum level. B, Differences at the family level. *** $P < .001$ compared with HCs. AR, allergic rhinitis; HC, healthy control.

Analysis of β -diversity based on nonweighted UniFrac distances confirmed that AR patients had gut microbiota that were distinct from those of the HCs. ANOSIM indicated that OTU separation at the genus level was highly driven by the overall composition of gut microbiome profile (Figure 4). Importantly, significant compositional differences between the AR and HC groups were found in NMDS plots based on Adonis analysis (AR vs HC: $R^2 = 0.05528$, $P < .001$) and ANOSIM (AR vs HC: $R^2 = 0.252$, $P < .001$).

Identification of Key Microbes Responsible for Differentiating AR From HC

LEfSe analysis between the AR and HC groups revealed the characteristic gut microbiota profile and bacterial biomarkers of AR patients. The gut microbiota of AR

patients was depicted in a cladogram (Figure 5). Several genera, such as *Escherichia-Shigella*, *Lachnoclostridium*, *Parabacteroides*, and *Dialister*, were revealed as key bacteria with high relative abundances that were relevant to AR ($LDA > 3$). In the HC group, tax from *Faecalibacterium* and *Roseburia* were found to be enriched in the gut microbiota ($LDA > 3$; Figure 5A and 5B).

KEGG Pathways of Gut Microbiota Differ Between AR Patients and HCs

To reveal the link between the gut microbiota and function in AR patients, we performed PICRUSt analysis to predict the KEGG functional orthologs that differed between the AR and HC groups (Figure 6). Compared with the HCs, the AR patients showed significant

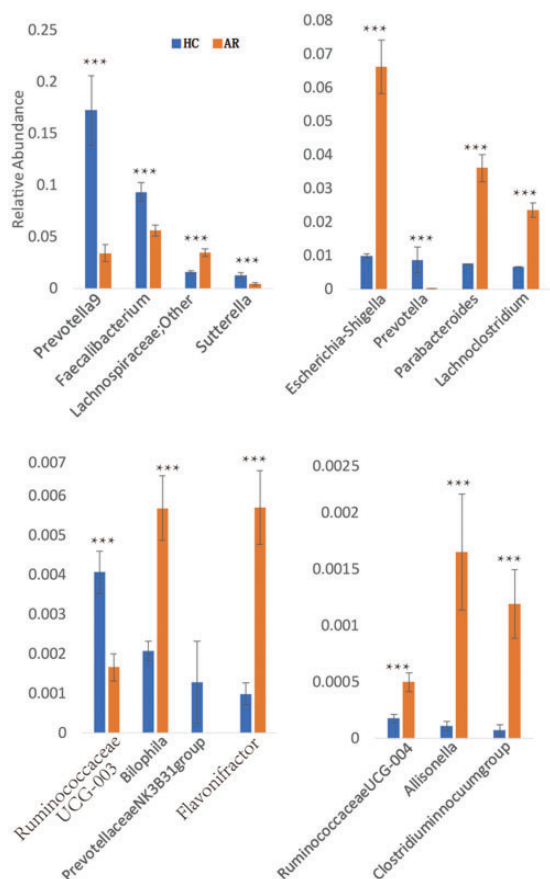


Figure 3. Relative abundances of representative taxa in the AR (orange) and HC (blue) groups. The top 15 genera with significant between-group differences on the Kruskal–Wallis H test are shown. *** $P < .001$ compared with HCs. AR, allergic rhinitis; HC, healthy control (For interpretation of the references to colours in this figure legend, refer to the online version of this article).

alterations in several important functional orthologs. In the AR group, the activities of the several pathways were increased including the transporter, ABC transporter, phosphotransferase, and 2-component pathways. The orthologs that were enriched in the AR patients included those involved in pyruvate, porphyrin, and chlorophyll metabolism, while the orthologs that were prevalent among the HCs included those involved in purine metabolism and peptidoglycan biosynthesis. In addition, marked microbial functional shifts were identified in AR patients when compared with the HCs including enriched orthologs related to electron transfer carriers; protein folding and associated processing; cell motility and secretion; tryptophan metabolism; transcription-related proteins; caprolactam degradation; valine, leucine, and isoleucine degradation; amino acid metabolism; alpha-linolenic acid metabolism; and lysine degradation ($P < .001$, Kruskal–Wallis test). The disease-related orthologs enriched in the AR group as compared with the HC group were those related to

amyotrophic lateral sclerosis, Huntington disease, *Staphylococcus aureus* infection, bladder cancer, pathogenic *Escherichia coli* infection, renal cell carcinoma, and African trypanosomiasis ($P < .001$, Kruskal–Wallis test).

Analysis of the β -diversity calculated using non-weighted UniFrac distances showed that the functional orthologs of the gut microbiota of the AR patients and HC subjects clustered apart in PC1 (93.91%) and PC2 (3.31%; Figure 7).

Discussion

This study demonstrated that compared with HCs, AR patients showed a marked alteration in the composition of the gut microbiome and a significant shift in microbial function, which indicated a significant heterogeneity in terms of disease association. Recently, many studies have confirmed that the gut microbiota is etiologically implicated in the development of allergic diseases.^{21,22} In humans, the gut microbiota during early life plays a crucial role in the development of the immune system, thereby affecting the development of allergic diseases.²³

In this study, we were able to identify certain microbes that distinguished AR patients from HCs. Certain microbes in the phylum *Proteobacteria* were enriched in the gut microbiota of AR patients as compared with the HCs. *Proteobacteria* include numerous pathogens such as *Salmonella*, *Escherichia*, *Helicobacter*, and *Vibrio* species. All proteobacteria are gram-negative and possess an outer membrane that is composed of mainly lipopolysaccharides (LPS). Higher levels of *Proteobacteria* may lead to an increase in LPS content and raise the risk of inflammation and immunological abnormality.²⁴ This finding is not consistent with a previous report which found that *Proteobacteria* were more abundant in infants without allergic manifestations at 12 months of age.²⁵ This difference may be mainly attributable to differences in the ages of the participants.

In this study, we found that taxa belonging to the phylum *Bacteroidetes* were enriched the gut microbiota of HCs. However, another study has demonstrated a high *Bacteroides* abundance in the gut microbiota of infants with early-onset atopic eczema, severe milk allergy, or a high risk of chronic allergies.⁹ Our result also contrast with findings from other food allergy-related studies, which have reported that gut colonization with *Bacteroides* corrects the Th1/Th2 imbalance and induces the development of regulatory T-cells.^{26–28}

In our study, the α -diversity was significantly lower in AR patients than in HCs. Low gut microbiota diversity has previously been associated with atopic eczema but not with food allergy.²⁵ The α -diversity of the gut microbiota during early life has been found to be increased in children with egg allergy as compared with HCs.²⁹ In one study, this association between microbiota

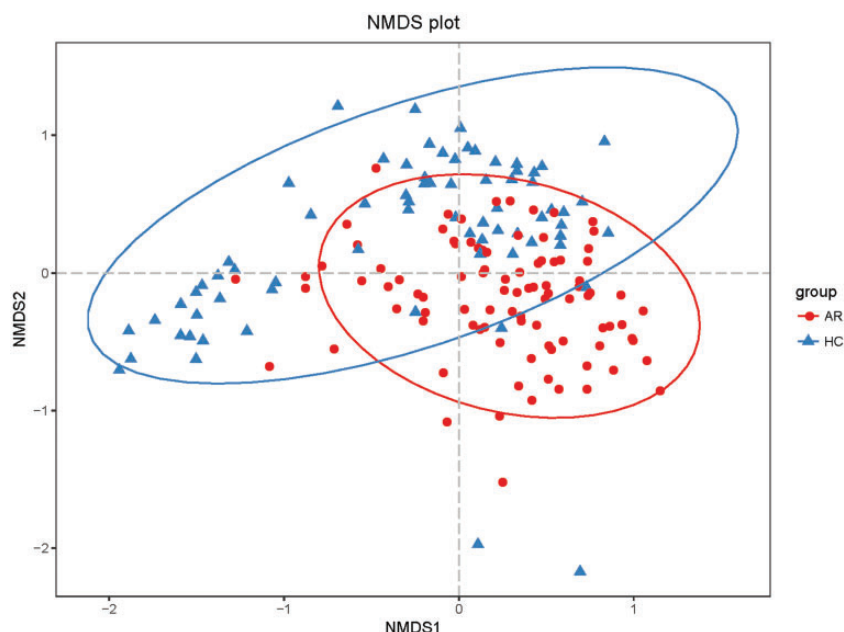


Figure 4. Gut microbiota community analysis. NMDS plots of bacterial β -diversity based on nonweighted UniFrac distances are shown. AR, allergic rhinitis; HC, healthy control; NMDS, nonmetric multidimensional scaling.

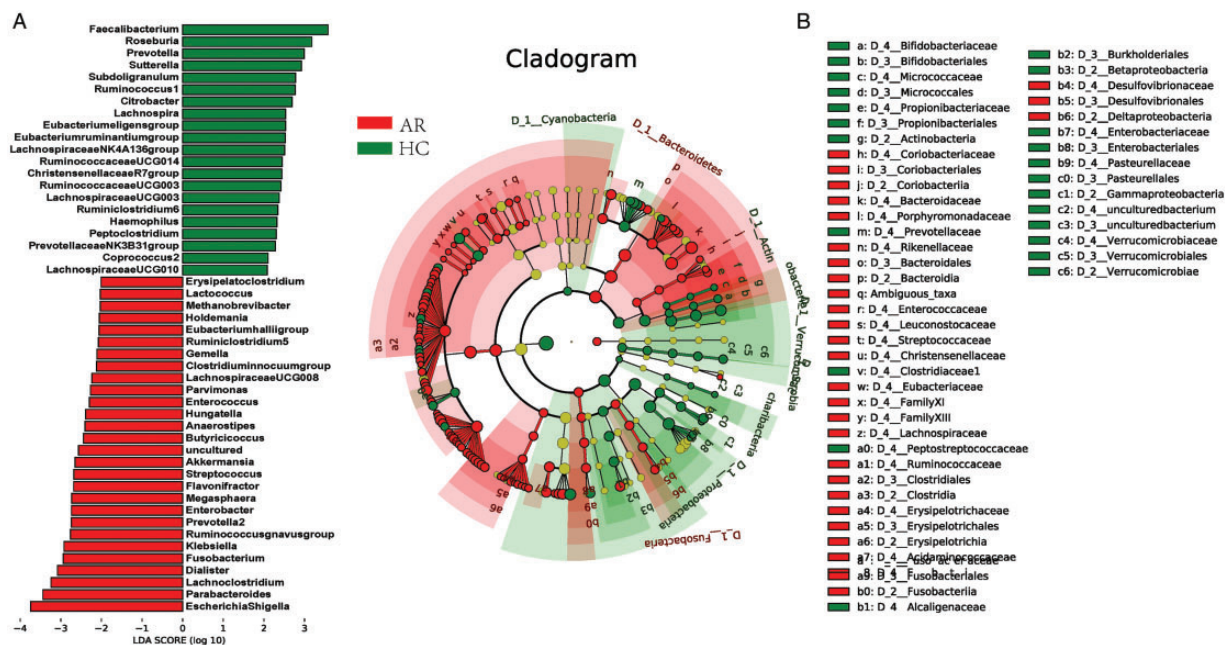


Figure 5. LEfSe analysis. A, Histogram of the LDA scores of the taxa enriched in the AR and HC groups. The LDA scores indicate the effect sizes and rankings of the taxa with different relative abundances between the AR and HC groups. B, A cladogram of the enriched taxa in the gut microbiota of AR patients and HCs. The point in the center represents the root of the tree (bacteria). The rings represent the taxonomic levels in descending order, from phylum to genus. The diameters of the circles represent the relative taxonomic abundances. AR, allergic rhinitis; HC, healthy control; LDA, linear discriminant analysis; LEfSe, LDA effect size.

composition and food allergy was present only in subjects sampled at an early age and was not found when sampling was repeated 6 months later.³⁰ The richness and diversity of the gut microbiota at the age of 1 month were not found to be associated with the

development of atopic dermatitis during the first 2 years of life.³¹ The above findings may indicate that the associations between the gut microbiota and allergy development are age dependent, and that the gut microbiota composition during early infancy may determine

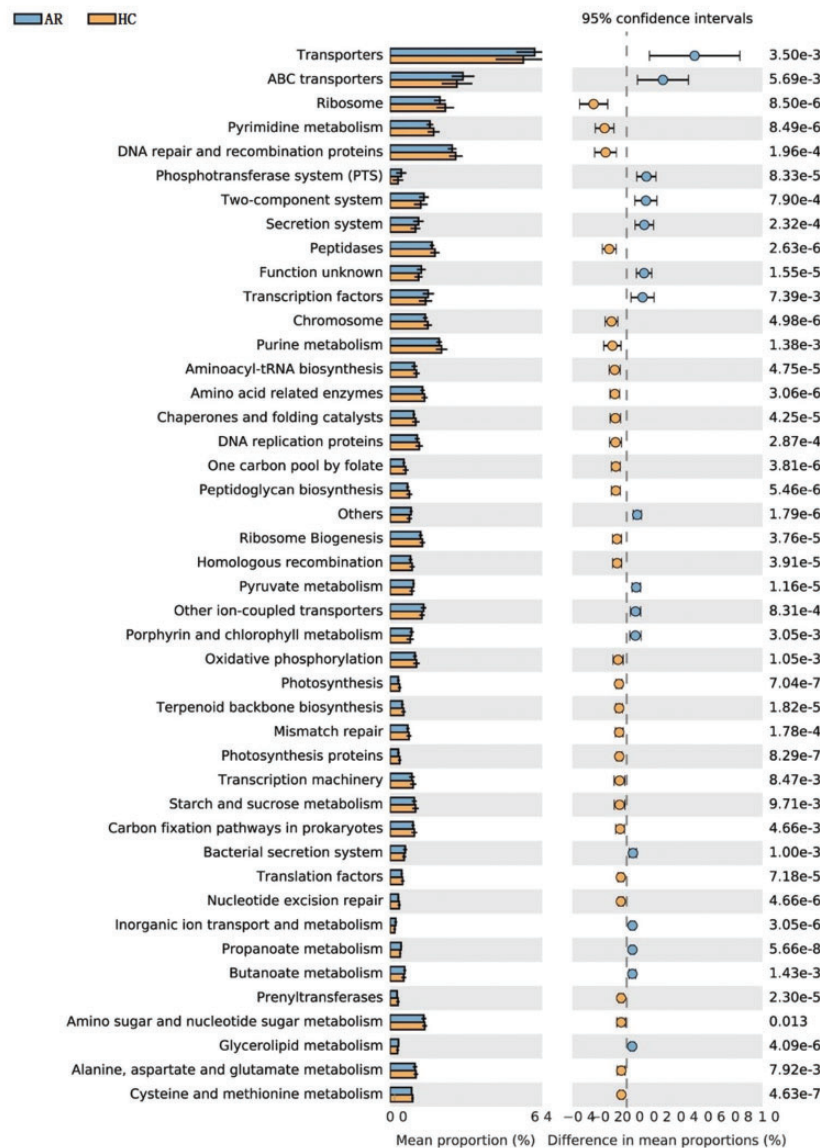


Figure 6. Prediction of gut microbial function based on KEGG pathway analysis. The error bars show the KEGG pathways that significantly differ between the AR and HC groups. AR, allergic rhinitis; HC, healthy control.

the course of food allergies in later life. A potentially dramatic change in the gut microbiota-allergic disease association may occur after solid foods are introduced in the infants' diet (usually at ~6 months of age).

The gut microbiota of AR patients may include distinct microbes such as *Prevotella*. Indeed, *Prevotella copri* has been detected in stool samples from patients with rheumatoid arthritis and may be involved in the pathogenesis of this disease.³² Furthermore, an increase in *Prevotella* abundance correlates with a decrease in the abundances of *Bacteroides* and other beneficial microbes. Mice inoculated with *Prevotella copri* show increased sensitivity to chemically induced colitis.³³ Consistent with the above findings, our study found

that *Prevotella* abundance was significantly increased in the AR group.

We also found that the AR group showed an increase in the abundance of the genus *Parabacteroides*, which consists of gram-negative, obligate anaerobes. This finding is consistent with another study which found that the abundance of the genus *Parabacteroides* was higher in fecal samples from children with autism spectrum disorder than in those obtained from related controls.³⁴ The precise link between the genus *Parabacteroides* and the development of AR remains unclear at present. *Faecalibacterium* and *Akkermansia* have been shown to play important roles in chronic and neurodegenerative diseases.^{35,36} These organisms produce short-chain fatty

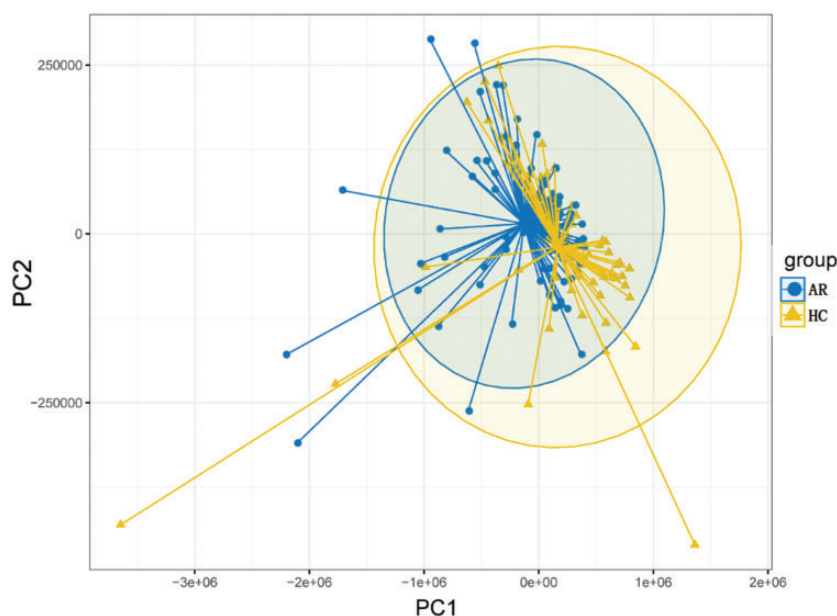


Figure 7. Gut microbiota functional ortholog analysis. PCA of bacterial functional orthologs based on β -diversity determined using nonweighted UniFrac distances. AR, allergic rhinitis; HC, healthy control; PCA, principal component analysis.

acids and other beneficial metabolic products. A decrease in the abundance of these beneficial gut microbes has been shown to be harmful to health.^{35,36} Decreases in the abundance of *Faecalibacterium* and *Akkermansia* may be related to the pathogenesis of AR.

Collectively, the above results show an increase in harmful microbes and a decrease in beneficial microbes in AR patients, which supports the hypothesis that dysbiosis of the gut microbiota is involved in the development of AR.

As an initial substrate, pyruvate can be utilized by commensal bacteria to produce butyrate, which is crucial for maintaining gut health in humans. However, in patients with oral diseases such as periodontal infection and oral cancer, butyrate shows cytotoxic effects.³⁷ PICRUSt-predicted KEGG pathway analysis showed that the bacterial functional pathways related to pyruvate, porphyrin, and chlorophyll metabolism were significantly enriched in AR patients. In contrast, functional pathways involved in purine metabolism and peptidoglycan biosynthesis were significantly decreased. This result suggests that increased pyruvate metabolism by the gut microbiota may be related to the development of AR. One study has reported that compared with HCs, patients with egg allergy show decreased purine metabolism.²⁹ Thus, an alteration in purine metabolism in the gut microbiota may be related to allergic diseases such as egg allergy and AR.

Given that the gut microbiome composition and function were conserved between the AR and control groups, we propose that gut microbiome-derived signatures may

have utility as biomarkers of AR. With these biomarkers, it may be possible to build a prediction model using artificial intelligence algorithms that can be used for the prediction, prevention, or therapy of AR and other allergic diseases.

In conclusion, gut dysbiosis may profoundly influence the occurrence of AR. Metagenomic functional prediction indicated that some functional metabolic pathways may be involved in the occurrence of AR. It is noteworthy that our study was limited to a relatively small sample size consisting of only patients from China. We are aware that the microbiome is modulated by a range of factors such as race, food, and age. Accordingly, the present results warrant further investigation and need to be confirmed in larger cohorts from different regions as well as in animal studies.

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Author Contributions

X. L. and Y. L. contributed to the study design, data collection, generation, and interpretation, and manuscript preparation. X. C. contributed to the data generation, analysis, and interpretation, and manuscript preparation. J. L. contributed to the preparation and critical revision of the manuscript. G. X. provided a critical revision of the manuscript.

Data Availability

The microbiota sequencing data have been deposited in the Bioproject database of the National Center for Biotechnology Information (NCBI; project number: PRJNA597724). All other data are available upon request from the authors.

Declaration of Conflicting Interests

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

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Supplemental Material

Supplemental material for this article is available online.

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