The Impact of Air Pollution on Intestinal Microbiome of Asthmatic Children: A Panel Study

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Abstract

Background

Intestinal microbiome can influence human health. Previous researches showed that the intestinal microbiome played an important role in immune responses associated with allergic disease. Colonization of intestinal microorganisms in the early life could affect future susceptibility to asthma. In China the incidence of childhood asthma raised sharply in recent years and the air pollution was serious in the meantime. Based on the above theories and conditions, the main objective of this article was to explore the impact of air pollution on intestinal microbiome of asthmatic children preliminary.

Results

A total of 42 fecal samples from 21 children, among whom 11 children with asthma and 10 children without asthma, were collected twice in a clean day and a polluted day respectively. Identifying the bacteria in gut by the method of high throughput sequencing for 16SrRNA gene. The results showed that Firmicutes, Bacteroidetes, Actinobacteria and Proteobacteria were the dominant phyla in all children. Proteobacteria (p=0.020) increased significantly and Bacteroidetes (p=0.072) decreased marginally significantly in asthmatic children compared with healthy children. Bacteroidetes (p=0.067) showed marginally significant increasing and Actinobacteria (p=0.001) showed significant decline in asthmatic children after air pollution. The variations of Firmicutes (p=0.106) and Proteobacteria (p=0.064) were marginally significant in healthy children after air pollution. Analyzed by multiple linear regression, the relative abundance of Firmicutes, Bacteroidetes at phylum level, Bacteroidia at class level, Clostridiumsensu stricto_1 and Terrisporobacter at genus level significantly correlated with environmental pollutants (p<0.05), including PM2.5, PM10, SO2, NO2 and O3.

Conclusion
Composition of intestinal microbiome in asthmatic children were different from healthy children significantly, and air pollution could impact on intestinal microbiome both in asthmatic and healthy children.

Background

Asthma is a heterogeneous disease, usually characterized by chronic airway inflammation. It always brings heavy burden to the family and society. In recent years, prevalence of asthma was increasing in many countries, especially among children [1]. In the past 30 years, incidence of childhood asthma increased sharply in China [2], and air pollution was series in China in the meantime. Just as some researchers’ opinions [3,4], the increasing of childhood asthma might be correlated with air pollution. The mechanisms of air pollutants trigging asthma were not completely clear now. UK’s Committee on the Medical Effects of Air Pollutants proposed that air pollution might contribute to the development and exacerbation of asthma through four main mechanisms: oxidative stress and damage, airway remodeling, inflammatory pathways and immunological responses, and enhancement of respiratory sensitization to aeroallergens [5,6].

On the other hand, many studies demonstrated that intestinal microbiome were correlated with development of asthma. Human intestinal microbiome is composed of approximately $10^{14}$ microorganisms, which is 10 times the number of cells making up human body [7,8]. These microorganisms not only provide energy for metabolism directly, but also interact with host immune system to influence human health. In 1980s, David Strachan’s study showed that lack of exposure to microbes in early life could increase the susceptibility to atopic disease including asthma, which was so called hygiene hypothesis [9,10]. In recent years, Jakob et.al. studied in a cohort with 690 children, and found 1-year-old children with an immature composition of intestinal microbiome had an increased risk of asthma at
age 5 years [11]. As the development of microbiology technology, more experimental evidences available suggested that there was a microbial-immune cell interface, in which microbes cross-talk with immune cells to effect on the immune system [12-15]. And the intestinal microbes influenced immune function of lung through the gut-lung axis [16-18]. All the information above illustrated that intestinal microbiome play an important role on development of childhood asthma.

The most interested finding was that air pollution was illustrated to effect on the intestinal microbiome [19]. And in an investigation process of a project, which was named “Epigenetic mechanism of black carbon on non-atopic asthma” and founded by National Natural Science Foundation of China, investigators found that more allergic children with asthma than non-allergic children with asthma came hospital to consulate in air polluted days. Viewed in connection of microbiome, asthma and air pollution, this article assumed that the polluted environmental factors might trigger the allergy mechanism of asthmatic children through impacting on intestinal microbiome.

Furthermore, there were many studies about the relationship between intestinal microorganism colonization in early life and the asthma incidence in adult [9-11,20-22], but little researches about intestinal microbiome composition during asthma was onset. When studying the effects of air pollution on intestinal microbiome in children with asthma, this article would also focus on the community structure of intestinal microbiome in children with asthma.

Results

Participants Characteristics

21 participants were enrolled in the panel study, including 11 asthmatic children (6 boys and 5 girls) and 10 healthy children (7 boys and 3 girls) without asthma, with a mean age of 7.8 (SD (Standard Deviation) 1.5) years old. The average value of BMI (Body Mass
Index) was 17.1 (SD 2.1). All participants were living in Beijing more than 6 months. There were 8 allergic and 3 non-allergic children in asthmatic children. All healthy children were not allergic. Dietary habit for each participant didn’t change between two-time samples collecting.

Air Pollutants Level

Samples for each child were collected twice. Firstly, samples were collected in a clean day with AQI (Air Quality Index)<100, the average value of AQI for the five days before sampling was 73. Subsequently, samples were collected in a polluted day with AQI>100, the average value of AQI for the five days before sampling was 120. The concentrations of air pollutants were shown in Table 1. The effect on intestinal microorganisms of environmental factors was evaluated by 3-day moving average concentration of the following pollutants excepted O₃. O₃ was evaluated by the maximum daily 8-hour mean concentration.

Table 1 Concentrations of Air Pollutants in Sampling Days

| Percentile | Clean Day (AQL<100) | Polluted Day (AQL>100) |
|------------|---------------------|------------------------|
|            | 0th  | 25th | 50th | 75th | 100th | 0th | 25th | 50th | 75th | 100th |
| PM₂.₅      | 16.3 | 26.3 | 29   | 37.3 | 84.7  | 38.3| 38.3 | 60.3 | 84.7 | 149.3 |
| PM₁₀       | 17.7 | 53.7 | 55.3 | 76.7 | 11.8  | 68.3| 68.3 | 102  | 138  | 162  |
| NO₂        | 17.7 | 33.3 | 43.3 | 44.7 | 55    | 25.7| 25.7 | 44.7 | 48.7 | 69.7 |
| SO₂        | 2.3  | 4    | 6.7  | 10   | 10.3  | 4   | 4    | 8    | 10   | 18   |
| O₃         | 40   | 40   | 48   | 77   | 94    | 30  | 62   | 119  | 151  | 177  |
| Temperature| 6.7  | 6.7  | 11   | 16.7 | 27    | 9.1 | 15.2 | 20.7 | 27.8 | 29.1 |

The unit of PM₂.₅, PM₁₀, NO₂, SO₂ and O₃ is ug/m³, Temperature is °C.

Operational Taxonomic Units) Number Variation before and after Air Pollution

In the 42 samples, a total of sequencing reads, ranging from 59193 to 99554, were
generated from the V4 hypervariable region of 16SrRNA genes. The average number of obtained tags was 71358 (SD 7897). Clustered according to 97% identity, a mean OTUs of 506 (SD 195) was identified. The average value of OTUs was 480 (asthma children:537, healthy children:416) before air pollution and 533 (asthma children:511, healthy children:582) after air pollution. The total counts of OTUs and common OTUs for the two measurements varied by child (Figure 1). AS for the difference of OTUs between the two measurements among all 21 children, thirteen children showed decreasing and eight children showed increasing. OTUs of two children with non-allergic asthma and two healthy children changed more than 40 percent. The other 17 children shared more than 90 percent OTUs before and after air pollution (Figure 1).

Analyzed by Wilcoxon Test, the counts of OTUs in all children didn’t show significantly changing before and after air pollution ($p=0.366$). However, the OTUs number of asthmatic children changed marginally significantly after air pollution ($p=0.068$). The number of OTUs were significantly different between asthmatic and healthy children (before air pollution: $p=0.002$, after air pollution $p=0.022$).

Figure 1

Variation of the Relative Abundance of Bacteria

At the phylum level

22 phyla were identified in samples of clean day (asthmatic children: 22, healthy children:14) and 36 phyla were identified in samples of polluted day (asthmatic children: 20, healthy children:35) in all children. The number of phyla varied before and after air pollution. The 22 phyla obtained in clean day were all identified in air polluted day. The 14 (38.9%) new identified phyla only found in 2 asthmatic and 2 healthy children and the relative abundance of them were range from 10-4~10-5.

The relative abundance of *Firmicutes, Bacteroidetes, Actinobacteria and Proteobacteria*
were 62.1%, 25.3%, 8.2% and 3.9%. They accounted for more than 99.0% of all bacteria before and after smog. Constituent ratio of the four major phyla didn’t show significant difference before and after air pollution in all children (Chi square test, \( p > 0.05 \)) and it didn’t show significant difference between asthmatic and healthy children (Chi square test, \( p > 0.05 \)) either.

The abundance of each phylum changed to a certain degree after air pollution in every child (Figure 2). As for the relative abundance of the four major phyla respectively in the asthmatic and healthy children, *Bacteroidetes* and *Actinobacteria* varied (marginally) significantly before and after air pollution (Wilcoxon Test, Table 2) in asthmatic children, in reverse, the relative abundance of *Firmicutes* and *Proteobacteria* changed marginally significantly in healthy children before and after air pollution (Wilcoxon Test, Table 2).

Taking the sequencing data of healthy children in the clean days as normal, *Bacteroidetes* decreased marginally significantly (Wilcoxon Test, \( p = 0.072 \)), and *Proteobacteria* increased significantly in asthmatic children (Wilcoxon Test, \( p = 0.020 \)).

**Figure 2**

**Table 2 Difference of the dominant phyla in investigated children affected by air pollution.**

| Phylum      | Clean day | Polluted day | \( p \) value |
|-------------|-----------|--------------|---------------|
|             | mean      | SD           | Mean          | SD           |               |
| Asthmatic Children |           |              |               |              |               |
| Firmicutes  | 63.777    | 0.175        | 59.942        | 0.107        | 0.365         |
| Bacteroidetes | 20.573    | 0.085        | 27.815        | 0.104        | 0.067*        |
| Actinobacteria | 9.899     | 0.107        | 7.848         | 0.068        | 0.001**       |
| Proteobacteria | 5.149     | 0.097        | 4.014         | 0.071        | 0.966         |
| Healthy Children |           |              |               |              |               |
| Firmicutes  | 64.884    | 0.088        | 59.714        | 0.104        | 0.106*        |
| Bacteroidetes | 25.841    | 0.084        | 27.319        | 0.135        | 0.846         |
| Actinobacteria | 7.903     | 0.050        | 6.870         | 0.048        | 0.770         |
| Proteobacteria | 1.235     | 0.004        | 5.083         | 0.081        | 0.064*        |

“*” was marginally significant difference, “**” was significant difference.
At genus level

294 genera were identified in clean day, 462 genera were identified in the polluted day in all children. Ten genera disappeared and 178 genera appeared after air pollution, but they only appeared in one or two samples and the relative abundance of them ranged from 10^-4 ~10^-5. In clean day 282 genera were identified in asthmatic children and 188 genera were identified in healthy children; in polluted day 266 genera were obtained from asthmatic children and 421 genera were obtained from healthy children. The number of genus changed more obviously in healthy children affected by air pollution.

The genera with a relative abundance over 1% were analyzed. Among these genera, *Clostridium_sensu_stricto_1* (Wilcoxon test, $p=0.01$) and *Bacteroides* (Wilcoxon test, $p=0.05$) changed significantly in asthmatic children,*Fusicatenibacter* (Wilcoxon test, $p=0.03$) and *Terrisporobacter* (Wilcoxon test, $p=0.03$) changed significantly in healthy team before and after air pollution. Composition of the dominant genera in each child, which with a relative abundance above 1%, changed to some extent after air pollution (Figure 3).

Figure 3

When taking the data in the clean days of healthy children as normal, *Prevotella_9* (decreased, Wilcoxon Test, $p=0.043$), *Eubacterium_hallii_group* (increased, Wilcoxon Test, $p=0.020$), *Lactobacillus* (increased, Wilcoxon Test, $p<0.001$) varied significantly, and *Bacteroides* (decreased, Wilcoxon Test, $p=0.099$), *Terrisporobacter* (decreased, Wilcoxon Test, $p=0.061$), *Eubacterium_coprostanoligenes_group* (increased, Wilcoxon Test, $p=0.085$), *Clostridium_sensu_stricto_1* (increased, Wilcoxon Test, $p=0.099$) and *Streptococcus* (increased, Wilcoxon Test, $p=0.091$) showed marginally significant variation in asthmatic children.
Diversity changing

Regarding alpha diversity of the bacteria (Figure 4), the mean of chao1 parameter was 478.5±102.9 (asthmatic children:536.3±98.7, healthy children:415.0±64.4) and the mean of Shannon index was 5.8±0.5 (asthmatic children:5.9±0.6, healthy children:5.8±0.3) in the sequencing for clean day; the value of chao1 parameter was 545.1±302.9 (asthmatic children:504.4±91.4, healthy children:589.7±436.3), and the value of Shannon index was 5.9±0.6 (asthmatic children:5.9±0.9, healthy children:5.9±0.7) in sequencing for air polluted day.

Analyzed by Wilcoxon test, Chao1 parameter \( p=0.584 \) and Shannon index \( p=0.533 \) of all samples didn’t show significant differences before and after air pollution, chao1 parameter (before air pollution: \( p=0.004 \), after air pollution: \( p=0.020 \)) was significantly different and Shannon index (before air pollution: \( p=0.121 \), after air pollution: \( p=0.349 \)) wasn’t significantly different between asthmatic and healthy children.

Figure 4

The microbial composition was evaluated by using principal coordinates analysis (PCoA, Figure 5), based on the unweighted UniFrac distance. It showed difference between asthmatic and healthy children. Variation wasn’t significant before and after air pollution. Adonis test with unweighted Unifrac distance confirmed that the microbial composition of the asthmatic children was different from that of the healthy children (before air pollution: \( R^2=0.302, p<0.05 \), after air pollution: \( R^2=0.222, p<0.05 \)).

Figure 5

Network Analysis

The network analysis showed the structure of intestinal microbiome of the four teams
(Figure 6). The networks between AC1 (asthmatic children in clean day) and HC1 (healthy children in clean day) was quite different. Both AC1 and HC1 changed after air pollution. The variation was mainly about the relative abundance of taxa and correlation among each other. Accompanied by the variation, the community structure of intestinal microbiome was different before and after air pollution.

The network analysis further demonstrated that the most dominant functional bacteria were from *Firmicutes*. And the main variation happened in *Firmicutes* when air pollution happened. *Firmicutes* must played the most important role in maintaining intestinal microbes balance. It could be seen that the composition of intestinal bacteria showed balance relatively in healthy children in clean day. When in asthmatic children the unbalance of microbiome appeared. After experiencing the polluted day, the relative abundance of some taxa varied, the correlation among taxa became more complex than before both in asthmatic and healthy children. Specifically speaking, after the polluted days, the relative abundance of bacteria from *Firmicutes* varied obviously compared with that before air pollution both in the two teams, such as *Rombotsia, Lachlostridium* and so on. Furthermore, the correlation between main bacteria varied, such as *Faecalibacterium*, which was found to be correlated with less kinds of bacteria after air pollution in asthmatic children. And more negative correlation among taxa appeared when after air pollution.

There were some changes in the other phylum. *Prevotella_9* from phylum of *Bacteroidetes* was correlated with *Staphylococcus* and *Fusicatenibacter* in team of HC1, then it influenced more bacteria after air pollution both in teams of AC2 (asthmatic children in polluted day) and HC2 (healthy children in polluted day). *Mannomonas, Polycyclovorans* and *Vibrio*, which were from phylum of *Preteobacteria*, were independent relatively before air pollution in team of AC1, then they correlated with more taxa after air pollution.
However, there were no significant correlation between them and other taxa in AC1.

Figure 6

Linear discriminant analysis (LDA) of effect size (LEfSe)

In group of healthy children, genus of *Fusicatenibacter* was the biomarker before air pollution happened (Figure 7). The function as biomarker of *Fusicatenibacter* disappeared after air pollution, which implied the impact of air pollution on it. In asthmatic children, phylum of *A0839*, genus of *chiayiivirga* and species of *Actinomyces_sp._oral_clone_GU009* were the biomarkers before air pollution; class of *Bacteroidia* and order of *Bacteroidales* were the biomarkers after air pollution (Figure 7). The bacteria biomarkers between asthmatic and healthy children based on clean days were seen detailly in Figure 7 (AC1 vs HC1).

Figure 7

Analysis for impact of environmental factors

Based on the above analysis result, the following bacteria varied significantly before and after smog, including *Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria* and *A0839* at phylum level, *Bacteroidia* at class level, *Bacteroidales* at order level, *Clostridium_sensu_stricto_1, Bacteroides, Fusicatenibacter, Terrisporobacter* and *Chinayiivirga* at genus level, *Bacteroides_uniformis* and *Actinmyces_sp_oral_ctone_Gu009* at species level. The relationship between relative abundance of these bacteria and environmental pollutants were analyzed by multiple level analysis and multiple linear regression analysis.

As a panel study, the result was analyzed by multiple level analysis firstly. There was no
significant difference between the twice sequencing of each child for every taxon ($p > 0.05$). Then the analysis for impact of polluted environment factors on intestinal microbiome was back to method of multiple linear regression. Backward method was used and the factors of age, male, BMI, environmental temperature and O3 were adjusted. The multiple linear regression showed that the relative abundance of *Firmicutes* were significantly related with SO2 and NO2, *Bacteroidetes* and *Bacteroidia* were significantly related with SO2, *Clostridium sensu stricto_1* were significantly related with SO2 and NO2, *Terrisporobacter* were significantly related with PM2.5, PM10, SO2 and NO2, *Fusicatenibacter_1* were significantly related with PM2.5, PM10 and NO2 (Table 3). Concentration of O3 was evaluated in every multiple linear regression model, it was correlated with every taxon above ($P < 0.05$).

Table 3 Impact of Environmental Pollutants on Intestinal Microbiome
| taxa               | Level | air pollutant | β value | p value | F value | p value | Adjusted $R^2$ |
|-------------------|-------|---------------|---------|---------|---------|---------|--------------|
| Firmicutes        | phylm | SO$_2$        | -0.0316 | 0.019   | 3.85    | 0.0103  | 0.218        |
|                   |       | NO$_2$        | -0.0095 | 0.029   | 3.67    | 0.0129  | 0.207        |
| Bacteroidetes     | phylm | SO$_2$        | 0.0259  | 0.033   | 2.32    | 0.0909  | 0.088        |
| Bacteroidia       | class | SO$_2$        | 0.0287  | 0.015   | 2.97    | 0.0439  | 0.126        |
| Clostridium_sensu_stricto_ | genus | SO$_2$        | -0.0027 | 0.004   | 3.84    | 0.0104  | 0.217        |
| Clostridium_sensu_stricto_ |       | NO$_2$        | -0.0006 | 0.075   | 2.13    | 0.0965  | 0.099        |
| Fusicatenibacter  | genus | PM$_{2.5}$    | -0.0009 | 0.019   | 4.61    | 0.0024  | 0.305        |
|                   |       | PM$_{10}$     | -0.0006 | 0.009   | 4.58    | 0.0025  | 0.304        |
|                   |       | NO$_2$        | -0.0021 | 0.015   | 4.30    | 0.0036  | 0.287        |
| Terrisporobacter  | genus | PM$_{2.5}$    | -0.0006 | 0.003   | 5.67    | 0.0003  | 0.406        |
|                   |       | PM$_{10}$     | -0.0004 | 0.001   | 7.18    | 0.0001  | 0.430        |
|                   |       | SO$_2$        | -0.0026 | 0.031   | 4.28    | 0.0037  | 0.286        |
|                   |       | NO$_2$        | -0.0013 | 0.001   | 6.22    | 0.0002  | 0.433        |

Adjusted the factors of age, sex, BMI, environmental temperature and $O_3$.

Discussion
This study confirmed that composition of intestinal microbiome in asthmatic children were different from healthy children significantly, and air pollution had effects on intestinal microbiome both in asthmatic and healthy children.

As same as previous studies, *Firmicutes, Bacteroidetes, Actinobacteria* and *Proteobacteria* were the dominant phyla in human gut [23], they accounting for >99% of the total phyla both in asthmatic and healthy children in this study. Composition of intestinal microbiome was significantly different between asthmatic and healthy children, which was confirmed by analysis of PCoA and Adonis test. Network analysis showed the difference of community structure of intestinal microbiome between asthmatic and healthy children too. Most previous studies indicated an association between the intestinal microbiome composition and atopic sensitization or symptoms in time of early life [9,11,20-22]. The discovery of this article showed that intestinal microbiome was significantly different between asthmatic and healthy children. It suggested that action on mucosal of gut bacteria was a sustained process, which may influence the immune response all the time, intestinal microbiome may play an important role during the development of asthma.

As for the relative abundance of some main bacteria *Firmicutes, Bacteroidetes,*
Actinobacteria and Proteobacteria), constituent ratio of them didn’t change significantly after the smog, but the relative abundance of them varied. Bacteroidetes and Actinobacteria changed significantly in asthmatic children, in verse Firmicutes and Proteobacteria varied significantly in healthy children (Table 2). It illustrated that air pollution could influence intestinal microbiome and the impact of air pollution on intestinal bacteria was different between asthmatic and healthy children.

Firmicutes with the biggest relative abundance (>60%), occupied a dominant position in human’s gut bacteria. Fujimura [24] had reported that Firmicutes was a protected factor for asthma. And Faecalibacterium, which came from Firmicutes, was enriched in the gut of healthy people [25]. The result of this article indicated that the variation of intestinal bacteria induced by asthma or air pollution all mainly happened on Firmicutes (Figure 6), the relative abundance of Firmicutes in asthmatic children was lower than it in healthy children, and it declined marginally significantly after smog (p < 0.106, Table 2). The statistical analysis showed that the relative abundance of Firmicutes was significantly negatively correlated with concentration of SO₂ and NO₂ in all participants. These findings suggested that reduction of Firmicutes would do bad on health, which agreed with previous studies, and air pollution could act on bacteria of Firmicutes to do harm to health.

Though the relative abundance of Firmicutes in asthmatic children also declined in our
study on the whole (Table 2), the relative abundance of some bacteria belonging to Firmicutes increased in genus level in asthmatic children, including Streptococcus (Wilcoxon test, \( p=0.091 \)), Lactobacillus (Wilcoxon test, \( p<0.001 \)), Clostridium_sensu_stricto_1 (Wilcoxon test, \( p=0.099 \)), Eubacterium_hallii_group (Wilcoxon test, \( p=0.020 \)) and Eubacterium_coprostanoligenes_group (Wilcoxon test, \( p =0.085 \)). It revealed that balance of Firmicutes in intestinal bacteria may be more important for human health. And there was a study reported that Lactobacilli and Eubacteria were more prevalent in a low prevalence of childhood allergy [26], which was different with this article. The reason may be that the research objects of these articles were different, the previous study focused on the allergic disease, and this article only aimed to study the asthma, and some asthmatics were not allergic in this study.

Bacteroidetes was another important phylum in this study, the relative abundance of it increased marginally significantly after air pollution in asthmatic children. The relative abundance of Bacteroidetes in phylum level and Bacteroidia in class level positive correlated with concentration of \( SO_2 \). Bacteroidates in Order level, Bacteroidia in class level and Bacteroides_uniformis were the biomarkers influenced by air pollution in asthmatic children. All of these results illustrated that Bacteroidetes could be influenced by air pollution. In Jakob’s article [11], children at 1-year-old, who had high risk for asthma, had more relative abundance Bacteroides.
Whether the air pollution could trigger asthma through making \textit{Bacteroidetes} increase was worthy of further study. In this study, the relative abundance of \textit{Bacteroides} was lower in asthma children compared with healthy children (Wilcoxon test, $p=0.072$). The reason may be related with a limitation of this study. The size of samples in this research was small. As mentioned before, there were 27% non-allergic children in asthmatic team, so the reason needed to be further researched. A larger relative abundance in each team was another important bacterium from phylum \textit{Bacteroidetes}. The relative abundance of \textit{Prevotella_9} declined in asthmatic children (Wilcoxon test, $p=0.043$), though multiple linear regression analysis didn’t show it correlated with environment pollutants, the correlation with other bacteria changed in all teams (Figure 6). The function of it was worthy of further research.

\textit{Actinobacteria} and \textit{Proteobacteria} were all varied before and after air pollution. \textit{Actinobacteria} declined significantly after air pollution in asthmatic children. \textit{Proteobacteria} was the biomarker of asthmatic children (Figure 7), the abundance of it increased after air pollution, and correlated with more other bacteria in asthmatic children (Figure 6). \textit{Proteobacteria} increased marginally significantly after air pollution in healthy children (Table 1). Air pollution may increase risk of asthma developing through \textit{Proteobacteria}.

Previous studies have found that the diversity of intestinal microbiome decreased in some allergic disease, such as atopic dermatitis, and low species diversity may
relate with disease development. Result of this study didn’t show a significant changing about species diversity in healthy and asthmatic team. The reason may be that some non-allergic asthma children (p9, p10 and p11) were in the asthmatic team, they have more OTUs than other asthmatic children, and they have higher Chao1 index than other asthmatic children. But size of them was small. We didn’t analyze them separately. Microbiome of non-allergic asthma deserves to be further studied. Species diversity of intestinal microbiome didn’t show significantly variation before and after air pollution. The impacts of polluted environmental factors were analyzed based on number and relative abundance of the identified species. There were some changings before and after air pollution. The number of OTUs identified from each child has changed ranging from 4.7%-79% after air pollution. More phyla and genera were identified after air pollution, they all account for less than $10^4$. However, this phenomenon may be the first step for the changing of composition of intestinal microbiome. This changing may disturb the balance of intestinal microflora. Just as it was shown in the network analysis, the taxa with significant correlations changed and the correlation among each taxon varied before and after air pollution both in healthy and asthmatic teams. The findings about no significant changing before and after air pollution might related with that the interval between sampling was short. Based on theories of previous researches, the difference between the
asthmatic

and healthy children was shaped from birth, even in uterus. Composition of intestinal microbiome maintain a dynamic balance. There were many factors can influence intestinal microbiome, such as age, gender, diet and environment and so on. But the influence of them didn’t work in a short time [27,28], maybe so did the environment. From the result of this article, changings induced by air pollutants had emerged. When put all samples together to analyzed using multiple linear regression, the relative abundance of some bacteria was significantly influenced by environment pollutants, which illustrated the environmental action on intestinal bacteria.

There were two limitations about this study. The first one was that the sample size was small. Because this study was part of a big project, which main aimed to study on non-allergic asthma. As well know, morbidity of non-allergic asthma accounts for a relatively low proportion among all asthma. This study was implemented at the beginning of the main project, when fewer patients were enrolled to the group. Compared with other article about study of composition of intestinal microbiome with 3-4 individuals, this research still had a reference value [29,30]. The second limitation was that the interval of sampling was short. This article aimed to explore the air-pollution’s effects on asthmatic children preliminary, few studies could be referenced. So that this article designed sampling interval depending on the growth cycle of microorganisms.
As shown in the result, too short time might not reflect the effect of air pollution significantly. Based on the result preliminary, project team will overcome the limitation to further study on impact of air pollution on children asthma.

Conclusion

Just as microbiome in asthma play an important role in pathogenesis, phenotype and response to treatment [31,32], gut microbiota had been as a target for preventing and treating allergic disease [33]. This study found the main variation in composition of intestinal microbiome in asthmatic children, it always found some microorganism biomarkers for asthma or air pollution, which may help to reduce and prevent children asthma through adjusted the composition of intestinal microbiome. And air pollution could impact on intestinal microbiome both in asthmatic and healthy children, personal protective measures for children should be strengthened in polluted day, which can help to research the mechanism of asthma influenced by air pollution and help to prevent asthma development. In addition, the effect of air pollution on allergic and non-allergic children may be different, we will further study on the character of intestinal microbiome of allergic and non-allergic asthma. Study of this article would have a quite significance for public health and clinic.

Methods

Study populations

This study was part of a panel study, founded by National Natural Science Foundation of China. 11 children with asthma and 10 healthy children without asthma among 176 child
participants of the study were selected and agreed to join the air pollution study. All the 21 participants aged from 5 to 12 years old were asked to offer information about the living environment, study environment, history of disease, family history of asthma or allergy, and diet with a questionnaire which was revised on the basis of ISAAC (The International Study of Asthma and Allergies in Childhood) Phase Three Environmental Questionnaire, ISAAC core questionnaires on asthma, and NHANES (National Health and Nutrition Examination Survey) Food Questionnaire. Asthmatic children were diagnosed according to the Global Initiative for Asthma (GINA, http://ginasthma.org) by physicians from Children Hospital of Capital Institute of Pediatrics in Beijing. This project was approved by ethical review committee of Institute for Environmental Health and Related Product Safety, Chinese Center for Disease Control and Prevention. All participants signed the written informed consents, which described the purposes, methods and expected results of the study detailly.

Samples collection

Feces collection were required for all participants. All participants were asked to meet the followed criteria, including no antibiotic using history and vaccination history in the past one month, no injury and diarrhea in the past two weeks. In addition, girls were not in the period of menstrual cycle when offering feces.

Feces samples were collected twice. The collections were performed at the time before and after the smog developed respectively. AQI of the five days before the day sampling for clean day were all less than 100. The samples collection for polluted day were at the 3rd to 5th day in the polluted days, AQI of these days were all more than 100. Feces samples were collected with a sterile 30 mL plastic tubes, then the tubes were transported to the laboratory in an anaerobic tank at 4°C within four hours. Before detecting, the samples were stored at temperature of -80°C.
Samples were divided into four teams. They were asthmatic children in clean days (AC1), asthmatic children in polluted days (AC2), healthy children in clean days (HC1) and healthy children in polluted days (HC2).

DNA extracting and Sequencing

Total genome DNA was extracted from 0.5g feces sample using a modified CTAB (Cetyltrimethylammonium Ammonium Bromide) method [34,35]. DNA was assessed by 1% agarose gel electrophoresis, then it was diluted into 1ng/μL using sterile water for sequencing. 16SrRNA genes were sequenced on Illumina platform at Novogene Bioinformatics Technology Co., Ltd. The V4 region of 16SrRNA genes were amplified by using the specific primers of 515F[GTTGAGCMGCCGCGGTAA] and 806R[GGACTACHVGGGTWTCTAAT] with the barcodes. PCR reactions were carried out in a 30 μL system with 15 μL of Phusion High-Fidelity PCR Master Mix (New England Biolabs, USA), 0.2 μM of forward and reverse primers, and 10 ng template DNA. Thermal cycling consisted of initial denaturation at 98 ℃ for 1 min, 30 cycles of denaturation at 98 ℃ for 10 s, annealing at 50 ℃ for 30 s, elongation at 72 ℃ for 30 s, and finally keeping at 72 ℃ for 5min (Bio-rad T100 Thermal Cycler, USA). Then mixed PCR products with same volume of loading buffer and operated electrophoresis on 2% agarose gel for detection. Samples with 400-450bp were chosen and purified (GeneJET Gel Extraction Kit, Thermo Scientific, USA) for further experiments. Then the sequencing libraries were generated using TruSeq® DNA PCR-Free Sample Preparation Kit (Illumina, USA) and sequenced on an Illumina HiSeq 2500 (Illumina, USA).

Sequence analysis

Paired-end reads from the original DNA fragments were merged using FLASH [36], which merged paired-end reads when there were overlaps between reads. Paired-end reads was assigned to each sample according to the unique barcode. Sequences were analyzed using
the Quantitative Insights into Microbial Ecology (QIIME). Reads were filtered by QIIME quality filters to get Operational Taxonomic Units (OTUs). Sequences with ≥97% similarity were assigned to the same OTUs. A representative sequence was picked for each OTU and the RDP Classifier used to annotate taxonomic information for each representative sequence. Species were annotated according to the SSU rRNA data base in SILA(http://www.arb-silva.de) using the MUSCLE software (Version 3.9.31).

Statistical analysis

Statistical analyses were performed by the R software (V 3.4.). The significance of OTUs, α-diversity index (including chao1 and Shannon Index) and the relative abundance of bacterial at level of phylum and genus among the four teams were tested using Wilcoxon test. The changing of microbial composition was showed using principal coordinates analysis (PCoA) based on the unweighted UniFrac distance [37]. Correlation of co-currency bacteria was analyzed by network analysis, in which a Spearman’s correlation between two genera was calculated [38,39]. Significant biomarkers were evaluated by the Linear Discriminant Analysis (LDA) of effect size (LEfSe) analysis [40]. Multiple level analysis and multiple linear regression analysis was used to analyzed the impact of air pollution on intestinal microbiome. All reported p values were two-tailed, and a p value of<0.05 was considered significant.

List Of Abbreviations
| Abbreviations | Full Name |
|---------------|-----------|
| AQI | Air Quality Index |
| AC1 | asthmatic children in clean day |
| AC2 | asthmatic children in polluted day |
| BMI | Body Mass Index |
| GINA | Global Initiative for Asthma |
| HC1 | healthy children in the clean day |
| HC2 | healthy children in polluted day |
| ISAAC | International Study of Asthma and Allergies in Childhood |
| LDA | Linear discriminant analysis |
| LEfSe | Linear discriminant analysis of effect size |
| OTUs | Operational Taxonomic Units |
| SD | Standard Deviation |

Declarations

Ethics approval and consent to participate

This project was approved by ethical review committee of Institute for Environmental Health and Related Product Safety, Chinese Center for Disease Control and Prevention at Feb 15, 2015.

Consent for publication

The consents to participate in this study were obtained from all participating children and their parents or legal guardians. All participants and their parents or legal guardians signed the written informed consents, which described the purposes, methods and expected results of the study detailly.

Availability of data and materials

The datasets generated or analyzed during the current study will be available in National Center for Biotechnology Information. https://www.ncbi.nlm.nih.gov/.

Competing interests

The authors declare that they have no competing interests.
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Authors' contributions
PZ, QW and XB were in charge of design the study. CL and LS performed the diagnosis of asthmatic children. PZ, KZ, XL, XN and NX were responsible for collecting feces samples. PZ analyzed and interpreted the data and wrote the paper. All authors read and approved the final manuscript.

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References
1. Global Initiative for Asthma. Global Strategy for Asthma Management and Prevention, 2016. Available from: www.ginasthma.org.

2. National Cooperative Group on Childhood Asthma Institute of Environmental Health and Related Safety, Chinese Center for Disease Control and Prevention. Third nationwide survey of childhood asthma in urban areas of China. Chinese J Pediatr. 2013; doi: 10.3760/cma.j.issn.0578-1310.2013.10.003.

3. O’Connor GT, Neas L, Vaughn B, et al. Acute respiratory health effects of air pollution on children with asthma in US inner cities. J Allergy Clin Immunol. 2008;121:1133-1139.
4. Gudrun W, Elisa R, Manuela DS, Weiland SK, Francesco F. Short-term effects of PM10 and NO2 on respiratory health among children with asthma or asthma-like symptoms: a systematic review and meta-analysis. Env Heal Perspect. 2010;118:449-457.

5. Guarnieri M, Balmes JR. Outdoor air pollution and asthma. Food Chem Toxicol An Int J Publ Br Ind Biol Res Assoc. 2014; doi: 10.1016/S0140-6736(14)60617-6.

6. Gowers AM, Paul C, Ayres JG, et al. Does outdoor air pollution induce new cases of asthma? Biological plausibility and evidence; a review. Respirology. 2012;17:887-898.

7. Mazmanian SK, Round JL, Kasper DL. A microbial symbiosis factor prevents intestinal inflammatory disease. Nature. 2008;453:620-625.

8. Penders J, Stobberingh E E, Brandt P A V D, et al. The role of the intestinal microbiota in the development of atopic disorders. Allergy (Oxford). 2007; 62:1223-1236.

9. Strachan DP, Taylor EM, Carpenter RG. Family structure, neonatal infection, and hay fever in adolescence. Arch Dis Child. 1996;74:422-426.

10. Stiemsma LT, Reynolds LA, Turvey SE, Finlay BB. The hygiene hypothesis: current perspectives and future therapies. Immunotargets Ther. 2015;4:143-157.

11. Stokholm J, MJ B, Thorsen J, et al. Maturation of the gut microbiome and risk of asthma in childhood. Nat Commun. 2018;9:141.

12. Lathrop SK, Bloom SM, Rao SM, et al. Peripheral education of the immune system by colonic commensal microbiota. Nature. 2011;478:250-254.

13. Macpherson AJ, Harris NL. Interactions between commensal intestinal bacteria and the immune system. Nat Rev Immunol. 2004;4:478-485.

14. Lyons A, O’Mahony D, O’Brien F, et al. Bacterial strain-specific induction of Foxp3+ T regulatory cells is protective in murine allergy models. Clin Exp Allergy. 2010;40:811-819.

15. Torsten O, Dingding A, Sebastian Z, et al. Microbial exposure during early life has persistent effects on natural killer T cell function. Science. 2012;336:489-493.
16. Stiemsma LT, Turvey SE. Asthma and the microbiome: defining the critical window in early life. Allergy Asthma Clin Immunol Off J Can Soc Allergy Clin Immunol. 2017;13:3.

17. Marsland BJ, Trompette A, Gollwitzer ES. The Gut-Lung Axis in Respiratory Disease. Annals of the American Thoracic Society. 2015;12 Suppl 2:S150.

18. Aurélien T, Gollwitzer ES, Koshika Y, et al. Gut microbiota metabolism of dietary fiber influences allergic airway disease and hematopoiesis. Nat Med. 2014;20:159.

19. Salim SY, Kaplan GG, Madsen KL. Air pollution effects on the gut microbiota. Gut Microbes. 2014;5:215-219.

20. Björkstén B, Sepp E, Julge K, Voor T, Mikelsaar M. Allergy development and the intestinal microflora during the first year of life. J Allergy Clin Immunol. 2001;108:516-520.

21. Ball TM, Castro-Rodriguez JA, Griffith KA, Holberg CJ, Martinez FD, Wright AL. Siblings, day-care attendance, and the risk of asthma and wheezing during childhood. N Engl J Med. 2000;343:538-543.

22. Ege MJ, Melanie M, Anne-Cécile N, et al. Exposure to environmental microorganisms and childhood asthma. N Engl J Med. 2011;364:701-709.

23. Iizumi T, Battaglia T, Ruiz V, et al. Gut Microbiome and Antibiotics. Archives of Medical Research, 2017:48:727-734.

24. Fujimura KE, Sitarik AR, Havstad S, et al. Neonatal gut microbiota associates with childhood multisensitized atopy and T cell differentiation. Nat Med. 2016;22:1187-1191.

25. Wang Q, Li F, Liang B, et al. A metagenome-wide association study of gut microbiota in asthma in UK adults. BMC Microbiol. 2018; doi:10.1186/s12866-018-1257-x.

26. Bjorksten B. The intestinal microflora in allergic Estonian and Swedish 2-year-old children. Clin Exp Allergy, 1999; doi:10.1046/j.1365-2222.1999.00560.x.

27. Turnbaugh PJ, Ridaura VK, Faith JJ, Rey FE, Rob K, Gordon JL. The effect of diet on the human gut microbiome: a metagenomic analysis in humanized gnotobiotic mice. Sci Transl
28. Wu GD, Christian H, Kyle B, et al. Linking long-term dietary patterns with gut microbial enterotypes. Science. 2011; doi:10.1126/science.1208344.

29. Shigemitsu T, Takako K, Prapa S, et al. Influence of antibiotic exposure in the early postnatal period on the development of intestinal microbiota. Fems Immunology & Medical Microbiology. 2009; 56:80-87.

30. Dethlefsen L, Relman D A. Incomplete recovery and individualized responses of the human distal gut microbiota to repeated antibiotic perturbation. Proc Natl Acad Sci USA, 2011;108:4554-4561.

31. Singanayagam A, Ritchie AI, Johnston SL. Role of microbiome in the pathophysiology and disease course of asthma. Curr Opin Pulm Med. 2017;23:41.

32. Ariangela J. Kozik, Yvonne J, Huang. The microbiome in asthma: Role in pathogenesis, phenotype, and response to treatment. Annals of Allergy, Asthma & Immunology 2019;122: 270-275.

33. Aitoro R, Paparo L, Amoroso A, et al. Gut Microbiota as a Target for Preventive and Therapeutic Intervention against Food Allergy. Nutrients. 2017; 9:672.

34. Simachew A, Lanzén A, Gessesse A, Øvreås L. Prokaryotic Community Diversity Along an Increasing Salt Gradient in a Soda Ash Concentration Pond. Microb Ecol. 2016; 71:326-338.

35. Archer SDJ, Mcdonald IR, Herbold CW, Lee CK, Cary CS. Benthic microbial communities of coastal terrestrial and ice shelf Antarctic meltwater ponds. Front Microbiol. 2015; 6:485.

36. Tanja M, Salzberg SL. FLASH: fast length adjustment of short reads to improve genome assemblies. Bioinformatics. 2011; 27:2957-2963.

37. Lozupone C, Lladser ME, Dan K, Stombaugh J, Knight R. UniFrac: an effective distance
metric for microbial community comparison. Isme J. 2011; 5:169-172.

38. Jiao S, Liu Z, Lin Y, Yang J, Chen W, Wei G. Bacterial communities in oil contaminated soils: Biogeography and co-occurrence patterns. Soil Biol Biochem. 2016; 98:64-73.

39. Junjie Q, Yingrui L, Zhiming C, et al. A metagenome-wide association study of gut microbiota in type 2 diabetes. Nature. 2012; 490:55-60.

40. Segata N, Izard J, Waldron L, et al. Metagenomic biomarker discovery and explanation. Genome biology. 2011; doi: 10.1186/gb-2011-12-6-r60.

Figures
OTUs number for each sample before and after air pollution. P1-P11 were ID number of asthmatic children, P1-P8 were children with allergic asthma, P9-P11 were children with non-allergic asthma. C1-C10 were ID number of healthy children. The numbers in the circle was the quantity of identified OTUs. A was the sequencing at clean day, B was sequencing at polluted day.
Figure 2

Bar plot of relative abundance of the dominant phyla for all samples. The figure showed the changings of phyla’s relative abundance before and after air pollution. P1-P11 were ID number of asthmatic children, C1-C10 were ID number of healthy children.
Figure 3

Bar plot of genera with a relative abundance over 1% for each sample. The figure showed the changings of genera’s relative abundance before and after air pollution. P1-P11 were the samples from asthmatic children, C1-C10 were the samples from healthy children.
Chao1 and Shannon Index for each team. Chao1 index as a measure of richness and Shannon index as a measure of both richness and evenness. Team of asthmatic children in clean day (AC1) was in orange, team of asthmatic children in polluted day (AC2) was in grass green, team of healthy children in clean day (HC1) was in green, team of healthy children in polluted day (HC2) was in pink.
PCoA plot

Figure 5

PCoA plot with unweighted Unifrec-distance of four teams. Team of asthmatic children in clean day (AC1) was in red, team of asthmatic children in polluted day (AC2) was in blue, team of healthy children in clean day (HC1) was in green, team of healthy children in polluted day (HC2) was in orange.
Network analysis of four teams. It was applied to exploring co-occurrence patterns between microbial taxa to explore the mathematical, statistical and structural properties of them. The network showed the correlation identified from pairwise comparison of the genera abundance, in which the node represents a genus, the connection stands for a strong (Spearman’s $r > 0.6$) and significant (p value $< 0.01$) correlation. The red connected line represented positive correlation.
and the blue ones represented negative correlation. It described the complex pattern of interrelationships between OTUs in every team. HC1 was healthy children in the clean day, HC2 was healthy children in polluted day, AC1 was asthmatic children in clean day, AC2 was asthmatic children in polluted day.
LEfSe analysis for biomarkers. It was used to realize the impact of species with significant differences, to explore the biomarker for impact on intestinal microbiome of air pollution. LEfSe analysis distinguishing the microbial communities of each groups by a LDA score above 3.5. HC1 was healthy children in the clean day, HC2 was healthy children in polluted day, AC1 was asthmatic children in clean day, AC2 was asthmatic children in polluted day.

Figure 7
