Effect of Xiaoning Liquid on Gut Microbiota in Asthmatic Mice by 16s RDNA High - Throughput Sequencing

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Research

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Abstract

Background: To investigate the effects of Xiaoning liquid on gut microbiota in mouse during asthma.

Methods: A total of 60 mice were randomly and averagely assigned to healthy control group, control group, budesonide group, and Xiaoning liquid group. The later three groups were used to establish an Ovalbumin (OVA) asthma model. The intestinal bacterial communities were compared among groups using 16S rRNA gene amplification. Analyzing the structure of gut microbiota with OTU analysis, Shannon–Wiener, PCA, PCOA, etc. 16s rDNA high-throughput sequencing.

Results: The abundance and diversity of the gut microbiota in asthmatic mice increased, most obviously in the control group. The Bacteroidetes and Firmicutes levels increased in all asthmatic mice. The level of Bacteroides increased most obviously, making Bacteroides a useful marker of gut microbiota changes in asthmatic mice. The levels of Proteobacterium, Deferribacteraceae and Mucispirillum dropped significantly in the Xiaoning liquid group.

Conclusions: Xiaoning liquid can reduce the species and numbers of pathogenic bacteria and restored the intestinal microecology of asthmatic mice. Xiaoning liquid has a positive effect on the function of gut microbiota.

Background

Asthma is a chronic inflammatory disease of the respiratory airways characterized by an inappropriate immune response resulting in reversible airflow obstruction, airway hyper-responsiveness (AHR), mucus overproduction, tissue eosinophilia, and intense airway wall remodeling [1]. Asthma is one of the most common chronic respiratory diseases worldwide. Epidemiological studies have estimated that 250,000 deaths can be linked to this disease each year, and more than 600 million people have asthma-related symptoms [2]. It incurred significant health care expenditure. Although incurable, asthma can be controlled with appropriate drugs, self-management education, and by avoiding exposure to allergens [3].

Gut microbiota, a population of microbes residing in the gastrointestinal tract, play a pivotal role in maintaining the host health. Gut microbiota can be affected by diet, age, antibiotic use and environmental factors [4–6]. The microflora hypothesis, proposed by Noverr and Huffnagle [7] in 2005, has been widely accepted as a potential explanation of the relationship between intestinal flora and asthma. This has led to the concept of the “gut–lung axis”. The gut microbiota is composed of hundreds to thousands of bacterial species belonging mostly to the Firmicutes and Bacteroidetes phyla [8]. Recent research found that asthma is correlated with the decrease of intestinal probiotics, in which Bifidobacterium and Lactobacillus are the most representative [9]. Arancha Hevia et al. found that long-term asthma patients demonstrated a lower level of Bifidobacteria compared with those with newly developed asthma [10]. Asthma can decrease the diversity and disrupt the composition of gut microbiota [11]. Several studies found Clostridium spp. are implicated in the increased risk of asthma. Stiemema et al. studied a population of children diagnosed with asthma at preschool age in whom they found...
evidence of gut bacterial dysbiosis [12], and a reduction of Lachnospira in favor of Clostridium spp. was potentially linked to asthma. This bacterial dysbiosis was confirmed in other studies of the same group of authors, in which they showed the relative abundance of the bacterial genera Lachnospira and the decrease of Veillonella, Faecalibacterium, and Rothia in children at risk of asthma [13].

A well-running gut microbiota is helpful to prevent allergic diseases, such as asthma. Studies have shown that the imbalanced gut microbiota can be resumed by sorts of traditional Chinese medicine (TCM). Xuet et al. found that Gegen Qinlian decoction alleviated type 2 diabetes (T2D) through enriching the beneficial bacteria in the gut [14]. SheganMahuang Decoction (Synopsis of the Golden Chamber) is a TCM prescription for the ‘coldness’ and ‘stagnation’ in the lung and throat. It removes phlegm and gas in acute and chronic bronchitis, pneumonia, bronchial asthma, allergic rhinitis, etc. Refined from SheganMahuang Decoction, Xiaoning liquid contains the effective components from ten medicinal herbs: Ephedra sinica, Belamcanda chinensis, Asarum heterotropoides, Lepidium apetalum, Tussilago farlara, Aster tataricus, Citrus aurantium, etc. Previously, we showed Xiaoning liquid cured children with asthma with its anti-inflammatory, anti-asthmatic, antitussive, antispasmodic and antiviral effects [15–17]. Some elements in Xiaoning liquid are anti-inflammatory and antiviral, such as the isoflavone compound in the Belamcanda chinensis, the flavonoid compound in the Ephedra sinica, the benzyl mustard oil in the Lepidium apetalum [18–20]. Ephedra sinica alkaloids can alleviate the spasm in bronchial smooth muscles. The volatile oil in Asarum heterotropoides and the cofed ketone in Tussilago farlara can inhibit the release of inflammatory factors [21]. All these substances co-worked to relieve the asthma in the mice [22]. Besides, it plays a dual role in restoring the imbalanced gut microbiota and coordinating with the host immune system [23–26]. However, the effects and mechanisms of Xiaoning liquid on the function of gut microbiota in asthma still unclear. In this study, 16s rDNA sequencing was used to clarify the effects on gut microbiota of Xiaoning liquid in asthma.

**Materials And Methods**

**Study Materials**

Xiaoning liquid and Pulmicort were prepared by the Hospital of Chengdu University of Traditional Chinese Medicine (Chengdu, China). Xiaoning liquid is a patented prescription (Patent no. ZL201010190966.8), specification, 10 ml/bottle [27]. The liquid consisted of the elements from Belamcanda chinensis (L.) DC., Ephedra sinica Stapf or Ephedra intermedia Schrenk et C.A.Mey. or Ephedra equisetina Bge., Aster tataricus L. f., Asarum heterotropoides Fr. Schmidt var. mandshuricum (Maxim.) Kitag. or Asarum sieboldii Miq. var. seoulense Nakai or Asarum sieboldii Miq., Descurainia sophia (L.) Webb. ex Prantl. or Lepidium apetalum Willd., Tussilago farlara L., Citrus aurantium L., Scutellaria baicalensis Georgi, Pheretima aspergillum (E.Perrier) or Pheretima vu1garis Chen or Pheretima guillelmi (Michaelsen) or Pheretima pectinifera Michaelsen, Platycodon grandiflorum (Jacq.) A. DC.. Pulmicort (budesonide suspension) (AstraZeneca, Australia) was used: 2 ml of suspension (containing 1 ml of budesonide) in each bottle; catalog number, LOT320776.
Ovalbumin (OVA) was obtained from Sigma (USA). Components: water (by Kal Fischer), ≤ 10%; nitrogen, ≥ 12.5%; agarose electrophoresis ovalbumin, 62–88%. The 2% agarose gel was purchased from Biowest (Spain), DNA extraction kit OMEGA-soil DNA Kit from Omega (USA), FastPfu Polymerase from TransG (China), AxyPrep DNA Gel Extraction Kit Axygen Biosciences from Axygen (USA), and Illumina MiSeq platform TruSeqTM DNA Sample Prep Kit from Illumina (USA).

**Mouse Model of Aronchial Asthma**

Sixty BALB/C female mice (SPF, age of 6–8 weeks, body weight of 18 ± 2 g) were obtained from Chengdu Dashuo Biotechnology Co., Ltd. Animal license number: SCXK (chuan) 2015-030. After adaptation to feeding, 15 mice were randomly assigned to the HC (healthy control) group, and the other 45 were used to establish an OVA asthma model with a method previously described and slightly modified by us [28–30].

First, at the 1st and 14th day after the experiment started, 200 µL of sensitizing solution (100 µg of OVA, 200 µg of dry aluminum hydroxide, and 200 µL of PBS) was injected into the right abdominal cavity of each mouse, the same amount of PBS was injected into each mouse in the HC group through the same route. The modeled mice were randomly divided into three groups (15 mice/group): XN (Xiaoning liquid), control and budesonide group.

During day 21 to 27 and at one hour before excitation experiment, the XN group was treated with gastric delivery of Xiaoning liquid (10 ml/kg), the HC group and the control group with normal saline (10 ml/kg), and the budesonide group with 5 ml of pulmicort (budesonide suspension containing 2 ml of pulmicort and 3 ml of normal saline) followed by a 30-min atomization. All the groups were treated once a day for 7 days. During the excitation experiment, the control group, XN group, budesonide groups were treated with 5 ml of 1% OVA (100 mg OVA + 100 ml saline) and 5 ml of atomized solution, and the HC group with an equal amount of atomized PBS. All the groups were treated simultaneously and the treatment continued for 30 minutes. The excitation was performed in a glass container (length*width*height: 20 cm*16 cm*13 cm) and pulmicort atomization in a compressor nebulizer (OMRON, China, standard number: YZB/Liao,0930–2011). After 7 days of treatment, the mice were fasted for 24 hours before their eyeballs were removed for blood sampling.

**Fecal DNA Extraction and PCR Amplification**

All procedures were performed aseptically. Sterile scissors were used to open the abdominal cavity of the mouse along the midline of the abdomen, till the colon was fully exposed. Then 1 cm of colon 4 to 10 cm to the anus was cut off and immediately stored in cryotubes at -80°C.

The total DNA was extracted from the mouse intestinal contents using the soil DNA Kit according to the manufacturer’s instructions. The extracted genomic DNA was detected by 2% agarose gel electrophoresis and ultra-micro spectrophotometer.

The V3, V4 regions of bacterial 16S rRNA in the intestine of mice were amplified by PCR using specific primers: 338F, 5’-ACTCCTACGGGAGGCAGCA-3’; 806R, 5’-GGACTACHVGGGTWTCTAAT-3’.
The FastPfu Polymerase (TransG, China) was used for amplification. To begin with, a 3-minute denaturation was performed at 95°C, followed by 27 three-step thermal cycles: denaturation (95°C, 30 seconds), annealing (30 seconds), extension (45 seconds). The PCR reactions were triplicated for each sample. The PCR products were isolated via gelatinization using the AxyPrep DNA Gel Recovery Kit. The PCR products of the same sample were mixed and detected by 1% agarose gel electrophoresis. The PCR products of each sample were quantified by QuantiFluor™-ST blue fluorescence quantitative system, then mixed according to the sequencing amount.

**High-throughput Sequencing**

Illuminate platform library construction and sequencing was performed using the MISEQ sequencer Illuminate MisEq. After sequencing was completed, the data was de-interleaved using Trimmomatic Manual V0.32 software.

**Bioinformatic Analysis**

Operational units (OTUs) were sorted out using Usearch v7.1 software. The sequences were grouped according to their similarity. One group was considered as an OTU. Non-repetitive sequences (excluding single sequences) were extracted from optimized sequences, and OTU-clustered at a 97% similarity. The chimera was removed during the clustering, and the representative OTU sequences were obtained. After being mapped into the OTU representative sequences, the optimized sequences with a similarity degree over 97% were screened out and converted into an OTU table for subsequent analysis.

The Rim classifier Bayesian algorithm was used on the Qiime platform to compare the SILVA 16S bacterial and archaeal ribosomal databases, the Unite fungal database, and the RDP's FunGene functional gene database. Taxonomic analysis was performed for the OTU representative sequences sharing a similarity over 97%. PCA and PCoA were used to compare the distances between groups, and LDA Effect Size to illustrate the difference in the main microbes between groups.

**Statistical Analysis**

All experiments were performed in triplicate. The data were presented as the $M \pm S$. One-way ANOVA and related charts were made using SPSS Statistics v17.0 software. The LSD method and Dunnett's T3 method were used for comparison analysis. Data not in normal distribution were analyzed using the Wilcoxon test. $P < 0.05$ was considered statistically significant, $P < 0.01$ was considered highly significant.

**Results**

**Xiaoning Liquid Restructured the Gut Microbiota in Asthma Mice**

After the treatment with Xiaoning liquid, the structural change in the gut microbiota was detected in the four groups. According to the rarefaction curve and Shannon-Wiener curve (Fig. 1A, B), the sequenced
data were sufficient to reflect the microbiota change. As shown in Fig. 1C, the four groups shared 541 overlapping clusters (OTUs). The number of overlapping OTUs was 566 between the HC group and the control group, 557 between the HC group and the budesonide group, 582 between the HC group and the XN group, 575 between the control group and the budesonide group, 582 between the control group and the XN group, and 575 between the budesonide group and the XN group.

According to the system clustering tree (Fig. 1D, E1), the levels of gut microbiota in the four groups showed obvious difference, and that in the XN group was close to the HC group. According to the weighted Unifrac clustering tree (Fig. 1E2), the microbial species and abundance in the control, XN and budesonide groups were similar.

According to the PCA analyses (Fig. 1F1, F2, F3 and G1, G2, G3), the microbial composition in the control group was different from that in the HC group. The PCoA analysis showed that microbial composition in the HC group and the control group was different from that in the other two groups, but that in the budesonide group and the XN group showed insignicant difference.

According to NMDS results (Fig. 1H), the HC group showed a certain tendency to separate from the remaining three groups. The budesonide group and the XN group were closer, indicating that the difference between the two groups was insignificant.

Taken together, PCA, PCoA, and NMDS analyses showed that the numbers and species of gut microbes in asthmatic mice were different from those in the HC group. Hierarchical cluster analysis and Unifrac cluster analysis showed that the numbers and species in the XN group were similar to those in the HC group, indicating that the microbial composition in Xiaoning-treated mice was similar to that in the healthy mice.

**Xiaoning Liquid Reset the Bacterial Proportions in Gut Microbiota in Asthma Mice**

The histogram revealed the microbial species and their relative abundance. As shown in Fig. 2A, C, E, at phylum level, Bacteroidetes and Firmicutes made up the majority, and Proteobacteria and Deferribacteres the minority of the fecal microbes in each group. Compared with the other three groups having a higher level of Bacteroidetes, the HC group showed a higher level of Firmicutes. However, the XN group exhibited lower levels of Proteobacteria and Deferribacteres compared to the other three groups.

As shown in Fig. 2B, D, F, at genus level, Bacteroides S24-7_group_norank made up the majority of the fecal microbes in each group. The HC group had a higher level of Staphylococcus, and the XN group showed a lower level of Mucispirillum than the other three groups. Finally, 23 genera were found in all samples.

**Xiaoning Liquid Inhibited the Growth of Certain Bacteria**
As shown in Fig. 3A, histograms of LDA scores were plotted to identify the biomarkers and the dominant microorganisms in the gut flora. According to the results of Wilcoxon rank sum test, *Bacilli*, *Leuconostocaceae*, *Alistipes* and *Ruminococcaceae* dominated the community. There were 13 taxa in the HC group, 7 in the control group, 14 in the budesonide group and 10 in the XN group. Among them, in the HC group, *Bacilli* scored the highest value, and *Staphylococcaceae* scored the second value. *Leuconostocaceae* scored the highest value, and *Leuconostoc* scored the second value in the control group. *Alistipes* scored the highest value, and *Helicobacter* scored the second value in the budesonide group. *Ruminococcaceae* scored the highest value in the XN group.

An evolutionary cluster analysis map was generated based on LDA scores to determine the important microbiota via taxonomy. As shown in Fig. 3B, *Bacilli, Bacillales* and *Micrococcales* dominated in the HC group; *Leuconostocaceae, Corynebacteriales* and *Aerococcaceae* in the control group; *Deferribacteres, Helicobacter* and *Campylobacterales* in the budesonide group (some being pathogenic); *Betaproteobacteria, Ruminococcaceae* and *Burkholderiales* in the XN group.

Taken the results of Fig. 4A-4D together, the abundance values of *Staphylococcus* in the HC group were significantly higher than those in the budesonide and XN groups, but it was opposite to *Clostridiales*. *Mucispirillum* had a lower and *Parasutterella* had a higher abundance value in the XN group than in the other three groups. The abundance value of *Corynebacterium* in the XN group significantly decreased compared with the control group, so did *Helicobacter* compared with the budesonide group. These results indicated that Xiaoning liquid changed the community structure of gut bacteria and inhibited the proliferation of some.

**Discussion**

Developed from Shegan Mahuang decoction, Xiaoning liquid can be used to treat asthmatic children which have their own physiological and pathological characteristics. Additionally, Xiaoning liquid plays a dual role in restoring the imbalanced gut microbiota. In this study, we investigated the relationship between intestinal flora and asthma with a TCM, Xiaoning liquid. Our results suggested that Xiaoning liquid can reduce the species and numbers of pathogenic bacteria and restored the intestinal microecology of asthmatic mice.

It has been found that the gut microbiota can guard the intra-gastrointestinal environment from foreign pathogens. The microbiota interacts with the host to dynamically regulate the gut physiology. As a disease invades the host, the gut microbiota is geared and immediately involved in sorts of biological processes that are intricately intertwined. According to the interpretation of TCM, a person's lung and large intestine cooperate closely, which may explain the modern 'lung-gut axis' theory to some extent. Since the alveolar and intestinal epithelium arise from the endoderm, the airway and the intestine may present a functional similarity [31]. Many studies have verified the interaction between pulmonary and intestinal immunity [32–34].
According to Fig. 1, the microbiota in the control group, budesonide group and XN groups was more diverse than that in HC group, suggesting that the intestinal microbial diversity and distribution uniformity of asthma mice were increased after treatment, especially in the control group. Venn, PCA, PCoA and NMDS analysis showed that the numbers and species of gut microbiota in asthmatic mice were different from those in HC group, and the changes in the control group were more obvious than those in the other three groups. Hierarchical clustering analysis and Unifrac clustering analysis showed an overlap between XN group and HC group.

Compared with HC group, taxonomic analysis showed that *Staphylococci* in asthmatic mice decreased and some *Ruminococcacea* increased significantly, which may be associated with the immune response of asthmatic mice. The pulmonary inflammation may disturb the immune system of intestinal mucosa, resulting in intestinal barrier dysfunction, microbiota imbalance, inherent bacterial reduction, and mass reproduction of opportunistic bacteria, foreign bacteria and passing bacteria. Relevant literature shows that *Staphylococcus* is vulnerable to changes in the micro-ecological environment [35]. Therefore, the asthma-disrupted intestinal microecology may inhibit the growth of normal *staphylococcus* in the present study. It has been found that mucin in the intestinal tract can be used as the binding site and nutrient source [36]. Inflammatory changes in the body can affect the immune response in intestinal mucosa. So the asthma-induced rise of mucus level may provide an ideal environment for the proliferation of bacteria in the present study. Besides, after modeling, the *Bacteroidetes* were the most abundant in the gut, followed by *Firmicutes, Proteobacteria* and *Deferribacteres*, which agrees with previous research results [37]. Shannon and Simpson (Table 1) detected that the microbial diversity in the budesonide and XN groups was greater than that in HC group. The present study also showed that the abundance and uniformity of intestinal flora increased, so did the ratio of *Bacteroidetes* to *Firmicutes*, all indicating that the opportunistic pathogens may arise from intestinal *Bacteroidetes* in the case of asthma.

### Table 1

|       | Reads  | OTU  | ace  | chao  | coverage       | shannon  | simpson       |
|-------|--------|------|------|-------|----------------|----------|---------------|
| **HC** | 328614 | 583  | 599  | 603   | 0.999900       | 4.23     | 0.0363        |
|       |        |      | (591,615) | (591,629) | (4.23,4.24)  | (0.036,0.0366) |
| **C** | 278058 | 620  | 628  | 631   | 0.999928       | 4.64     | 0.019         |
|       |        |      | (623,639) | (624,651) | (4.62,4.65)  | (0.0189,0.0191) |
| **B** | 328614 | 594  | 602  | 617   | 0.999933       | 4.69     | 0.0195        |
|       |        |      | (597,615) | (602,659) | (4.69,4.7)   | (0.0194,0.0196) |
| **X** | 328614 | 602  | 612  | 610   | 0.999936       | 4.7      | 0.0212        |
|       |        |      | (606,625) | (605,625) | (4.7,4.71)   | (0.021,0.0213) |

HC: healthy control group, C: control group, B: budesonide group, X: Xiaoning group
We also found that after drug treatment, the diversity of gut microbiota in asthmatic mice decreased. The results of characteristic group analysis showed more pathogenic bacteria in the intestinal tract of asthmatic mice, especially in the control group. In budesonide group and XN group, a few pathogenic bacteria were found, but the gut flora was still dominated by normal. Staphylococcus, Escherichia-Shigella of XN group decreased significantly compared with HC group. Mucispirillum, abundant in the intestinal mucus layer, can regulate the expression of intestinal immunity-related genes in the case of inflammatory diseases, and its large-scale proliferation may trigger intestinal diseases [38]. Compared to the other three groups, Mucispirillum decreased significantly, suggesting that Xiaoning liquid could reduce the pathogenic and revive the probiotic bacteria in the gut, both supporting an intestinal microbiological balance.

In the present study, we did not detect the microflora in the mouse lung, so the interaction on the bacterial level between the lungs and the gut remains unclear, which should be resolved by more research.

In conclusion, Xiaoning liquid can reduce the species and numbers of pathogenic bacteria and restored the intestinal microecology of asthmatic mice. Xiaoning liquid has a positive effect on the function of gut microbiota.

Declarations

Ethics approval and consent to participate:

The study was approved by the Xiaoning liquid and Pulmicort were prepared by the Hospital of Chengdu University of Traditional Chinese Medicine (Chengdu, China). Sixty BALB/C female mice (SPF, age of 6–8 weeks, body weight of 18±2 g) were obtained from Chengdu Dashuo Biotechnology Co., Ltd. Animal license number: SCXK (chuan) 2015-030.

Consent for publication:

Not applicable.

Availability of data and material:

Not applicable.

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Competing interests:

There are no potential conflicts of interest to disclose.

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Not applicable.

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Figures
Figure 1

Xiaoning Liquid Restructured the Gut Microbiota in Asthma Mice. (A) Rarefaction curves determined at a 97% similarity level. (B) Shannon–Wiener curves of samples. (C) Venn diagram of OTUs in the four groups. (D) Multiple sample similarity tree. (E) System clustering tree of unweighted unifrac and weighted unifrac. (F) Multiple sample PCA analysis. (G) Multiple sample PCoA analysis. (H) Non metric multidimensional scaling analysis based on beta diversity distance (NMDS).
Figure 2

Xiaoning Liquid Reset the Bacterial Proportions in Gut Microbiota in Asthma Mice. (A) Heat map of classification at phylum level. (B) Heat map of classification at genus level. (C) Constitutional diagram of
Figure 3

Xiaoning liquid changed the community structure of gut bacteria. (A) Distribution histogram based on LDA. (B) Cladogram.
Figure 4

Xiaoning liquid inhibited the proliferation of some. (A) Histogram of group abundance distribution between HC and budesonide. (B) Histogram of group abundance distribution between HC and XN. (C) Histogram of group abundance distribution between XN and control. (D) Histogram of group abundance distribution between XN and budesonide.