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Effect of Xiaoning liquid on gut microbiota in asthmatic mice by 16S rDNA high-throughput sequencing

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Abstract

To investigate the effects of Xiaoning liquid on gut microbiota in mouse during asthma. 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. 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 *Bacteroide*s a useful marker of gut microbiota changes in asthmatic mice. The levels of *Proteobacterium*, *Deferribacteraceae* and *Mucispirillum* dropped significantly in the Xiaoning liquid group. 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.

Keywords: Xiaoning liquid; asthma; gut microbiota; high-throughput sequencin; pathogenic bacteria.

Practical Application: Xiaoning liquid plays a dual role in restoring the imbalanced gut microbiota.

1 Introduction

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 (Noval Rivas et al., 2016). 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 (Mathew et al., 2012). It incurred significant health care expenditure. Although incurable, asthma can be controlled with appropriate drugs, self-management education, and by avoiding exposure to allergens (Kliegman et al., 2007).

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 (Dethlefsen et al., 2006; Hopkins et al., 2002; Swann et al., 2011). The microflora hypothesis, proposed by Noverr & Huffnagle (2005) 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 (Human Microbiome Project Consortium, 2012). Recent research found that asthma is correlated with the decrease of intestinal probiotics, in which Bifidobacterium and Lactobacillus are the most representative (Shen et al., 2016). Hevia et al. (2016) found that long-term asthma patients demonstrated a lower level of *Bifidobacteria* compared with those with newly developed asthma. Asthma can decrease the diversity and disrupt the composition of gut microbiota (Blaser, 2011). Several studies found Clostridium spp. are implicated in the increased risk of asthma. studied a population of children diagnosed with asthma at preschool age in whom they found evidence of gut bacterial dysbiosis (Watson et al., 2019), 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 (Arrieta et al., 2016).

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). Xu et al. (2015) found that Gegen Qinlian decoction alleviated type 2 diabetes (T2D) through enriching the beneficial bacteria in the gut. Shegan Mahuang Decoction (Synopsis of the Golden Chamber) is a TCM prescription for

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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 Shegan Mahuang 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-allergic, anti-asthmatic, antitussive, antispasmodic and antiviral effects (Liu et al., 1999, 2001; Qin, 2010). 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 (Zhang & Zhang, 2014; Jiang et al., 2007; Wang & Gong, 2008). 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 (Liang et al., 2001). All these substances co-worked to relieve the asthma in the mice (Wu et al., 2005). Besides, it plays a dual role in restoring the imbalanced gut microbiota and coordinating with the host immune system (Tian, 2005; Zhang et al., 2011a, b; Lei, 2007). 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.

2 Materials and methods

2.1 Study materials

Xiaoning liquid and Pulmicort were prepared by our university (Chengdu, China). Xiaoning liquid is a patented prescription (Patent NO.ZL201010190966.8), specification, 10 mL/bottle (Chengdu University of Traditional Chinese Medicine, 2010). 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).

2.2 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 (Forsythe et al., 2007; Leigh et al., 2002; Du et al., 2008). 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.

2.3 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.

2.4 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.

2.5 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.

2.6 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.

3 Results

3.1 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 (Figure 1A, B), the sequenced data were sufficient to reflect the microbiota change. As shown in Figure 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 (Figure 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 (Figure 1E2), the microbial species and abundance in the control, XN and budesonide groups were similar.

According to the PCA analyses (Figure 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 insignificant difference.

According to NMDS results (Figure 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.

3.2 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 Figure 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 Figure 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.

3.3 Xiaoning liquid inhibited the growth of certain bacteria

As shown in Figure 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*

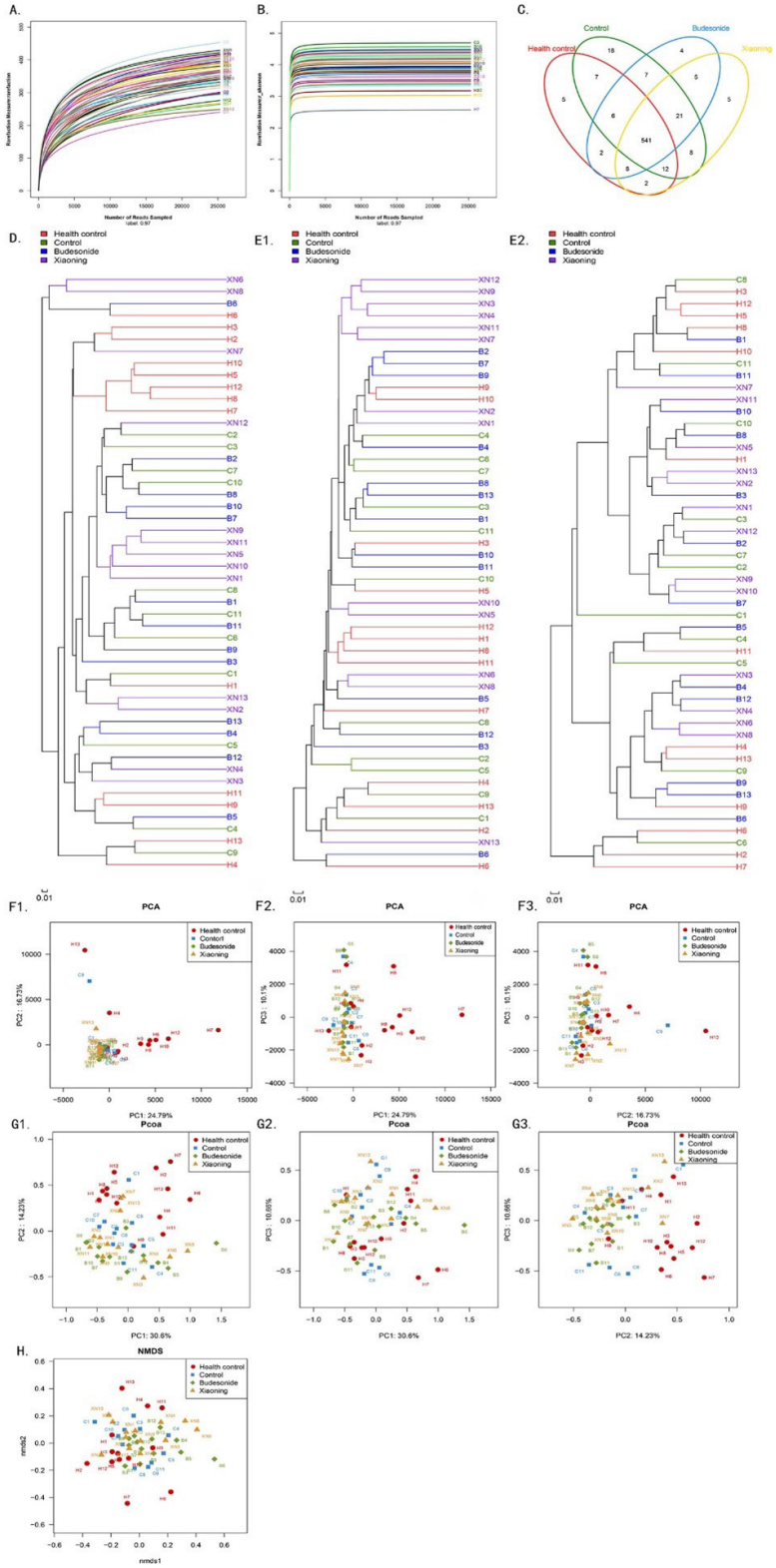

Figure 1. Xiaoning Liquid Restructured the Gut Microbiota in Asthma Mice. (A) Rarefaction curves determined at a 97% similarity level; (B) Shannon-Wiener curves of samples; (C) Venn diagram of OTUs in the four groups; (D) Multiple sample similarity tree; (E) System clustering tree of unweighted unifrac.and weighted unifrac; (F) Multiple sample PCA analysis; (G) Multiple sample PCoA analysis; (H) Non metric multidimensional scaling analysis based on beta diversity distance (NMDS). PC1 and PC2 respectively represent the suspected influencing factors for the deviation of microbial composition of the two groups of samples, which need to be summarized in combination with the sample characteristic information. For example, if the samples of group A healthy control group (in red) and group B model group (in blue) are separated in the direction of PC1 axis, it can be analyzed that PC1 is the main factor leading to the separation of group A and group B (which can be two locations or different acid and alkali). At the same time, it is verified that this factor has high possibility and affects the composition of samples.

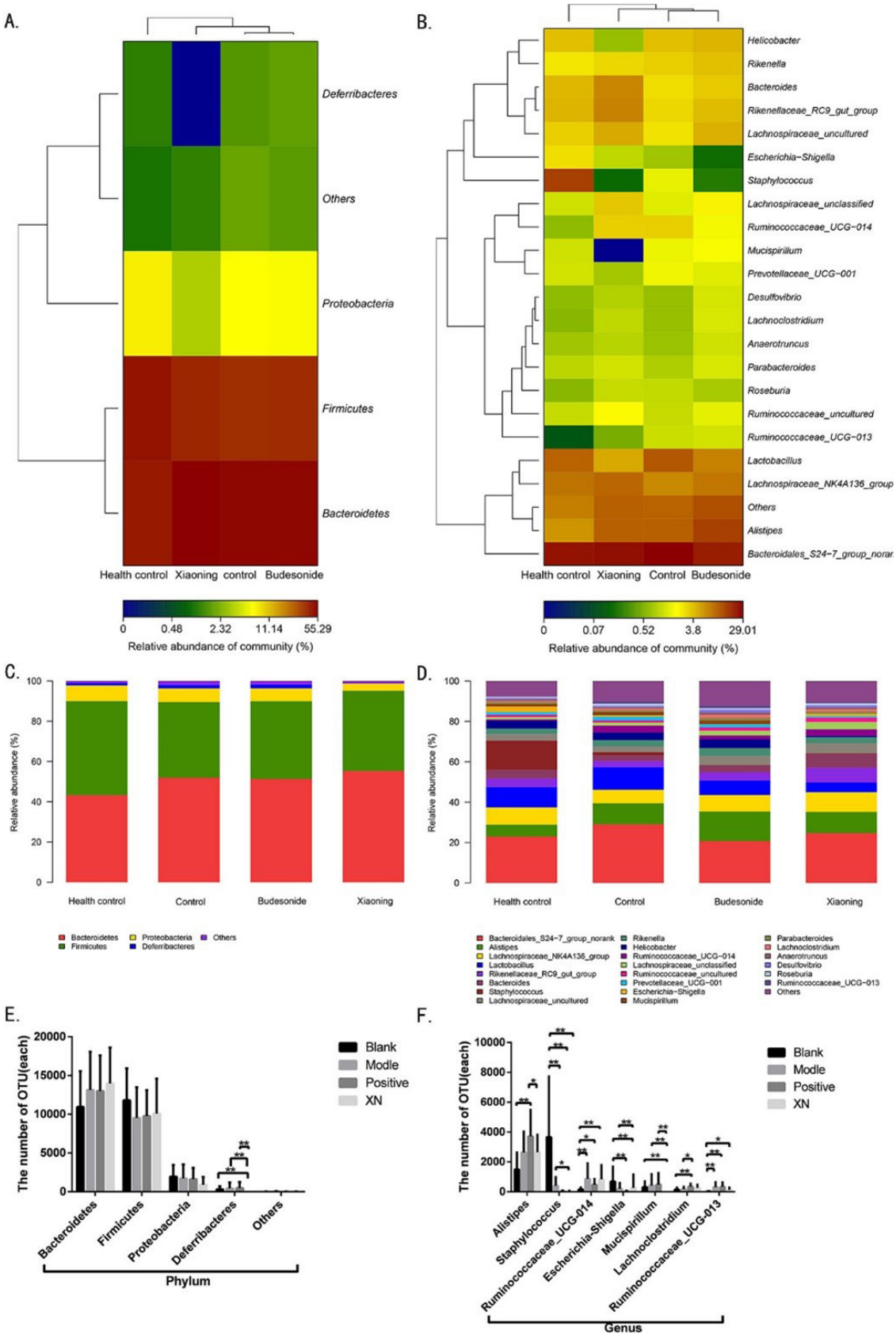

Figure 2. Xiaoning Liquid Reset the Bacterial Proportions in Gut Microbiota in Asthma Mice. (A) Heat map of classification at phylum level; (B) Heat map of classification at genus level; (C) Constitutional diagram of phylum-level community structure; (D) Constitutional diagram of genus-level community structure; (E) Quantity comparison chart of classification level phylum; (F) Quantity comparison chart of classification level genus. *P < 0.05, **P < 0.01. Data are presented as mean ± SD of fifteen samples in each group (E-F, n = 15).

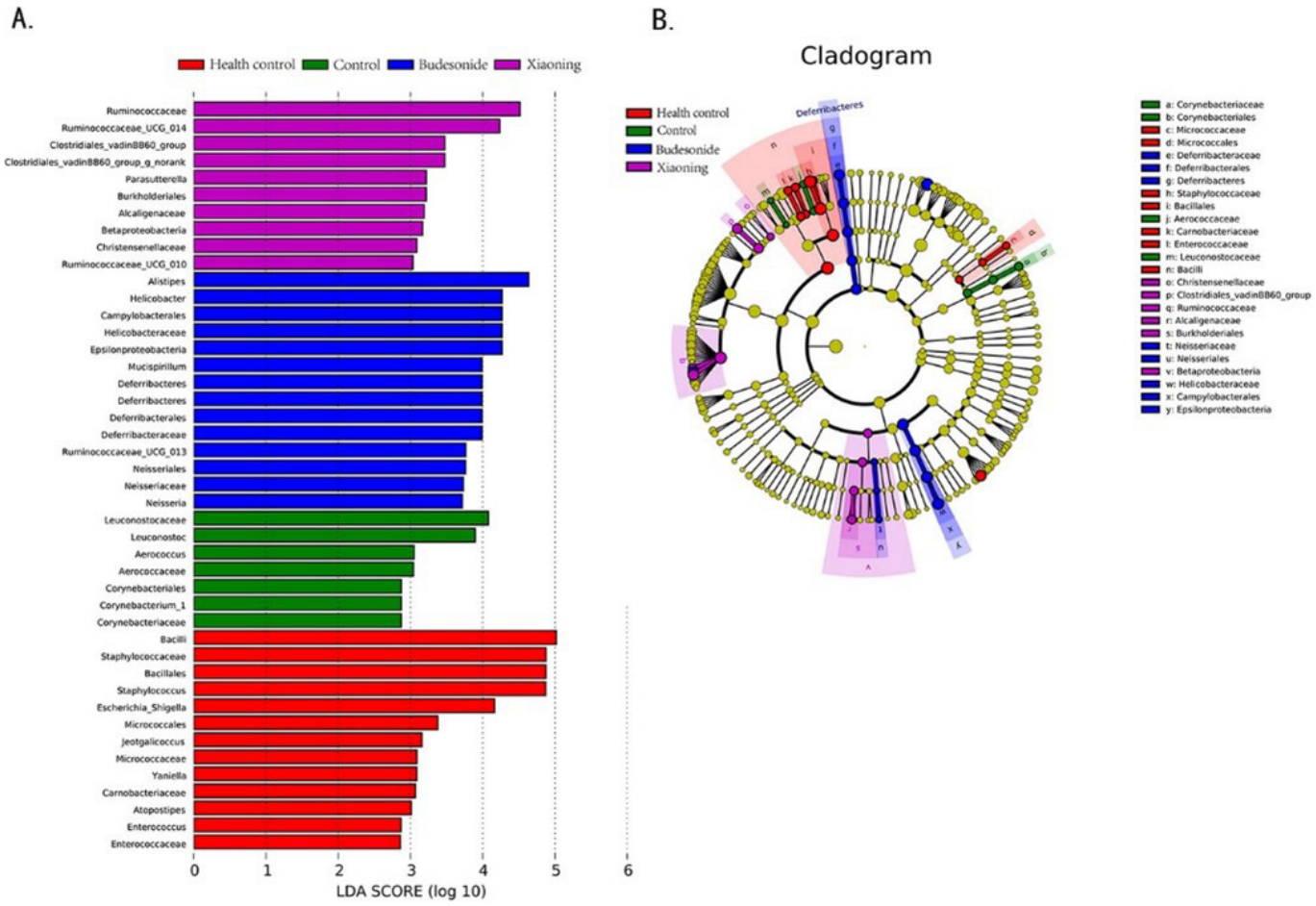

Figure 3. Xiaoning liquid changed the community structure of gut bacteria. (A) Distribution histogram based on LDA; (B) Cladogram.

scored the second valuein 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 Figure 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 Figure 4A-D 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.

4 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 (Bourbon & Chailley-Heu, 2001). Many studies have verified the interaction between pulmonary and intestinal immunity (Huang et al., 2014; Xu et al., 2012; Wang, 2012).

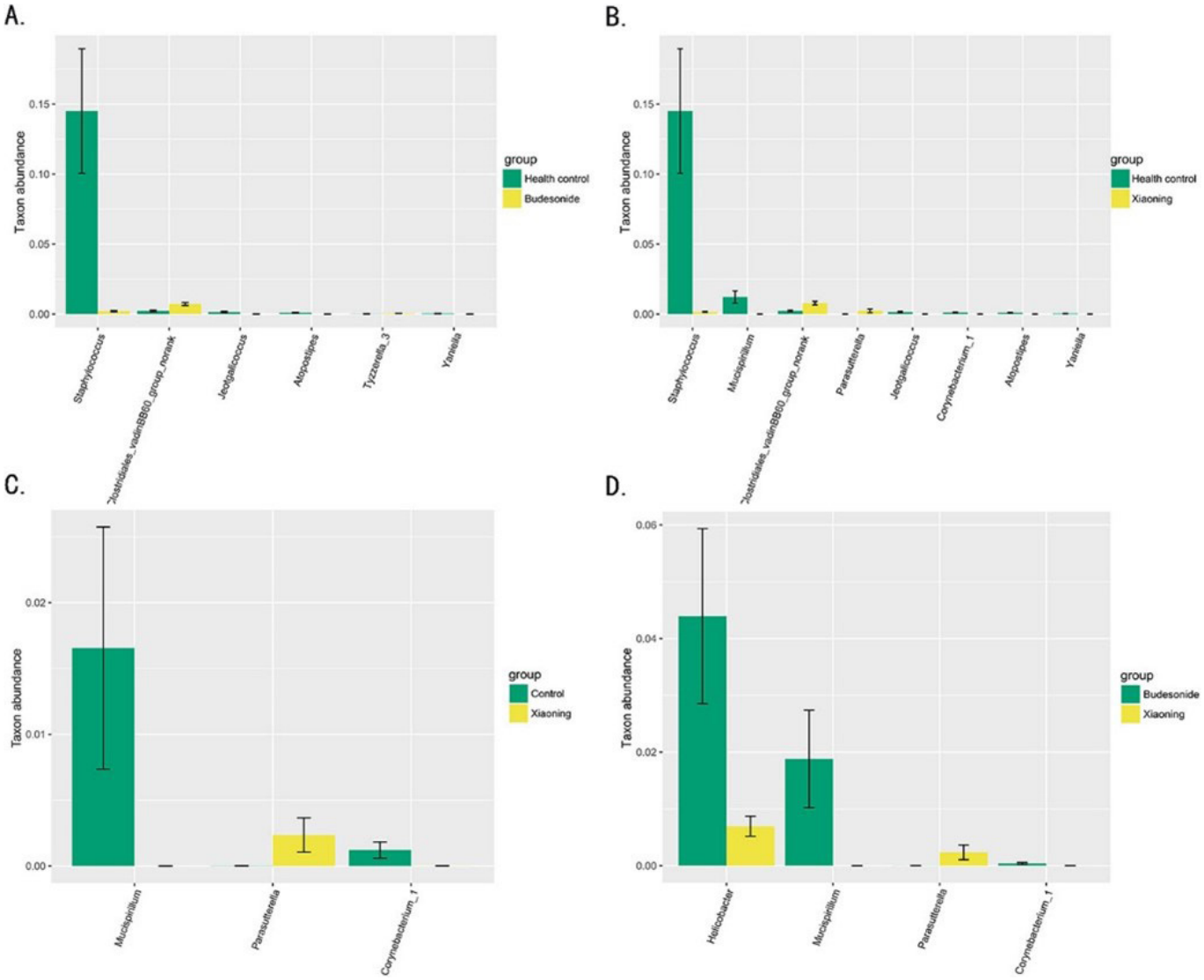

Figure 4. Xiaoning liquid inhibited the proliferation of some. (A) Histogram of group abundance distribution between HC and budesonide; (B) Histogram of group abundance distribution between HC and XN; (C) Histogram of group abundance distribution between XN and control; (D) Histogram of group abundance distribution between XN and budesonide.

According to Figure 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 sepecies 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 (Kastman et al., 2016). 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 (Crost et al., 2013). 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 (Schwab et al., 2014). Shannon and Simpson (Table 1) detected that the microbial diversity in the budesonide

Table 1. Diversity indices of the various groups.

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

HC: healthy control group; C: control group; B: budesonide group; X: Xiaoning group.

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.

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 (Loy et al., 2017). 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 micro-ecological 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.

5 Conclusion

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.

Ethical approval

The study was approved by the Xiaoning liquid and Pulmicort were prepared by the Hospital of Chengdu University of Traditional Chinese Medicine (Chengdu, China) [P20200409]. 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.

Conflict of interest

There are no potential conflicts of interest to disclose.

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Author contributions

YYH is resposible for the guarantor of integrity of the entire study, literature research, experimental studies, data acquisition & analysis, statistical analysis, manuscript preparation & editing; YTL is resposible for the literature research, experimental studies, data acquisition, statistical analysis, manuscript preparation & editing; QWH is resposible for the manuscript review; MQ is resposible for the study concepts & design, experimental studies, data acquisition & analysis, statistical analysis; HYZ, ZWG and QWL are resposible for the data analysis, statistical analysis; QZ is resposible for the definition of intellectual content, manuscript review. All authors read and approved the final manuscript.

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