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Study of The specificity of gut microbiota in adult patients with delayed-onset of atopic dermatitis

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KEYWORDS
adult-onset atopic dermatitis;
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Abstract
Background: Atopic dermatitis (AD) is a common and recurrent skin disease. The first onset of AD in adults is known as adult-onset atopic dermatitis (AOAD). Gut microbiota is closely associated with AD, and the "gut-skin" axis is considered as a novel target for prevention of AD. However, only a few studies have analyzed AOAD, particularly the studies that compared differences in intestinal flora between AOAD and persistent AD patients.
Objective: To investigate main specificities of intestinal microbiota in AOAD patients, particularly comparing with persistent AD patients.
Methods: A comprehensive taxonomic and functional analysis of gut microbiota in 10 healthy, 12 AOAD, and 10 persistent AD patients was done by using bacterial 16S ribosomal RNA (rRNA) gene analysis. Chao1 and Shannon diversity indices were measured to analyze alpha diversity, and the linear discriminant analysis (LDA) effect size (LEfSe) algorithm was applied to identify differences in genus.
Results: The alpha diversity of gut microbiota in AOAD patients was decreased, with *Escherichia-shigella* (15.8%) being the predominant genus of AOAD group. *Agathobacter* and *Dorea* in AOAD patients were significantly reduced, whereas the relative level of *Bacteroides pectinophilus* group was remarkably elevated compared with healthy volunteers and persistent AD patients.
Conclusion: The present study revealed differences in intestinal flora between AOAD, healthy adults, and non-adult onset of AD, and explored differential dominant bacteria between AOAD and persistent AD patients.

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Introduction

Atopic dermatitis (AD) is a common and recurrent skin disease characterized by impaired skin barrier function, severe itching, inflamed dry skin, erythema, and abnormal immune response.[1,2] With the growing industrialization and urbanization, morbidity of AD has also increased gradually. In addition, recent reports have demonstrated that special nutritional components or microbiome metabolites could regulate the epigenetic effects related to immune functions and allergic manifestations.[3–5] Recent epidemiological studies have reported the global prevalence of AD in adults as 10%.[6,7] It has been demonstrated that genetic and environmental factors, diet, emotional health, and skin care are significantly associated with AD.[8] The cause of AD is complex, and the pathogenic mechanism has not been fully elucidated.[9]

In recent years, the relationship between abnormal intestinal flora and allergic diseases has attracted increasing attention. Intestinal microecology is a dynamic ecosystem influenced by gut microbial diversity and composition and is closely associated with immune responses and different disease.[10,11] Numerous studies have reported that gut microbiota is a potential target for regulating allergic asthma, AD, and other immune-related diseases.[12] The gut and skin share several similar features, and the "gut-skin" axis is considered as a novel target for preventing AD.[13] The composition and diversity of gut microbiota is closely associated with cutaneous lesional manifestations, implying relationship between intestinal flora and AD.[14,15] Therefore, targeting intestinal microbiome is a potential approach to alleviate inflammatory skin manifestations in AD patients.

Previously, AD was considered as a disease with onset in childhood; however, epidemiological studies have revealed that one in four adults had first onset of AD in adulthood, which is known as adult-onset AD (AOAD).[16] Compared to child onset of AD, the lesional distribution and morphology are different in AOAD, with the most commonly reported body regions being the hands, eyelids, neck, and flexural surfaces of upper limbs.[17] It was found that the skin barrier function is impaired by age and AD, resulting in poor elasticity values in older AD patients.[18] Based on the current situation, factors influencing AOAD are complex, highlighting a requirement for further investigations into the pathogenesis and optimal treatment of AOAD.[19] Although immune dysregulation and cutaneous barrier dysfunction have been reported as critical etiologies of AD, the exact mechanism of the disease pathogenesis remains ambiguous.[20] Moreover, limited research has been reported concerning AOAD, particularly analyzation of differences in intestinal flora between AOAD and AD persisting into adulthood (persistent AD) patients.

In order to analyze differences in intestinal microbiome in AOAD and persistent AD patients, a comprehensive taxonomic and functional analysis of gut microbiota was done in 10 healthy volunteers, 12 AOAD patients, and 10 persistent AD patients by using bacterial 16S ribosomal RNA (rRNA) gene analysis.

Materials and Methods

Subjects

A total of 32 participants, which included 10 healthy volunteers, 12 AOAD patients, and 10 persistent AD patients, were enrolled at Affiliated Hospital of North Sichuan Medical College. All the participants were at least 18 years old and had complied with the international diagnostic Hanifin and Rajka criteria for the diagnosis of AOAD.[21] AOAD and non-AOAD patients were identified according to their age at the first onset of AD. Specifically, patients whose age at the first onset of AD was less than 18 years were classified as non-AOAD patients; others were classified as AOAD patients. All healthy participants had no personal or family history of allergic susceptibility. The following groups of patients were excluded from the study: having a history of medical illnesses such as diarrhea and gastroenteritis; congenital genetic diseases; endocrine and metabolic diseases; blood system diseases; heart, liver, and kidney diseases or dysfunction; or having taken probiotics or antibiotics within 1 month before the start of this study. The clinical details of all participants are given in Table 1. The study was approved by the Medical Ethics Committee of Affiliated Hospital of North Sichuan Medical College. Written informed consent was obtained from all participants or a legally authorized representative(s) for anonymized patient information published in this paper. The protocol and experiments were conducted in accordance to the World Medical Association Declaration of Helsinki.

The AD severity was evaluated by investigator's global assessment (IGA) score, eczema area and severity index (EASI) scoring, and life quality score, as described previously.([22]) Both IGA and EASI scores were evaluated based on the severity of skin lesions, the size of injured area, and the proportion of injured area to the whole body.[22] The life quality score, including the dermatology life quality index (DLQI), was evaluated by the overall severity of the disease.[23,24]

Fecal sample collection and 16S rRNA gene amplicon sequencing

Fecal sample collection was done as described previously. ([25]) Briefly, the fecal samples were obtained from AD patients and healthy subjects using the standard method of placing a piece of feces in a Falcon tube, storing it at -80°C until further biochemical analysis. For deoxyribonucleic acid (DNA) extraction from fecal sample, cetyltrimethyl ammonium bromide/sodium dodecyl sulfate method was used. 16S rRNA gene amplicon sequencing was performed according to the published protocols. Specifically, the V3–V4 region of the 16S rRNA gene was amplified by polymerase chain reaction (PCR) and used for amplicon library construction following Illumina (San Diego, CA, USA) instructions. The PCR product was extracted from 2% agarose gel and purified using the AxyPrep DNA gel extraction kit (Axygen Biosciences, Union City, CA, USA)

Table 1 Clinical characteristics of delayed onset in atopic dermatitis patients.

	Control (n = 10)	AOAD (n = 12)	non-AOAD (n = 10)	P (AOAD vs. non-AOAD)
Age (years)	27.2 ± 10.53	28.83 ± 9.21	26.8 ± 7.19	0.476
Gender n (%)				0.77
Men	4 (40%)	3 (25%)	4 (40%)	
Women	6 (60%)	9 (75%)	6 (60%)	
Age at onset	–	24.58 ± 5.8	6 ± 3.18	<0.001***
mEASI score	–	22.08 ± 4.34	20.7 ± 4.27	0.462
IGA score n (%)				0.565
2 (Mild)	–	2 (17)	3 (30%)	
3 (Moderate)	–	7 (58)	6 (60%)	
4 (Severe)	–	3 (25)	1 (10%)	
DLQI score	–	16.58 ± 3.40	15.1 ± 2.51	0.267
Smoke n (%)				
Yes	3 (30%)	6 (50%)	4 (40%)	0.969
No	7 (70%)	6 (50%)	6 (60%)	

Abbreviations: AOAD: adult-onset atopic dermatitis; non-AOAD: non-adult onset of atopic dermatitis (onset at the age of less than 18 years); DLQI: dermatology life quality index; IGA: investigator's global assessment; mEASI: modified eczema area and severity index

according to standard protocols, and quantified using Quantus™ Fluorometer (Promega Corp, Madison, WI, USA).

The purified amplification product was pooled in equimolar and pair-end sequenced on an Illumina MiSeq PE300 platform/NovaSeq PE250 platform (Majorbio Bio-Pharm Technology Co. Ltd., Shanghai, China). The raw 16S rRNA gene sequencing reads were de-multiplexed, quality-filtered by fastp version 0.20.0 (FASTQ data pre-processing tool) and merged by FLASH version 1.2.7. Operational taxonomic units (OTUs) with 97% similarity cutoff[6] were clustered using UPARSE version 7.1, and chimeric sequences were identified and removed. Taxonomy of each OTU representative sequence was analyzed by RDP Classifier version 2.2 against the 16S rRNA database using a confidence threshold of 0.7.

Statistical and bioinformatic analysis

All statistical analyses were conducted with IBM SPSS version 23.0 (IBM SPSS Inc., Chicago, IL, USA).The significant differences were compared using the one-way analysis of variance (ANOVA) followed by general linear model procedures using a univariate approach. The level of statistical significance was set at $P < 0.05$. The alpha diversity (Chao1 richness estimator and the Shannon diversity index) was determined at the level of amplicon sequence variants (ASV) using vegan library from the R package #62. The linear discriminant analysis (LDA) effect size (LEfSe) algorithm was applied to identify differences in genus.

Results

A total of 32 fecal samples were collected for 16S rRNA gene sequencing, of which 10 samples were from healthy participants, classified as the control group, 12 samples were from AOAD patients, and 10 samples were from persistent AD patients. A total of 3,640,390 paired-end 16S rRNA gene sequence readings were obtained from all the samples in these three groups, with an average of 113,762.188 readings per sample, ranging from 61,000 to 231,307 per sample, and assigned into 1248 OTUs. There were 111 unique OTUs for the healthy control group, 215 unique OTUs for the AOAD group, 128 unique OTUs for the persistent AD patients, and 504 OTUs shared by the three groups (Figure 1A).

The rank abundance curves (Figure 1B) and the rarefaction curves (Figure 1C) demonstrated the species richness and evenness of 32 samples. With increase in sample size, the number of observed species was stabilized, with no further remarkable increase or fluctuation. The results depicted that the curve had reached a plateau and the sequencing data were reasonable. The size of samples in this study was sufficient to analyze the gut microbial diversity of AOAD and persistent AD patients.

In order to determine differences in overall gut microbial diversity of AOAD patients, the alpha diversity analysis was used to evaluate microbial community diversity and richness. Chao1 and Shannon diversity indices revealed that the relative abundance of the AOAD group decreased compared to the persistent AD group or healthy control group, although this decrease was not statistically significant (Figure 2A). Changes in the abundance of gut microbiota at the level of phylum, class, order, family, genus, and species were also evaluated (Figures 2B–G). In the AOAD group, *Escherichia-shigella* (15.8%) was the predominant genus. Besides, *Faecalibacterium* (19.4%) and *Subdoligranulum* (13.8%) were predominant genera in the persistent AD group and the control group, respectively.

Results of the ANalysis of Similarities (ANOSIM) demonstrated that differences in the gut microbiota structure between groups were significantly greater than variation within groups ($R = 0.197$, $P < 0.05$; Figure 3A). The microbial species abundance data at the genus level were analyzed using the Metastat method. As shown in Figures 3B and C,

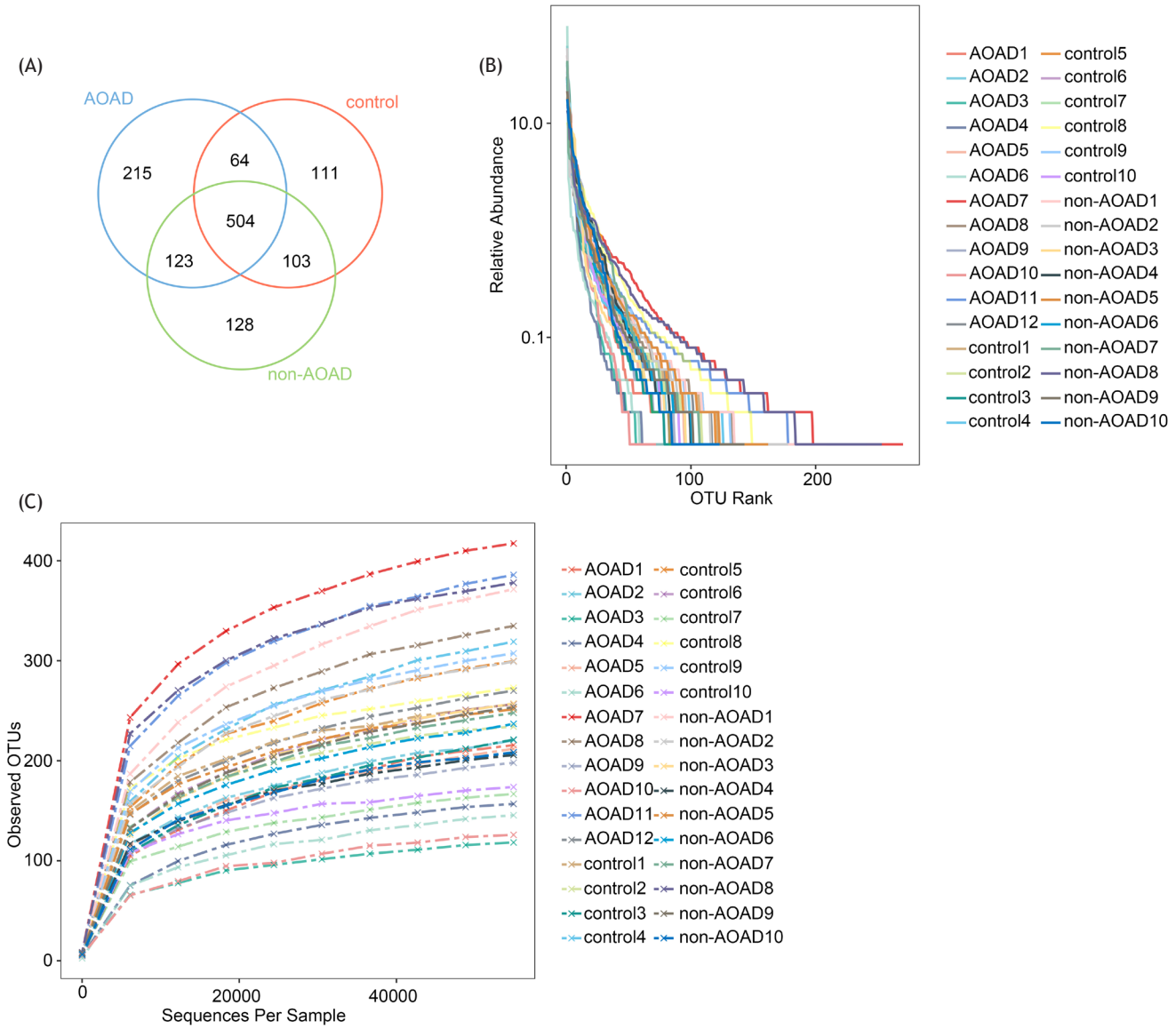

Figure 1 Overall assessment of intestinal microbiota in control, AOAD, and non-AOAD groups. (A) Venn diagram, (B) rank abundance curve, and (C) rarefaction curves of the observed OTUs in control, AOAD, and non-AOAD groups. OTUs: operational taxonomic units.

significant differences were observed between AOAD, control, and persistent AD groups. *Agathobacter* and *Dorea* in the samples of AOAD patients were reduced remarkably compared with the samples of healthy volunteers and persistent AD patients. In addition, the relative level of [*Bacteroides*] *pectinophilus* in the AOAD group was remarkably elevated than that in the control and persistent AD groups.

LEfSe analysis was applied to investigate taxonomic differences between three groups (Figures 4AB). A total of 13 differentially abundant microbial taxa (1 class, 2 order, 3 families, and 7 genra) appeared in three groups. At the genus level, abundance of *Escherichia-shigella* was observed in the AOAD group, while abundance of *Agathobacte*, *Bifidobacterium*, and *Roseburia* were observed in the control group. Moreover, *Lachnospiraceae NK4A136*, *Dorea*, and *Clostridia UCG-014* genra were abundant in the persistent AD group.

Discussion

Over the past 30 years, prevalence of AD has indicated an upward trend worldwide, and seriously, it is continuously rising in many developing countries.[26] AD usually starts in childhood; however, the most recent research has demonstrated that the prevalence of AOAD is underestimated and is higher than stated previously.[27] In addition, middle-aged people are the main labor force, but the long course of the disease affects and becomes a financial burden on family and the society. Thus, it is necessary to explore its pathological mechanism and develop specific treatments.[28,29]

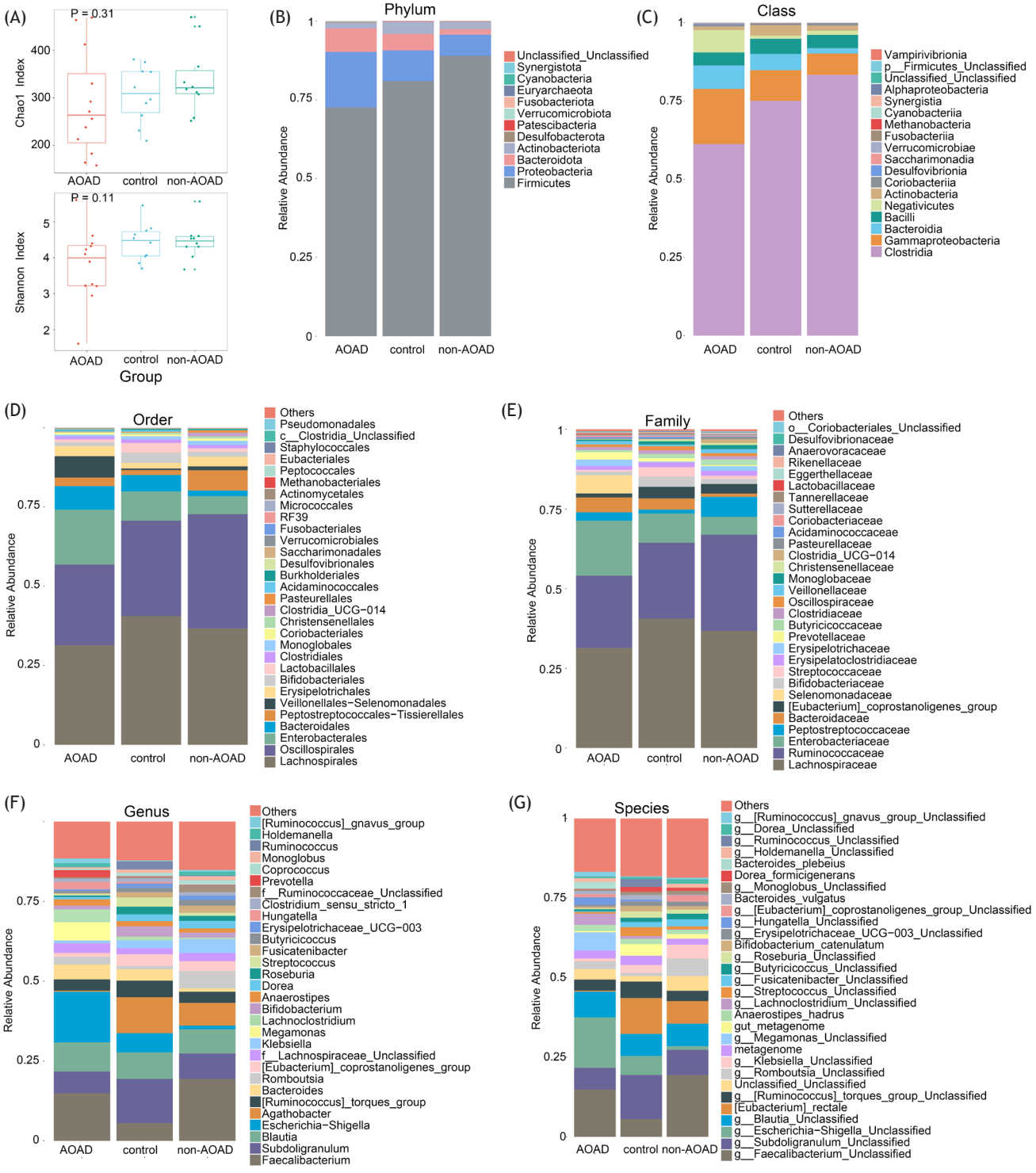

Figure 2 Changes in the diversity and composition of gut microbiota in control, AOAD, and non-AOAD groups. (A) Chao 1 and Shannon diversity index in control, AOAD, and non-AOAD groups. Relative abundance at the levels of (B) phylum, (C) class, (D) order, (E) family, (F) genus, and (G) species in control, AOAD, and non-AOAD groups.

Recently, some studies have elaborated the important role of gut flora in signaling of the mucosal immune system, and that intestinal microecological dysbiosis alters immune regulation related to the pathological process of AD.[12,30] However, the detailed underlying mechanism of intestinal microecology in the development of AOAD has not been reported. Some studies illustrated that administration of probiotics, and other therapeutic approaches to gut microbiota are expected to be available treatments for AD.[31,32] A prospective controlled trial comprising 35

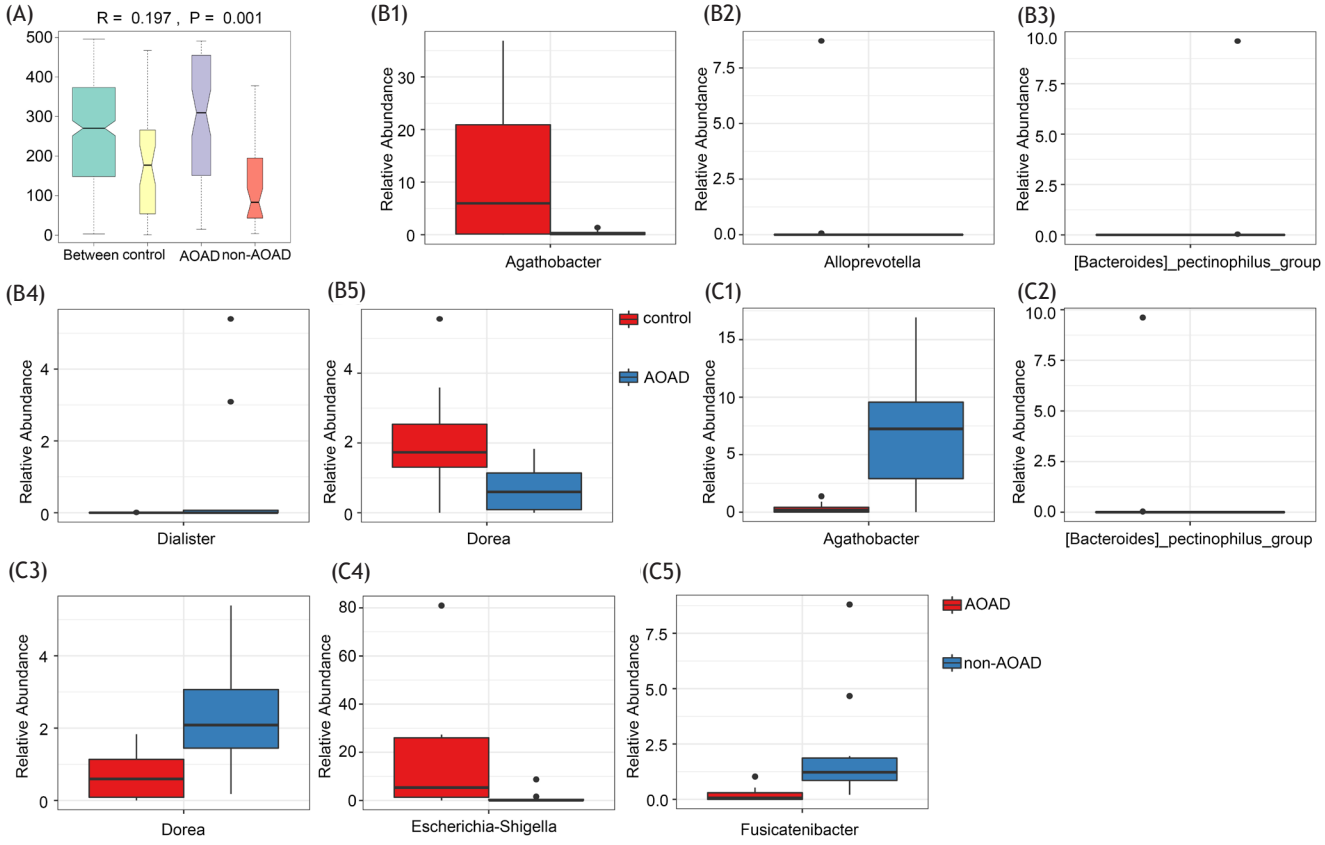

Figure 3 Differences of microbial species between control, AOAD, and non-AOAD groups. Differences between control, AOAD, and non-AOAD groups were assessed using the one-way ANOSIM analysis. (A) R-values and P-values demonstrated community variation between the compared groups. (B1–B5) Box diagrams of differences in species between AOAD and control groups; and (C1–C5) persistent atopic dermatitis (AD) group. ANOSIM: ANalysis of Similarities analysis.

subjects confirmed that probiotic treatment established a general decline in AD symptoms.[33] Furthermore, a recent clinical study on prevention of AD indicated that continuous probiotics treatment for more than 3 months ameliorated the diversity of intestinal flora.[25] The present study has demonstrated a decreasing trend in the alpha diversity of intestinal flora in AOAD patients, indicating that there could be a significant difference when expanding the sample size. In addition, reduction in microbial diversity of the intestines contributed to the onset of AD in adulthood, but this could be improved by administration of probiotics. This hypothesis requires confirmation by the further research.

Recently, several methods have been developed to detect the composition of microbial community from natural and clinical samples, and 16S rRNA gene amplicon sequencing is one of the typical approaches to elucidate the composition of gut microbiota.[34] 16S rRNA gene amplicon sequencing has been used widely in disease diagnosis or clinical analysis. For example, pathogenic bacteria in the blood of sepsis patients could be detected using 16S rRNA gene amplicon sequencing analysis.[35] In addition, 16S rRNA gene amplicon sequencing has been used to identify microbial diversity in fecal microbiota following transplantation therapy for chronic intractable constipation.[36] Changes in the diversity and composition of gut microbes have been reported in AD patients compared to healthy individuals, including decreased abundance of *Streptococcus*, and altered proportions of *Escherichia coli*, *Shigella*, *Acinetobacter*, *Pseudomonas*, and *Enterococcus*.[37,38] Therefore, it is reasonable to analyze the specificity of gut microbiota in late-onset AD using 16S rRNA gene amplicon sequencing.

Several studies have demonstrated that the relative abundance of clostridia is positively associated with the risk of AD.[39-41] Interestingly, the opposite or no association has been reported as well.[42-44] Methodological differences in research design and microbiological techniques limit the informational value of comparing results of these studies. The present data indicated that *Clostridia UCG-014* was more abundant in the persistent AD group than in AOAD patients. Previous research merely compared AD patients with healthy persons, and overlooked distinctions between AOAD and persistent AD patients, which could be a possible reason for the onset of AD in adulthood.

Conclusion

The present study established differences in the intestinal flora of AOAD patients and healthy adults. Furthermore,

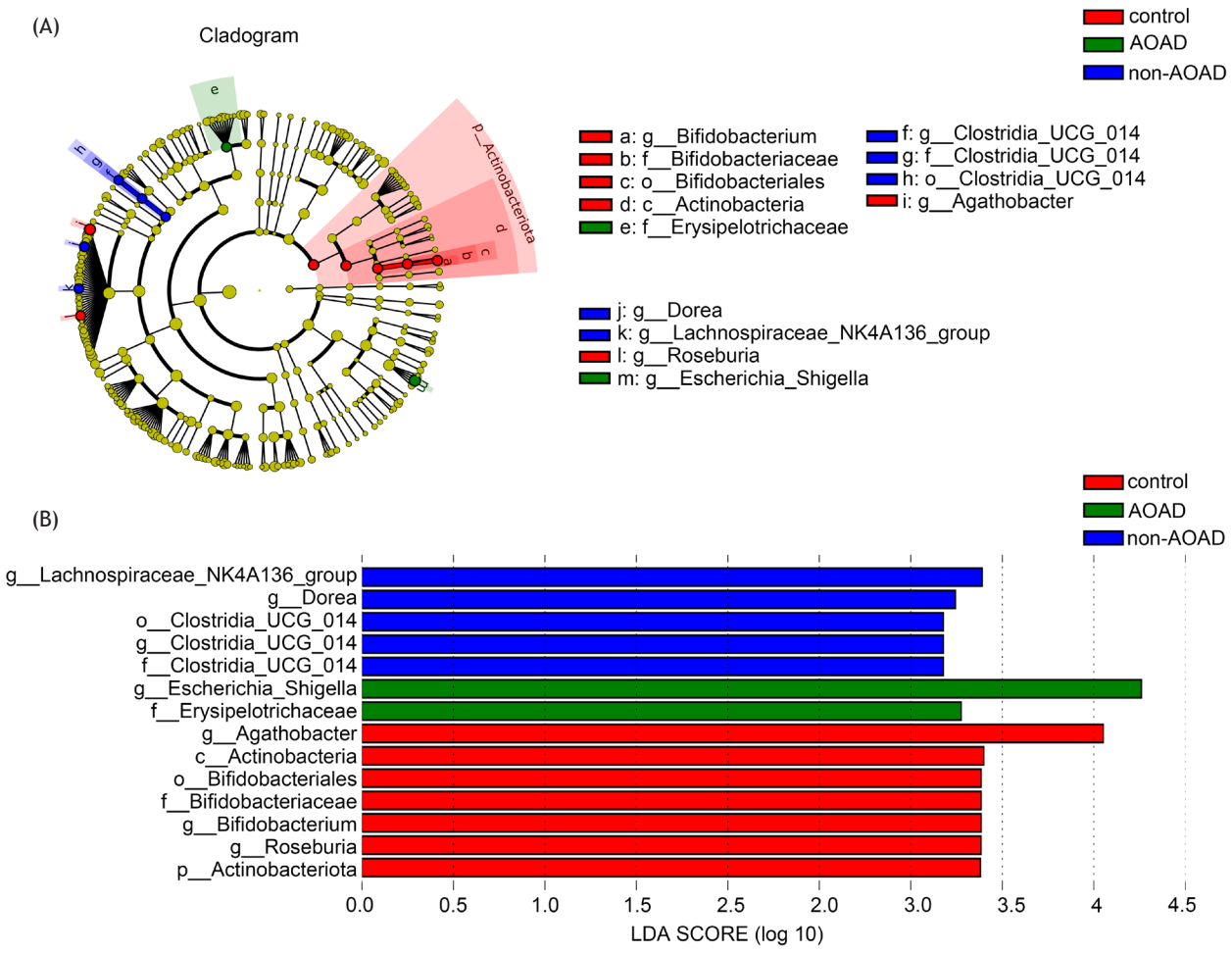

Figure 4 Specific microbial taxa differences in the gut microbiota of control, AOAD, and non-AOAD groups. (A) LEfSe analysis compared differentially abundant taxa between control, AOAD, and non-AOAD groups. (B) LDA of microbiota composition between control, AOAD, and non-AOAD groups. LDA: linear discriminant analysis; LEfSe: LDA effect size algorithm.

the diversity and composition of gut microbiota in AOAD and non-AOAD patients were compared, and the differential dominant bacteria between AOAD and persistent AD patients were explored. In this study, because of limited sample size, some parameters require improvement, such as limited applicability of results of this study to patients consuming different diets in different geographic areas. Therefore, the future studies must explore whether changes in intestinal flora lead to the onset of AD in adulthood, and whether there are specific gut bacteria that can be used as biomarkers to predict the onset of AD. Finally, the present study provided a foundation to predict the incidence of AOAD through intestinal bacteria and to improve AOAD by bacteriotherapy in the future.

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Competing Interests

The authors stated that there were no conflict of interest to disclose.

Data Availability

The authors declare that all data supporting the findings of this study are available within the paper, and any raw data can be obtained from the corresponding author upon request.

Author Contributions

Ting Liu, Chuan Yang, and Jia He designed and carried out the experiments. All the authors analyzed and interpreted the data. Ting Liu prepared the manuscript, and in the end, all the authors read and approved the final manuscript.

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