Comparison of Gut Microbiota Between Infants with Atopic Dermatitis and Healthy Controls in Guangzhou, China

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Purpose: Evidence on the role of the gut microbiota in atopic dermatitis is inconsistent as human intestinal microbiota is influenced by geography. This cross-sectional study therefore aimed to compare differences in the gut microbiota of infants with atopic dermatitis and healthy infants in Guangzhou, China, by analyzing their stool.

Patients and Methods: The composition of the intestinal microbiota was analyzed from the stool samples of 20 infants with atopic dermatitis (AD group) and 25 healthy infants (non-AD group) (1–6 months old), using full-length 16S rRNA gene sequencing. The Wilcoxon test was used to analyze the relative abundance of bacteria by phylum, family, genus, and species between groups; microbial community richness and diversity were compared between the two groups.

Results: There were no significant differences in the microbial community richness and diversity between the two groups. At the phylum level, 11 bacterial phyla were found; most sequences belonged to one of the three dominant bacterial phyla – Firmicutes, Proteobacteria, and Bacteroidetes. The top 10 microbes at the phylum, family, and genus levels showed no significant changes in their composition within the gut microbiota between the AD and non-AD groups. A decrease in the ratio of the Streptococcus genus was found in atopic dermatitis group when compared with healthy controls (p=0.048).

Conclusion: A decrease in the abundance of Streptococcus was found in children with AD. The role of Streptococcus in the development of AD needs to be confirmed in a large cohort study.

Keywords: allergy, infant, gut microbiome, Streptococcus

Introduction

Atopic dermatitis (AD) is characterized by recurrent, eczematous lesions and intense itching, with a prevalence of 10% in adults and 20% in children in developed countries, and the highest disease burden among skin diseases. The pathogenesis of AD is affected by barrier dysfunction and immune dysregulation; microbial dysbiosis, which affects the development of AD, has therefore gained more attention. However, reports regarding the influence of changes in intestinal microbiota on the development and progression of AD are inconsistent. Abrahamsson et al reported that low intestinal microbial diversity early in life was associated with subsequent AD. Wang et al found that in infants with eczema, Deinococcaceae and Bacteroidaceae were significantly enriched, while the co-abundance network of Clostridiaceae, Veillonellaceae, and Lactobacillaceae were reduced.
Intestinal microbiota is affected by various factors, including age and geographical location, due to different traditions, lifestyles, and genetic backgrounds, there are differences in the human intestinal microbiomes from different geographical locations. Few studies have focused on the characteristics of the intestinal microbiota of infants with AD in Guangzhou, China; therefore, we conducted this case-control study to compare the abundance and characteristics of the intestinal microbiota between infants with and without AD.

Patients and Methods
This case-control study and its protocols were approved by the Medical Research Ethics Committee of the Guangdong Women and Children Hospital (No. 201,701,068), and carried out in accordance with the principles of the Helsinki Declaration. Written informed consent was obtained from the parents of each participant.

Healthy children who underwent routine physical examination and children with AD, aged between 1 and 6 months, were recruited through convenience sampling. The diagnosis of AD included pruritus, typical morphology and distribution, chronic or chronically relapsing course, and atopic personal or family history according to the Hanifin and Rajka criteria. All healthy control infants had no personal or family history of allergic disease. Exclusion criteria for research subjects were: intestinal diseases such as diarrhea and gastroenteritis; congenital genetic diseases; endocrine and metabolic diseases; blood system diseases; heart, liver, and kidney diseases or dysfunction; and having taken pro- or antibiotics within 1 month before the start of the study.

Stool samples were freshly collected and stored at −80°C until extraction. Total genome DNA was isolated from fecal samples using the cetyltrimethyl ammonium bromide/sodium dodecyl sulphate method. DNA samples were amplified by polymerase chain reaction (PCR) using primers 5′-AGAGTTTGATCCTGCGCTCAG-3′ and 5′-GNTACCTTGTTACAGCTT-3′ specific for the full-length of the 16S ribosomal RNA (rRNA) gene. Each PCR was performed in TransStart<sup>®</sup> FastPfu DNA Polymerase (TransGen Biotech). Same volume of 1X loading buffer was mixed with the PCR products which were then detected by 2% agarose gel electrophoresis. QIAquick® Gel Extraction Kit (QIAGEN) was used to purify the mixture of PCR products. We generated sequencing libraries using SMRTbell<sup>™</sup> Template Prep Kit (PacBio) and assessed the library quality by using the Qubit<sup>®</sup> 2.0 Fluorometer (Thermo Scientific) and FEMTO Pulse system. At last, the full-length16S rRNA sequencing using a PacBio Sequel platform was conducted after library construction, as previously described. Processing of the raw data included filtering for a minimum of three passes, deleting the circular consensus sequence, removing sequences outside the expected ampli-con size (min length: 1340 bp, max length: 1640 bp), and removing chimeric sequences. Clean reads were finally obtained. UPARSE software v7.0.1001 (Robert Edgar, <http://drive5.com/uparse/> ) was used to assign sequences with a similarity of ≥97% to the same operational taxonomic units (OTUs). Based on the Mothur algorithm, SSU rRNA datasets from the Silva database (<https://www.arb-silva.de/> ) were used to annotate taxonomic information, obtain taxonomic information, and count each sample at each classification level, i.e., kingdom, phylum, class, order, family, genus, and species community composition. The raw sequences reported in this article have been deposited in the NCBI BioProject: PRJNA718759.

To identify community richness and diversity, the Chao1 and Shannon indices were calculated, whereas to evaluate differences in species complexity, the weighted UniFrac algorithm was used for beta diversity. We then assessed the similarity of the bacterial community compositions among samples using a Venn diagram. Continuous variables were presented as means and standard deviations, while categorical variables were presented as absolute values and relative frequencies. The Chi-squared test was applied to evaluate the characteristics of infants with and without AD. Alpha and beta diversity index differences between groups were analyzed using the t-test or Wilcoxon test. The relative bacterial abundance with respect to phylum, family, genus, and species between groups was analyzed using the Wilcoxon test; p-values <0.05 were considered statistically significant. All indices were calculated using QIIME (v1.9.1), and displayed with R software (v2.15.3, <http://www.R-project.org>).

Results
We recruited 45 children aged between 1 and 6 months – 20 children with AD and 25 healthy children; their characteristics are presented in Table 1. Stool samples were collected for 16S rRNA sequencing, and a total of 393,391 optimized reads were obtained. There were 131 unique OTUs for infants without AD, 123 unique OTUs for infants with AD, and 101 OTUs shared by both groups.
Table 1 Characteristics of Infants with and without Atopic Dermatitis

|                          | Non-AD Group (N=25) | AD Group (N=20) | p-value |
|--------------------------|----------------------|-----------------|---------|
| Sex                      |                      |                 | 0.396   |
| Male (%)                 | 7 (28)               | 8 (40)          |         |
| Female (%)               | 18 (72)              | 12 (60)         |         |
| Delivery                 |                      |                 | 0.38    |
| Vaginal (%)              | 13 (52)              | 13 (65)         |         |
| Cesarean section (%)     | 12 (48)              | 7 (35)          |         |
| Feeding method           |                      |                 | 0.363   |
| Breastfeeding (%)        | 15 (60)              | 9 (45)          |         |
| Formula feeding (%)      | 1 (4)                | 3 (15)          |         |
| Breast and formula feeding (%) | 9 (36) | 8 (40) |         |

Abbreviation: AD, atopic dermatitis.

(Figure 1). The species accumulation boxplot suggests that the boxplot tends toward a more gradual position, indicating that the sample size was sufficient for analysis of fecal microbiota (Figure 2).

The top 10 microbes at the phylum (Figure 3A), family (Figure 3B), genus (Figure 3C) and species (Figure 3D) levels showed no significant changes in composition within the gut microbiota between the non-AD and AD groups (Table 1S). At the phylum level, most sequences belonged to one of the three dominant bacterial phyla, ie, Firmicutes, Proteobacteria, and Bacteroidetes. These three bacterial phyla accounted for 97.44% and 97.34% of the total sequence in infants with and without AD, respectively. Other bacterial phyla, such as Verrucomicrobia, Actinobacteria, Fusobacteria, Nitrosirinae, Rokubacteria, and Planctomycetes accounted for a much lower proportion than the dominant bacterial phyla; however, there were no differences in the proportions of Firmicutes, Proteobacteria, and Bacteroidetes between the two groups (p>0.05).

A total of 41 bacterial families were found in infants both with and without AD. The top 10 shared families included Streptococaceae, Enterobacteriaceae, Bacteroidaceae, Enterococaceae, Veillonellaceae, unidentified_Clostridiales, Staphylococaceae, Campylobacteraceae, Peptostreptococaceae, and Lachnospiraceae; these accounted for 74.51% and 83.48% of the total sequence in infants with and without AD, respectively. Additionally, children with and without AD were found to have nine and six unique bacterial families, respectively.

In total, 51 bacterial genera were shared between the two groups; 19 unique bacterial genera were identified in infants with AD, and 21 unique bacterial genera were identified in infants without AD. The top 10 shared bacterial genera included Streptococcus, unidentified_Enterobacteriaceae, Klebsiella, Bacteroides, Enterococcus, Citrobacter, Veillonella, unidentified_Clostridiales, Staphylococcus, and Campylobacter, accounting for 68.34% and 79.67% of the total DNA sequence in infants with and without AD, respectively. Excluding Staphylococcus, Citrobacter, and Campylobacter, the shared bacterial genera accounted for greater than 1% of the total DNA sequence in the two groups. Of the 41 bacterial families and 51 bacterial genera in the gut microbiota, the ratio of Streptococcaceae family and the Streptococcus genus between the two groups were significantly different (Figure 4); we found significant decreases in the ratio of Streptococcus in the AD group (p=0.048).

A total of 52 bacterial species were found in infants with and without AD. In addition, children with and without AD each had 26 unique bacterial species. Among the shared bacterial species, the most abundant in the AD group were Escherichia coli, Bacteroides fragilis, and Enterococcus faecalis; the most abundant bacterial species in the non-AD group were Escherichia coli, Streptococcus galgaloticus subsp pasteurianus, and Klebsiella pneumoniae. No significant differences were
found between the two groups at the species level (p>0.05).

The Shannon and Chao1 indices were used to evaluate microbial community diversity and richness. According to the OTUs distribution, differences in the Chao1 index were insignificant, with averages of 28.5±22.9 and 25.0±15.4 in children without and with AD, respectively (p=0.5497). The mean Shannon index values of the infants without and with AD...
were 1.63±0.95 and 1.63±0.85, respectively (p=0.9867). The Chao1 and Shannon indices indicated that there was no significant difference in microbial diversity between infants with and without AD (Figure 5), while the analysis of differences in beta diversity indicated that there was no significant difference between the two groups (Figure 6).

Discussion

Although the pathogenesis of AD is both complex and multifactorial, recent evidence has implicated the gut microbiota. Similar to previous observations, the findings of this study demonstrated that at the phylum level, the intestinal microbiota mainly comprises Firmicutes, Proteobacteria, and Bacteroidetes.\textsuperscript{14,15}

Chen et al analyzed the stools of children with food allergies and revealed that compared with healthy infants, the abundance of Firmicutes was higher in infants with food allergies, whereas the abundance of Bacteroidetes was lower.\textsuperscript{16} Abrahamsson et al reported that children with IgE-associated AD had a lower diversity of Bacteroidetes in the first month of age, and a lower diversity of Proteobacteria at 1 year of age.\textsuperscript{4} Inconsistent with these reports, our study did not reveal dramatic changes in the microbial composition of infants with AD in contrast to healthy infants at the phylum level.

Geographical location is a key factor that influences intestinal microbiota. For example, compared with children from central Thailand, children from northeastern Thailand have a greater abundance of Bacteroides fragilis, Clostridium leptum, Prevotella, Clostridium cocoides, Eubacterium rectale, and lactobacillus.\textsuperscript{17} Lin et al explored the gut microbiota of 394 healthy subjects from seven different cities in China; they found that the influence of ethnicity on the diversity and composition of the gut microbiota was less than that of geographical location.\textsuperscript{18} Fontana et al studied the intestinal microbiota of healthy volunteers of the same ethnicity from Apulia, Lazio, and Lombardy (three different regions of Italy), and found that both alpha and beta diversity did not change.\textsuperscript{19} This notion was consistent in our study, in which we showed that the gut microbial community richness in infants with and
without AD remained similar; recruiting both infants with AD and healthy controls within the same geographic area may have contributed most to these results.

Colonization of the intestinal flora is a dynamic and complex process that continues to develop in the first few years of life. A previous study suggested that low intestinal microbial diversity in infants at one month of age is related to subsequent atopic eczema; however, we did not observe a change in the diversity of gut microbes in the AD group. Lee MJ compared the intestinal microbial composition of stool samples from healthy infants and infants with AD at the age of 6 months; no significant differences in OTUs, PCoA plot, and Shannon diversity indices were found between the two groups.

Zhang et al assessed the relationship between the gut microbiome profile of healthy infants and infants with AD aged <3 years. They found that the decreased abundance of *Bifidobacterium* was not significant in children under 6 months, while the decline in microbial diversity was most obvious in children aged 2–3 years. A longitudinal study by Galazzo et al suggested that fecal microbiota diversity did not differ significantly between children 5–13 weeks of age, but increased at 21–31 weeks; the different ages of the research subjects are believed to have caused these inconsistent results.

Interestingly, the abundance of *Streptococcus* was significantly decreased in the AD group, compared with the healthy controls in our study. Similarly, Chen et al reported significant decreases in the number of streptococci in the food sensitization group. Park et al studied the intestinal microbiota of infants with AD and found that high levels of *Akkermansia* and low levels of *Streptococcus* were evident in transient AD cases. Although there were no significant differences in the microbial community richness and diversity between the AD and non-AD groups, the abundance of *Streptococcus* was underrepresented in children with AD. The reduction *Streptococcus* may be a sign of dysbiosis associated with impending AD onset and might contribute to the prevention of AD development in infancy. Streptococci are widely distributed in the oral cavity, nasal cavity, and intestinal tract. How changes in streptococci abundance affect the occurrence of AD is not clear; however, previous studies have suggested that streptococci might be involved in the intestinal Th17 response.

**Figure 6** Gut microbial β diversity in the non-AD (NG) and AD group (AG) infants.
The present study has several major limitations; first, this was a case-control study that did not analyze stool samples before the onset of AD, making it difficult to prove a time connection. Second, the samples were selected by convenience in our study, which can lead to bias. Third, our research subjects were infants aged 1–6 months; as intestinal microbiota varies greatly among individuals, the intestinal microbiota of this age group is still in the process of dynamic development. Fourth, many factors that affect the intestinal microbiota, including maternal prebiotic and antibiotic exposure, number of siblings, and animal exposure; since this information was not collected, the description of potential confounding factors was insufficient. Finally, our analysis of the intestinal microbiota was limited to species-level analysis of the bacterial communities; fungi were not identified. Nevertheless, studies have maintained the importance of gut fungal communities during the early stages of life with respect to allergic diseases.²⁶,²⁷

Conclusion
In summary, our study suggests that diversity may not be as important as specific microorganisms in the pathogenesis of AD. The genus and family differences between infants with AD and healthy controls observed in our study may provide insight regarding the link between the gut microbiome and atopic dermatitis. Still, the role of *Streptococcus* in the development of AD needs to be confirmed in a large cohort study.

Abbreviations
AD, atopic dermatitis; OTU, operational taxonomic unit; 16SrRNA, 16S ribosomal RNA.

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Disclosure
The authors declare that they have no competing interests.

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