A low abundance of *Bifidobacterium* but not *Lactobacillus* in the feces of Chinese children with wheezing diseases

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**Abstract**

**Background:** The intestinal microbiota is linked with allergic reaction diseases. However, the difference in the fecal microbiota composition between sensitized wheezy and nonsensitized subjects in Chinese children remains unknown. The aim of this study was to quantitate the amounts of fecal microbiota in wheezy children, and to explore the correlation between fecal microbiota and serum Th1/Th2/Th17-type cytokines and total IgE in these patients.

**Methods:** The amounts of *Bifidobacterium* and *Lactobacillus* were determined using a 16S-RNA real-time polymerase chain reaction (PCR) method in wheezy children (cases) and nonwheezy controls. Serum Th1/Th2/Th17-type cytokines levels were measured using flow a cytometric bead array assay. In addition, the concentrations of total serum IgE was also determined.

**Results:** In comparison with that in the healthy control (HC), significantly lower abundance of *Bifidobacterium* and lower levels of Th1 cytokines (IFN-γ and TNF-α), but higher levels of Th2-type cytokines (IL-4, IL-5) and Th17-type (IL-17A) cytokine were detected in children with bronchiolitis and asthma. But there was no significant difference in the amounts of *Lactobacillus*. Interestingly, the amounts of fecal *Bifidobacterium* were correlated positively with serum Th1 cytokines IFN-γ, and correlated negatively with serum Th17 cytokines IL-17A, Th2 cytokines IL-4 and serum total IgE in these patients.

**Conclusions:** Our findings demonstrated that lower quantity of *Bifidobacterium*, but not *Lactobacillus*, may be correlated with asthma and bronchiolitis in Chinese children. These results also may provide guidance in choosing the proper probiotics for wheezing children.

**Abbreviations:** IFN-γ = interferon-γ, IL-17A = interleukin-17A, IL-4 = interleukin-4, IL-5 = interleukin-5, Th cell = helper T cell, TNF-α = tumor necrosis factor-α.

**Keywords:** asthma, *Bifidobacterium*, bronchiolitis, IgE, *Lactobacillus*, Th1/Th2/Th17-type cytokines

1. **Introduction**

Bronchiolitis is a common pediatric respiratory infection characterized by respiratory syncytial virus (RSV) or other respiratory viruses induced inflammation of the bronchioles. Infants hospitalized with bronchiolitis are at increased risk of both recurrent wheezing and childhood asthma characterised by airway hyperreactivity (AHR) and airway remodeling.\(^{[1]}\) Although many genetic and immune factors, such as Toll-like receptors (TLRs) are associated with the development of wheezing diseases, other factors contributing to the pathogenic process of wheezing diseases remains unclear. Therefore, a better understanding of the pathogenic process will be of great significance in the management of patients with bronchiolitis and subsequent asthma.

It is generally believed that immunological dysfunction play a crucial role in the development of asthma.\(^{[2–6]}\) Th2 cells enhance B cell differentiation and stimulate immunoglobulin E (IgE) production by secreting the cytokines interleukin (IL)-4, IL-5, and IL-13.\(^{[4,5]}\) Th1 cells secrete the cytokines IFN-γ and induce increased production of neutrophil attracting chemokines, which enhance neutrophil infiltration.\(^{[5–7]}\) Moreover, Th17 cells secrete the cytokines IL-17 and IL-22, which indirectly regulate epithelial function and promote eosinophilic infiltration, a hallmark of wheezing.\(^{[8]}\) However, whether a change in the frequency of Th cells exists in patients with bronchiolitis have not been clarified.
The microbiota, forming an extremely complex microecological system that affects nutrition, metabolism, and immunity, is regarded as the largest immune organ. For example, recent studies have revealed that dysbiosis of the intestinal microbiota can alter the steady-state immunologic balance to cause asthma and other atopic diseases. Hence, it is one potential method of treating bronchial asthma through administration of proper probiotics. Bifidobacterium and Lactobacillus, which are genus of gram-positive anaerobic bacteria, are known to have anti-inflammatory activities through the production of acetic and lactic acid. While the fecal microbiota in sensitized wheezing and nonsensitized nonwheezy children has been studied, no difference in the fecal microbiota composition between sensitized wheezy and nonsensitized, nonwheezy children aged 3 to 5 years was found in a population of children from a western country. However, whether this is the same case in Chinese children with asthma or bronchiolitis remains unknown.

In the current study, we sought to quantitate the amounts of fecal Bifidobacterium and Lactobacillus in wheezy children, and to explore the correlation between fecal microbiota and serum Th1/Th2/Th17-type cytokines and total IgE in these patients.

2. Materials and methods

2.1. Ethics statement

From February 2016 to December 2017, a total of 30 children with asthma, 30 children with bronchiolitis, and 30 healthy controls (HC) from the Department of Pediatrics, the Second People’s Hospital of Changzhou were recruited in this study. All eligible candidates for this survey and their parents received written information detailing the intent of the study. As the study only included a noninvasive sampling procedure for which the participants’ willingness to provide samples was nonmandatory, the Ethics Committee approved the verbal informed consent obtained from the parents of participating children.

2.2. Patient selection criteria

The experimental group selection criteria were as follows: all the wheezy children satisfied the diagnostic criteria of bronchiolitis or asthma, had the common symptom of wheezing, and lengthened expiratory phase by a stethoscope. The control group selection criteria were as follows: all the healthy controls had no history of allergic reactions, had no family history of wheezing or asthma, had the common symptom of wheezing, and had a normal feces examination.

All subjects (experimental group and control group) had no diarrhea or intestinal diseases within 4 weeks prior to sample collection, and had a normal feces examination.

2.3. Enzyme-linked immunosorbent assay (ELISA)

Individual subjects were subjected to routine laboratory tests for serum IgE by a commercially available enzyme-linked immunosorbent assay (ELISA) kit (human IL-IgE ELISA, AdipoGen, Switzerland).

2.4. Real-time PCR

2.4.1. Sample collection and DNA extraction. Fecal samples were collected from patients with wheezing diseases after their parents gave informed consents to participate in the study. The samples were put immediately on ice and processed within 1 hour after defecation and stored at –80°C until further analysis. The DNA extraction was performed according to the instructions of the QIAamp DNA Stool Mini Kit.

2.4.2. 16S ribosomal RNA primer design. The homology of 16s rRNA sequences of all subtypes of the 2 bacteria in NCBI was compared by BIODIF software to find the conserved region of the 16s rRNA gene. Species-specific sequences were used to design primers for the Bifidobacterium and Lactobacillus 16s rRNA genes using OLIGO software. The PCR primers used are listed:

| Bifidobacterium | qPCR Forward | 5’-CGGGTGAATGATGCTTGACC-3’ |
| Lactobacillus | qPCR Forward | 5’-GGCAAGTAGGAAATCTTCAC-3’ |
| Lactobacillus | qPCR Reverse | 5’-GGGCTTTGACGATAGTTAG-3’ |

2.4.3. Amplification of the 16s rRNA genes of Bifidobacterium and Lactobacillus. The 50 µL classical PCR reaction system consisted of forward and reverse primers (2 µL each; 10 µM), genomic DNA (2 µL), distilled water (19 µL), and 2 × EasyTaqPCR Supermix (25 µL). The amplification reaction conditions were 30 cycles of 2 min at 94°C, 30 seconds at 94°C, 60 seconds at 62°C, and 1.5 minutes at 72°C, followed by 10 minutes at 72°C. The PCR products were separated on an agarose gel, purified using an agarose gel DNA Extraction Kit, and submitted to Shanghai Biotecnology (Shanghai, China) for sequencing.

2.4.4. Real-time PCR of Bifidobacterium and Lactobacillus. The absolute amounts of the 2 bacteria in the fecal samples were determined by real-time PCR by the SYBR Green I dye method, a calibration curve was constructed based on a serial dilution of DNA from 1:10^3 to 1:10^7 (Bifidobacterium: 74,440 copies to 7,44,000 copies; Lactobacillus: 14,200,000 copies to 142 copies), and then the quantities of the test bacteria were calculated. All the genomic DNA of the test samples was diluted to 10 ng/µL with distilled water as a template negative control. The population change of Bifidobacterium and Lactobacillus in the fecal samples was tested by real-time PCR. The reaction volume was 12.5 µL, containing forward and reverse primer (0.25 µL each; 10 µM), passive reference dye (0.25 µL), template DNA (1 µL), distilled water (4.5 µL), and Top Green qPCR SuperMix (6.25 µL). The real-time PCR conditions consisted of initial denaturation at 95°C for 30 seconds, followed by 40 cycles of denaturation for 5 seconds at 95°C, annealing, and elongation at 62°C for 31 seconds.

2.4.5. Cytometric bead arrays of serum cytokines. The serum levels of Th1 cytokines (IFN-γ and TNF-α), Th2 cytokines (IL-4, IL-5) and Th17 cytokine (IL-17A) in patients were determined by cytometric bead array (CBA), according to the manufacturer’s protocol (BD Biosciences, San Jose). In brief, 50 µL of individual serum were used in duplicate for analysis, as described previously. The serum concentrations of Th1/Th2/Th17-related cytokines were quantified using the CellQuestPro and CBA software (Becton Dickinson) on a FACsCalibur cytometer (BD Biosciences).

2.5. Statistical analysis

Data are expressed as median and range or individual mean values. The difference between the groups was analyzed by Mann–Whitney U nonparametric test using the SPSS 18.0
software. The relationship between variables was evaluated using the Spearman rank correlation test. A 2-sided P value of < .05 was considered statistically significant.

3. Results

3.1. Clinical characteristics of wheezy children

Thirty fecal samples of asthma or bronchiolitis patients and 30 age- and sex-matched healthy volunteers were included in this study. As shown in Table 1, there were no significant differences in the distribution of the mode of delivery and feeding pattern between the patients and HC. As expected, the serum levels of total IgE in children with bronchiolitis or asthma were higher than that in the control group (P < .05), whereas the quantity of Lactobacillus in children with bronchiolitis or asthma was not significantly different (P > .05) compared with the control group.

3.2. The amounts of fecal Bifidobacteria and Lactobacillus in children with bronchiolitis and asthma

The amounts of Bifidobacteria and Lactobacillus in the fecal samples were quantified using the real-time PCR method (Fig. 1). The amounts of the 2 bacteria demonstrated that the quantity of Bifidobacteria in children with bronchiolitis or asthma was obviously lower than that in the control group (P < .05), whereas the quantity of Lactobacillus in children with bronchiolitis or asthma was not significantly different (P > .05) compared with the control group.

3.3. Serum Th1/Th2/Th17-type cytokines in children with bronchiolitis and asthma

Th cell plays a key role in the progression of the wheezing diseases. Our study further detected serum levels of Th1/Th2/Th17-secreted cytokines in children with bronchiolitis and asthma. The results indicated higher levels of Th17-type (IL-17A) and Th2-type cytokines (IL-4, IL-5), and lower levels of Th1 cytokines (IFN-γ and TNF-α) in children with bronchiolitis and asthma than the HC. Moreover, asthma patients displayed higher levels of Th2-type cytokines (IL-4, IL-5) and Th17-type (IL-17A) cytokine, and lower levels of Th1 cytokines (IFN-γ and TNF-α) compared to bronchiolitis patients (Fig. 2).

3.4. Correlation of fecal Bifidobacteria and Lactobacillus with serum total IgE in children with bronchiolitis and asthma

IgE is thought to be one of the important indicators to assess the severity of wheezing diseases. We further determined the potential association between the amounts of fecal Bifidobacterium and Lactobacillus and serum total IgE in bronchiolitis and asthma patients. We found that the amounts of fecal Bifidobacterium were correlated negatively with the level of serum total IgE in bronchiolitis and asthma patients (Fig. 3A and B). In contrast, we did not observe any significant correlation between fecal Lactobacillus and serum total IgE level in these patients (P > .05).

3.5. Correlation of fecal Bifidobacteria and Lactobacillus with serum Th1/Th2/Th17-type cytokines in children with bronchiolitis and asthma

The relationship between fecal Bifidobacteria and Lactobacillus and serum Th1/Th2/Th17-type cytokines in wheezing diseases

| Parameters | Bronchiolitis | Asthma | Healthy controls |
|------------|--------------|--------|-----------------|
| Number     | 30           | 30     | 30              |
| Age, years | 0.8±0.3      | 4.6±0.6| 2.9±1.2         |
| Gender (M/F) | 16/14       | 17/13  | 17/13           |
| Mode of delivery (%) | 13(43)     | 14(47) | 14(47)          |
| Cesarean section | 17(57)     | 16(53) | 16(53)          |
| Feeding pattern (%) | 11(38)      | 10(33) | 10(33)          |
| Breast feeding | 14(47)    | 15(50) | 14(47)          |
| Formula feeding | 11(36)     | 10(33) | 10(33)          |
| Mixed feeding | 5(17)      | 5(17)  | 5(17)           |
| Total IgE, IU/mL | 73.37±16.37 | 106.52±21.57 | 26.92±6.78 |

The data are presented as mean±standard deviation or number (%). 

* P<.05 vs HCs, † P<.05 vs bronchiolitis.

Figure 1. Amounts of Bifidobacteria and Lactobacillus in children with asthma or bronchiolitis compared to controls. (A) Amounts of fecal Bifidobacteria; (B) Amounts of fecal Lactobacillus. The horizontal lines indicate the mean values of the different groups.
has not yet been investigated. We analyzed the correlations and found that the amounts of fecal *Bifidobacterium* were correlated negatively with Th17 cytokine IL-17A and Th2 cytokine IL-4 and correlated positively with Th1 cytokines IFN-\(\gamma\) in the serum of asthma patients (Fig. 4A–C). Moreover, another group of bronchiolitis also showed the same trend (Fig. 4D–F). In contrast, we did not observe any significant correlation between fecal *Lactobacillus* and serum Th1/Th2/Th17-type cytokines in these patients (\(P > .05\)).

### 4. Discussion

Asthma and bronchiolitis are common wheezing and chronic airway allergic diseases in children. Approximately 30% to 40% of those with bronchiolitis can develop asthma.[1–3] Previous studies have shown that Th1/Th2-related cytokines are thought to play critical roles in the pathogenesis of wheezing diseases.[4–7] In good agreement with previous studies, we found increases in Th2-related cytokines (IL-4, IL-5) and decreases in Th1-related cytokines (IFN-\(\gamma\), TNF-\(\alpha\)) in children with asthma at the
wheezing stage as well as in children with bronchiolitis, suggesting that imbalanced Th1/Th2 cell may contribute to the development of wheezing diseases. More interestingly, Th2 cytokines (IL-4, IL-5) levels were higher in asthma patients than that in bronchiolitis, suggesting that an early viral infection may lead to Th2-oriented immunity and further to increased asthma susceptibility.[2,3] In addition, our study reveals an increase of Th7-related cytokine IL-17A in children with asthma and bronchiolitis, suggesting that IL-17 is also a pivotal cytokine in the pathogenesis of wheezing disease, which is consistent with that of previous studies.[8] These studies showed that regulation of the expression of Th1/Th2/Th17-type cytokines secretion may be a promising strategy for the development of novel anti-wheeze therapies.

Many literatures have reported that *Bifidobacterium*, which are a part of the intestinal microbiota, were effective in inhibiting allergic airway response and suppressing autoimmune responses in murine models.[13,14] These effects are related to the ability of *Bifidobacterium* to reduce the number of activated DCs and CD4+ T cells and the production of Th2-associated cytokines IL-4 and IL-5.[13,14,18] Moreover, *Bifidobacterium longum* dampen host pro-inflammatory responses by inducing the production of Treg cell and repressing local Th17 responses.[19,20] However, the differences in the amounts of fecal *Bifidobacterium* in children with wheezing diseases during the acute exacerbation phase is still not clear. Our findings revealed that the intestinal microbiota was disturbed in children with asthma at the wheezing stage as well as in children with bronchiolitis; the wheezing patients were less likely to have *Bifidobacterium*, suggesting that *Bifidobacterium* may have a negative effect or preventive role in the induction of bronchiolitis and asthma in children. These findings were consistent with previous studies[13,14], but were different from another report[15]. Conflicting results may be due to differences in a region-specific fashion, including lifestyle and eating habits. Moreover, these inconsistencies are partly due to enrollment of patients regardless of the phase of their disease. In addition, we found a negative correlation between fecal *Bifidobacterium* and serum total IgE levels, indicating that the deficiency of *Bifidobacterium* may be related to clinical severity.[17] More importantly, consistent with previous studies, we found that the amounts of fecal *Bifidobacterium* were correlated negatively with Th17 cytokine IL-17A and Th2 cytokine IL-4, and correlated positively with Th1 cytokines IFN-γ in the serum of asthma patients, suggesting that the deficiency of the *Bifidobacterium* may caused a shift of the immune system.[18,20] In contrast, in terms of the amounts of *Lactobacillus*, no significant differences between the children with asthma or bronchiolitis and the healthy control group or between the children with asthma and the children with bronchiolitis were found. Taken together, our findings suggest that *Bifidobacterium*, but not *Lactobacillus*, may be correlated with wheezing diseases in chinese children, and balance the quantity and activity of these T-helper cells through administration of *Bifidobacterium* may be one potential method of treating bronchial asthma.[21]

In conclusion, the difference between children with asthma or bronchiolitis in terms of the quantity of *Bifidobacterium* but not
Lactobacillus was found in feces, suggesting Bifidobacterium but not Lactobacillus may be the leading force to repress wheezing. These findings also implicate that Bifidobacterium deficiency may be one candidate who influences the Th1/Th2/Th17 cell balance. Therefore, further longitudinal studies of amounts and function of Bifidobacterium and Lactobacillus in the pathogenic process of asthma or bronchitis with a bigger population are warranted.

Author contributions

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