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ORIGINAL RESEARCH

Role of the Gut-Lung Microbiome Axis in Airway Inflammation in OVA-Challenged Mice and the Effect of Azithromycin

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Objective: This study aimed to investigate the role of the gut-lung microbiome axis in airway inflammation in asthma and to evaluate the effect of azithromycin on this axis, with a focus on the potential mechanism by which azithromycin reduces allergic airway inflammation.

Methods: Haematoxylin and eosin (H&E) and periodic acid-Schiff (PAS) staining were used to assess pathological changes in the lung tissues of asthmatic mice. Leukocyte cell types in bronchoalveolar lavage fluid (BALF) samples were quantified following Wright-Giemsa staining. Total IgE, OVA-specific IgE, IL-4, IL-6, and IL-17A levels in BALF and total IgE in serum were measured by ELISA. The respiratory and gut microbiota were analysed using 16S rRNA gene sequencing and subsequent taxonomic analysis.

Results: OVA-challenged asthmatic mice with gut microbiota dysbiosis exhibited alterations in the respiratory microbiota, resulting in further aggravation of airway inflammation. Following faecal microbiota transplantation (FMT) to restore gut microbiota, respiratory microbiota dysbiosis was partially improved, and airway inflammation was significantly alleviated. Furthermore, azithromycin reduced airway inflammation in asthmatic mice, particularly non-eosinophilic inflammation, for which low-dose azithromycin combined with budesonide proved more effective. Azithromycin significantly enhanced the diversity and microbial composition of the gut microbiota and also affected the respiratory microbiota. At the phylum level, azithromycin decreased the abundance of Proteobacteria in the gut microbiota. At the genus level, azithromycin reduced the abundance of Pseudomonas in the respiratory microbiota.

Conclusion: The gut-lung microbiome axis plays a crucial role in airway inflammation in asthma. Azithromycin may reduce airway inflammation in asthma through modulation of the gut-lung microbiome axis.

Keywords: asthma, gut-lung microbiome axis, azithromycin, budesonide, airway inflammation

Introduction

Bronchial asthma is a complex chronic inflammatory disease characterized by wheezing, cough, and chest tightness. Recently, its incidence has increased annually and is projected to reach 400 million cases worldwide by 2025.[1] Approximately 250,000 asthma-related deaths are reported each year.[2] Airway hyperresponsiveness, inflammation, and remodelling are key features of asthma, with airway inflammation considered the primary cause.[3] Studies to date indicate that asthma is associated with T helper (Th)-2 inflammation and non-Th2 inflammation.[4] Th2 inflammation is characterized by elevated levels of cytokines, such as IL-4, IL-5, and IL-13, while non-Th2 inflammation is associated with high levels of cytokines such as IL-17 and IL-6. Risk factors associated with the development of airway inflammation in asthma include epigenetic factors, exposure to tobacco smoke, airway microbes, and viruses, among which the dysbiosis of the human body's microbiome is considered to be a very important factor.[5]

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Recent findings suggest that vital crosstalk exists between the gut microbiota and the lungs, known as the gut–lung axis.[6] It is now understood that the gut microbiota fulfils various physiological roles, including the production of short-chain fatty acids (SCFAs), enhancement of local mucosal immunity, and promotion of immunotolerance.[7] Early-life exposure to antibiotics negatively impacts respiratory health and is linked to an increased risk of childhood asthma. Certain gut microbiota profiles have been found to protect against asthma. Similarly, the versatility and efficacy of SCFAs in airway inflammation have been investigated extensively.[8] Specifically, a lower abundance of Lachnospira, Veillonella, and Rothia in the gut is associated with a higher risk of developing allergies and asthma.[9] However, in recent years, several studies have shown that the respiratory microbiome is also closely associated with the occurrence and development of asthma. In asthma patients, an increased abundance of Proteobacteria, including genera such as Haemophilus, Pseudomonas, Neisseria, and Fusobacterium, is observed in the respiratory tract.[10,11] Additionally, the respiratory microbiota differs significantly between asthma patients with different types of inflammation.[12] Patients with eosinophilic airway inflammation show a higher abundance of Actinobacteria, while those with non-eosinophilic airway inflammation have higher levels of Proteobacteria.[13,14] Thus, the gut and respiratory microbiota play an important role in airway inflammation in asthma. However, the mechanisms underlying communication between the lungs and gut remain unclear. The microbiota may play a crucial role in mediating interactions between the gut and respiratory tract. Studies have shown that chronic inflammatory airway diseases alter the airway microbiome and can significantly affect gut microbiota.[15] Although there is growing research on the gut-lung microbial axis in asthma, its impact on airway inflammation remains unclear. This study aimed to investigate the role of the gut-lung microbiome axis in airway inflammation in asthma.

Azithromycin, widely used in the treatment of respiratory infections, regulates immunity, reduces airway hyperresponsiveness, and decreases airway inflammation.[16–18] It is reported that azithromycin's immunomodulatory effects are utilized to treat asthma. Azithromycin, being an antibiotic, obviously has significant interplay with the microbiome.[5] Park et al reported that azithromycin can modulate the gut microbiota.[19] Thorsen et al reported that azithromycin effectiveness was mediated by respiratory microbiota richness.[20] However, its effect on the gut–lung microbiome axis remains unknown. Therefore, this study aimed to observe the effects of azithromycin on the gut–lung microbiome axis to further explore the mechanism by which azithromycin alleviates allergic airway inflammation.

Materials and Methods

Mice

Female BALB/c mice (n = 60), aged 4–6 weeks, were obtained from Shanghai Laboratory Animal Co., Ltd. (SLAC, Shanghai, China) and maintained under specific pathogen-free conditions. Initially, the mice were randomly divided into two groups: control (n = 12) and Ovalbumin (OVA) (n = 48). To establish a mouse model of asthma, 48 mice were intraperitoneally sensitized with 20 μg of OVA (Grade V, Sigma-Aldrich, St. Louis, MO, USA) in 0.1 mL saline, combined with 0.1 mL Imject Alum (Thermo Fisher Scientific, Rockford, IL, USA) on days 0, 7, and 14. The mice were subsequently challenged with 2.5% OVA in saline using a nebulizer for 30 minutes on days 21–24 and 28–31. Mice in the control group (n = 12) were injected intraperitoneally with saline and Imject Alum emulsion, then exposed to sterile saline following the same schedule.

Mice in the gut microbiota disorder group received drinking water containing vancomycin (0.5 mg/mL) and neomycin (1 mg/mL) until the end of the challenge period. The other groups of mice were given only water.

Fresh faeces from donor mice (the control group) were collected and frozen at −80°C before faecal microbiota transplantation (FMT). An appropriate amount of faeces was added to sterile normal saline and mixed well, then centrifuged at 25 °C at 5000 ×g for 5 minutes. The supernatant was discarded, and the concentration of the microbiota solution in normal saline was adjusted to 0.3 g/mL. Recipient mice were administered 200 μL of the supernatant containing faecal microbiota via gavage 30 minutes before the OVA challenge.

Mice in the budesonide treatment group were nebulized with budesonide (1 mg in 3 mL normal saline) for 30 minutes prior to the OVA challenge.

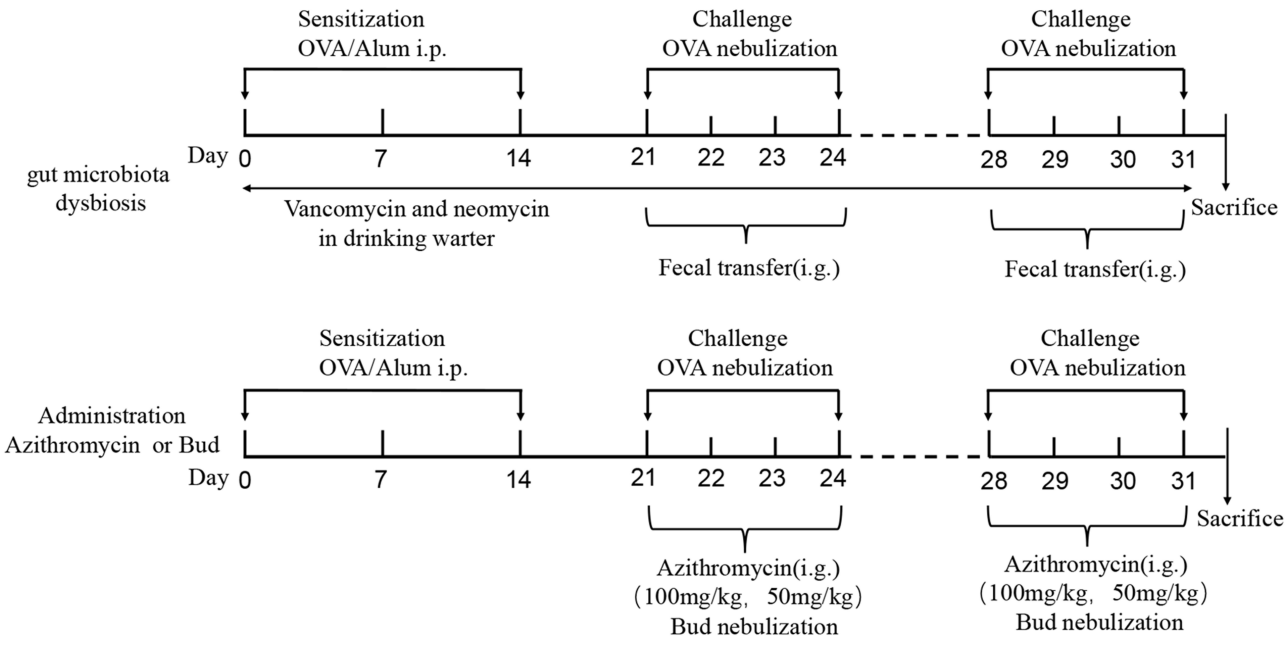

Figure 1 Experimental protocols. To establish a mouse model of asthma, 48 mice were intraperitoneally sensitized with 20 μg of OVA in 0.1 mL saline, combined with 0.1 mL Imject Alum on days 0, 7, and 14. The mice were subsequently challenged with 2.5% OVA in saline using a nebulizer for 30 minutes on days 21–24 and 28–31. Mice in the gut microbiota disorder group received drinking water containing vancomycin (0.5 mg/mL) and neomycin (1 mg/mL) until the end of the challenge period. Recipient mice were administered 200 μL of the supernatant containing faecal microbiota (0.3 g/mL) via gavage 30 minutes before the OVA challenge. Mice in the budesonide treatment group were nebulized with budesonide (1 mg in 3 mL normal saline) for 30 minutes prior to the OVA challenge. Mice in the azithromycin treatment group received either 100 mg/kg (high dose) or 50 mg/kg (low dose) of azithromycin daily through intragastric administration 30 minutes before the OVA challenge.

Mice in the azithromycin treatment group received either 100 mg/kg (high dose) or 50 mg/kg (low dose) of azithromycin daily through intragastric administration 30 minutes before the OVA challenge.

In this study, 48 mice from the OVA group were randomly assigned to eight groups: OVA (no treatment, n = 6), KOL (OVA with vancomycin and neomycin intervention, n = 6), KFL (OVA with vancomycin and neomycin intervention and FMT treatment, n = 6), OFL (OVA with FMT treatment, n = 6), AHL (OVA with high-dose azithromycin, n = 6), ALL (OVA with low-dose azithromycin, n = 6), BUD (OVA with budesonide treatment, n = 6), and ABL (OVA with low-dose azithromycin and budesonide treatment).

The mice were sacrificed within 24 hours of the final nebulization. After euthanasia, blood samples, bronchoalveolar lavage fluid (BALF), gut contents, and left lung tissues were collected and preserved. The experimental protocols used in this study are summarized in Figure 1.

Lung Histology

The left lungs were fixed in 4% paraformaldehyde for 24 hours and embedded in paraffin. Sections (4 μm thick) were stained with haematoxylin and eosin (H&E) and periodic acid-Schiff (PAS). All slides were examined in a randomly blinded manner by three independent investigators. A semi-quantitative scoring system was used to grade the extent of lung inflammation and goblet cell hyperplasia, as described previously.[21,22]

Collection of Bronchoalveolar Lavage Fluid and Gut Contents

Following euthanasia of the mice by cervical dislocation, the lungs were washed four times with sterile saline through endotracheal intubation of the lower respiratory tract (0.5 mL) to obtain BALF samples. BALF was filtered using a filter with an aperture of 0.22 μm. The filtered BALF was centrifuged at 1000 rpm for 15 minutes, and the supernatants were collected for cytokine detection. The filter membrane was preserved for subsequent DNA extraction. Gut contents from the anus to the cecum of the mice were collected under sterile conditions. All specimens collected were temporarily stored at −80°C.

BALF Cells, IgE, and Cytokines

A portion of the unfiltered BALF was centrifuged, and the precipitated cells were counted after staining with Wright–Giemsa, following the manufacturer's instructions.

All BALF specimens were collected as described in Collection of Bronchoalveolar Lavage Fluid and Gut Contents. The levels of total IgE and OVA-specific IgE (BioLegend, San Diego, CA, USA), IL-4, IL-6, and IL-17A (R&D Systems, Minneapolis, MN, USA) in BALF were measured using ELISA kits, according to the manufacturer's instructions.

Blood samples were collected from the heart after mice were anesthetized with 1% pentobarbital, which was left at 4°C for 2 h and centrifuged at 2000 rpm for 15 mins. Serum was separated and stored at −80°C. The levels of total IgE in serum were measured using ELISA kits, according to the manufacturer's instructions.

DNA Extraction, 16S rRNA Gene Sequencing, and Bioinformatics Analysis

Total genomic DNA from BALF and gut contents was extracted using the OMEGA Soil DNA Kit (Omega Bio-Tek, Norcross, GA, USA) according to the manufacturer's instructions and stored at −80°C. PCR amplification was performed using specific primers. The forward primer 338F (5'-ACTCCTACGGGAGGCAGCA-3') and reverse primer 806R (5'-GGACTACHVGGGTWTCTAAT-3') were used to amplify the V3–V4 region of the bacterial 16S rRNA genes. After the individual quantification step, the amplicons were pooled in equal quantities, and paired-end sequencing (2 × 300 bp) was performed using Illumina MiSeq (Illumina, San Diego, CA, USA) at Shanghai Personal Biotechnology Co., Ltd. (Shanghai, China). Procedures for DNA extraction, PCR amplification, and 16S rRNA gene sequencing were based on previous studies by our team.[23]

Microbiome bioinformatics analysis was performed using QIIME 2 with minor modifications.[24] The specific analysis scheme is based on a previous study.[23] We used the q2-diversity plugin to compute various α diversity metrics using Pielou's evenness indices and β diversity metrics using weighted UniFrac distance matrices. Principal coordinate analysis (PCoA) with weighted UniFrac distance matrices was conducted to study community composition. A bar chart of the microbiota composition was constructed using Wekemo Bioincloud (https://www.bioincloud.tech). Linear discriminant analysis effect size (LEfSe)[25] was used to detect differentially abundant taxa across groups using default parameters.

Data Analysis

The expression levels of cytokines and cell counts were compared using analysis of variance with GraphPad Prism 8.0 and IBM SPSS Statistics for Windows, version 24.0 (IBM Corp., Armonk, NY, USA). Differences among more than three groups were assessed using one-way analysis of variance (ANOVA), while variance between two groups was compared using the SNK-q test.

The Kruskal–Wallis test was employed to estimate intergroup differences in α diversity metrics, β diversity metrics, and LEfSe analysis. The Wilcoxon test was used to compare subclasses. All statistical tests used were two-sided, with a p-value ≤ 0.05 considered statistically significant.

Results

Vancomycin and Neomycin Altered the Diversity of the Respiratory and Gut Microbiota in Mice With Ovalbumin (OVA)-Challenged Asthma

The alpha rarefaction curves shown in Figure 2A and B indicate that the sequencing depth was sufficient. The α diversity represents the diversity of microbial groups in the model, while the β diversity analysis indicates the microbial diversity across different groups of mice. There was no significant difference in Pielou's evenness indices of the respiratory microbiota (BALF samples) among the control, OVA, KOL, KFL, and OFL groups ($p > 0.05$) (Figure 2C). The gut microbiota showed no significant difference in α diversity between OVA-challenged mice and control mice ($p > 0.05$), as determined using Pielou's evenness. The gut microbiota of OVA-challenged mice treated with vancomycin and neomycin, or FMT differed significantly from that of untreated OVA-challenged mice (Figure 2D).

The β diversity among the different groups was evaluated using the weighted UniFrac distance. The scatter plot based on the PCoA scores showed a clear separation of the community composition of the respiratory and gut microbiota between the control and OVA-challenged mice. PCoA demonstrated that the β diversity of the respiratory and gut microbiota of OVA-induced mice treated with vancomycin and neomycin, or FMT was significantly different from that of untreated OVA-challenged mice (Figure 2E and F). These results indicate that vancomycin and neomycin can affect the β diversity of the respiratory and gut microbiota in OVA-induced asthmatic mice.

Vancomycin and Neomycin Changed the Composition of the Respiratory and Gut Microbiota in Mice With OVA-Challenged Asthma

To understand the effects of vancomycin and neomycin on the composition of the respiratory and gut microbiota in OVA-challenged asthmatic mice, the microbial composition was analysed at the phylum and genus levels.

Proteobacteria, Firmicutes, and Bacteroidetes were identified as the three dominant phyla in the respiratory microbiota across all groups. Compared to control mice, Proteobacteria were more abundant in OVA-challenged asthmatic

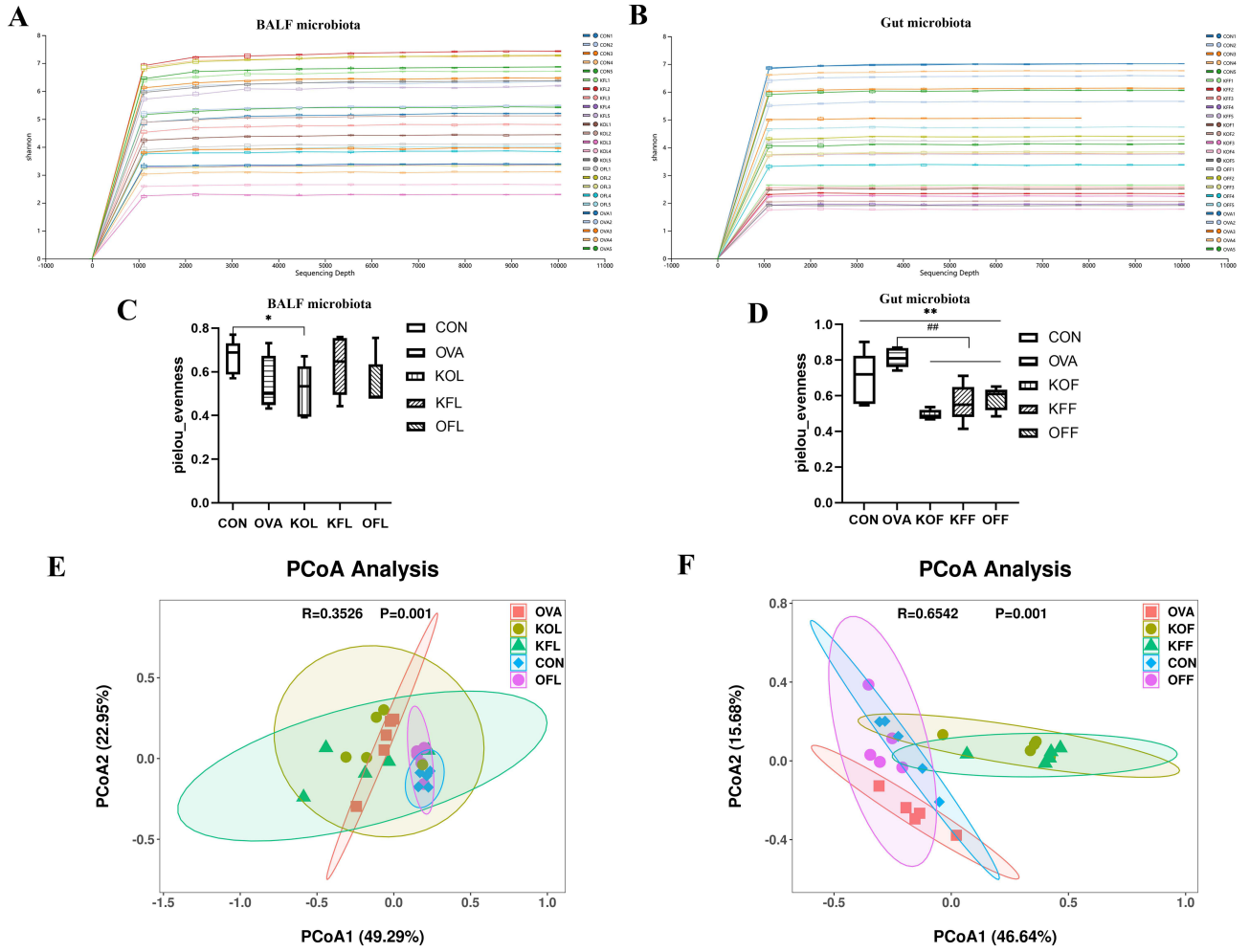

Figure 2 Effects of vancomycin and neomycin on the diversity of the respiratory and gut microbiota in ovalbumin (OVA)-challenged asthmatic mice. (**A**) α rarefaction curve in bronchoalveolar lavage fluid (BALF) microbiota. (**B**) α rarefaction curve in gut microbiota. (**C**) α diversity analysis (using the Pielou's evenness) of BALF microbiota. (**D**) α diversity analysis (using the Pielou's evenness) of the gut microbiota. (**E**) PCoA plot showing the β diversity of BALF microbiota (P = 0.001). (**F**) PCoA plot showing the β diversity of gut microbiota (P = 0.001). PCoA of all samples using weighted UniFrac distance. PCoA, principal coordinates analysis. (n = 5 per group, *P ≤ 0.05 vs the CON group; **P ≤ 0.01 among all the groups; ##P ≤ 0.01 vs the OVA group).

Abbreviation: CON, control mice; OVA, OVA-induced asthmatic mice; KOL, respiratory samples of OVA-induced asthmatic mice with vancomycin and neomycin intervention; KFL, respiratory samples of OVA-induced asthmatic mice with vancomycin and neomycin intervention and FMT treatment; OFL, respiratory samples of OVA-induced asthmatic mice with FMT treatment; KOF, gut samples of OVA-induced asthmatic mice with vancomycin and neomycin intervention; KFF, gut samples of OVA-induced asthmatic mice with vancomycin and neomycin intervention and FMT treatment; OFF, gut samples of OVA-induced asthmatic mice with FMT treatment.

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mice, regardless of vancomycin and neomycin intervention. Proteobacteria were significantly reduced in OVA-challenged asthmatic mice treated with FMT. In contrast, Bacteroides significantly decreased in OVA-challenged asthmatic mice with or without the intervention of vancomycin and neomycin (Figure 3A). At the genus level, the top three genera in the respiratory microbiota were Anoxybacillus, Cupriavidus, and Pseudomonas. Anoxybacillus was more abundant in both control and OVA-challenged asthmatic mice treated with FMT, while Cupriavidus was more abundant in OVA-challenged asthmatic mice with or without vancomycin and neomycin (Figure 3B). To further identify the effects of vancomycin and neomycin on the microbiota composition in OVA-challenged asthmatic mice, the different abundances of bacterial communities were analysed using LEfSe (Figure 3C). The analysis revealed that, among the respiratory microbiota, Bacteroidales were present in control mice, Pseudomonas and Rhizobiales in OVA-challenged mice, Caulobacteraceae and Cupriavidus in OVA-challenged mice treated with vancomycin and neomycin, and Anoxybacillus and Bacillaceae in OVA-challenged mice treated with FMT.

Unlike the respiratory microbiota, Bacteroidetes, Firmicutes, and Proteobacteria were the three dominant phyla in the gut microbiota across all groups. Compared to control mice, Proteobacteria were more abundant in OVA-challenged asthmatic mice, with or without vancomycin and neomycin intervention. Proteobacteria were significantly reduced in the OVA-challenged asthmatic mice treated with FMT (Figure 3D). At the genus level, the top three genera in the gut microbiota were Bacteroides, Lactobacillus, and Enterobacteriaceae. Bacteroidales were more abundant in control and OVA-challenged asthmatic mice treated with FMT, while Enterobacteriaceae were more abundant in OVA-challenged asthmatic mice with or without vancomycin and neomycin (Figure 3E). The analysis of the microbial composition using LEfSe (Figure 3F) revealed that, among the gut microbiota, Bacteroidales were present in control mice, Clostridiales and S24-7 in OVA-challenged mice, Enterobacteriaceae in OVA-challenged mice treated with vancomycin and neomycin, and Erysipelotrichaceae and Allobaculum in OVA-challenged mice treated with FMT. These results indicate that vancomycin and neomycin affect the composition of the respiratory and gut microbiota in asthmatic mice to some extent.

Azithromycin Altered the Diversity of the Respiratory and Gut Microbiota in Mice With OVA-Challenged Asthma

The alpha rarefaction curves shown in Figure 4A and B indicate that the sequencing depth was sufficient. Significant differences were observed in Pielou's evenness indices of the respiratory ($p < 0.05$) and gut ($p < 0.01$) microbiota among control mice, untreated OVA-challenged mice, and azithromycin and/or budesonide-treated mice (Figure 4C and D). PCoA demonstrated that the β diversity of the respiratory and gut microbiota of OVA-challenged mice treated with azithromycin and/or budesonide was significantly different from that of untreated OVA-challenged mice (Figure 4E and F). These results indicate that azithromycin affects the β diversity of the respiratory and gut microbiota in OVA-challenged mice.

Azithromycin Altered the Composition of the Respiratory and Gut Microbiota in Mice With OVA-Challenged Asthma

To understand the effects of azithromycin and budesonide on the composition of the respiratory and gut microbiota in OVA-challenged asthmatic mice, the microbial composition was analysed at the phylum and genus levels.

Proteobacteria, Firmicutes, and Bacteroidetes were the three dominant phyla in the respiratory microbiota across all groups. Notably, Proteobacteria were more abundant in OVA-challenged asthmatic mice than in control mice. The abundance of Proteobacteria significantly decreased in OVA-challenged asthmatic mice treated with azithromycin and budesonide, particularly with the combination of low-dose azithromycin and budesonide. Compared to control mice, the levels of Firmicutes and Bacteroidetes were reduced in OVA-challenged asthmatic mice, but these levels improved significantly following treatment with azithromycin and budesonide (Figure 5A). At the genus level, the three most abundant genera in the respiratory microbiota were Pseudomonas, Burkholderia, and Anoxybacillus. Pseudomonas was more prevalent in OVA-challenged asthmatic mice than in control mice, and its abundance significantly decreased in OVA-challenged asthmatic mice treated with azithromycin and budesonide (Figure 5B). To further assess the impact of azithromycin and budesonide on the microbiota composition in OVA-challenged asthmatic mice, we analysed the

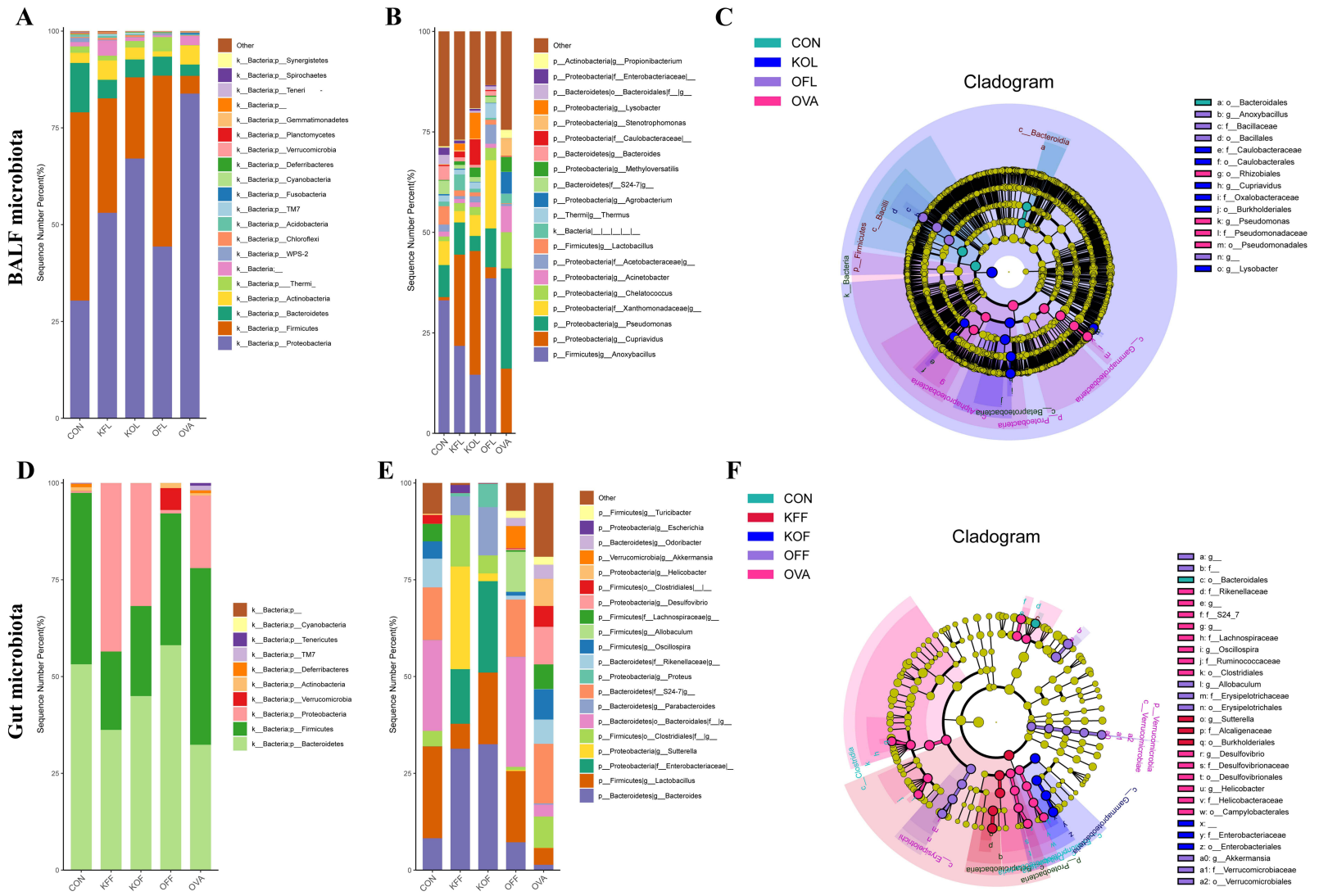

Figure 3 Effects of vancomycin and neomycin on the composition of the respiratory and gut microbiota in ovalbumin (OVA)-challenged asthmatic mice. (**A**) Composition of bronchoalveolar lavage fluid (BALF) microbiota at the phylum level. (**B**) Composition of BALF microbiota at the genus level. (**C**) Different abundances of bacterial communities in the BALF samples, as indicated in LEfSe analysis. The circles represent phylogenetic levels from phylum (innermost circle) to genera (outermost circle). LDA scores > 4.5. (**D**) Composition of the gut microbiota at the phylum level. (**E**) Composition of the gut microbiota at the genus level. (**F**) Different abundances of bacterial communities in the gut samples, as indicated in LEfSe analysis. LDA scores > 4.5. (n = 5 per group, adjusted p values ≤ 0.05).

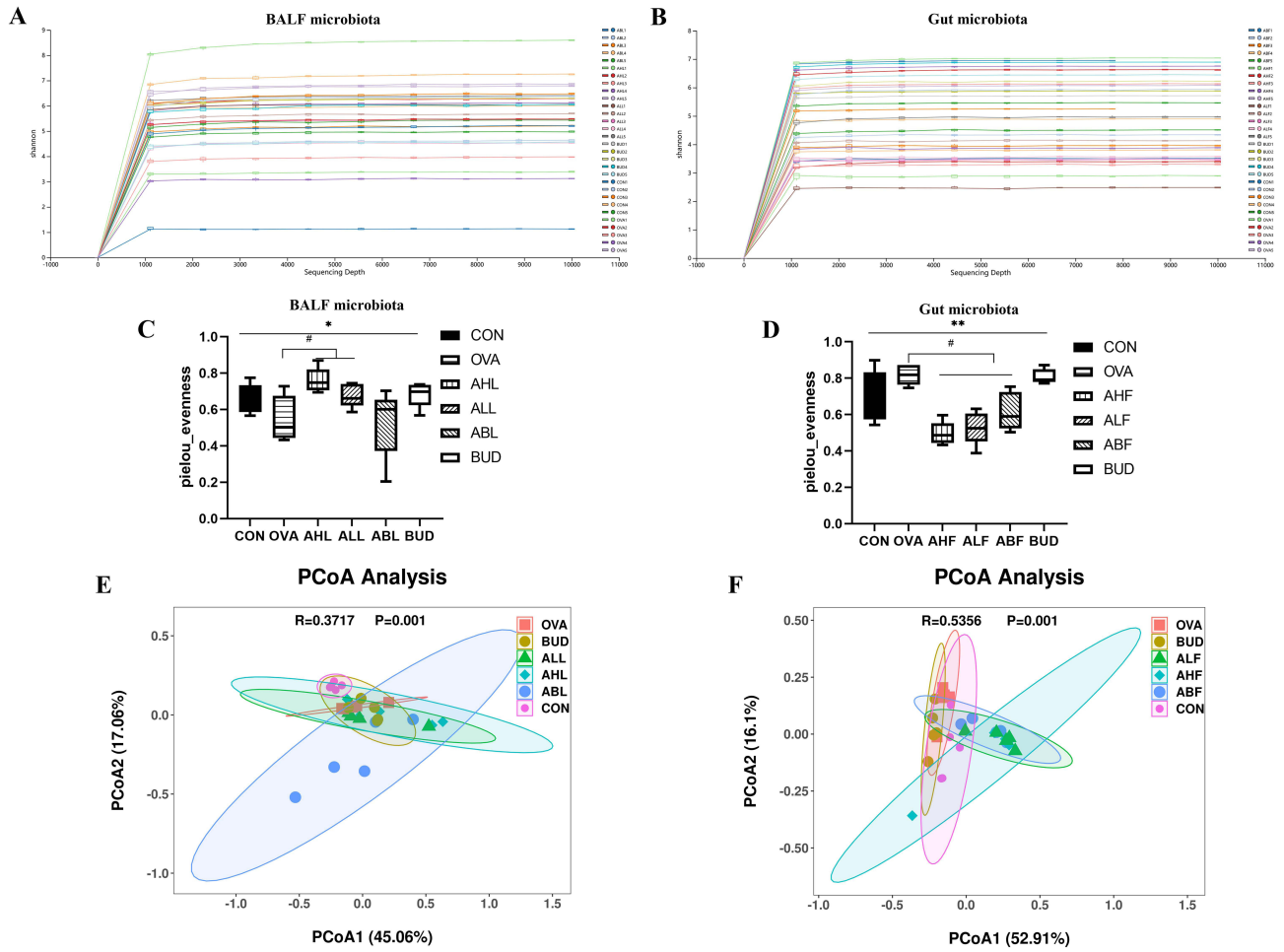

Figure 4 Effects of azithromycin on the diversity of the respiratory and gut microbiota in ovalbumin (OVA)-challenged asthmatic mice. (**A**) α rarefaction curve in bronchoalveolar lavage fluid (BALF) microbiota. (**B**) α rarefaction curve in gut microbiota. (**C**) α diversity analysis (using the Pielou's evenness) of BALF microbiota. (**D**) α diversity analysis (using the Pielou's evenness) of the gut microbiota. (**E**) PCoA plot showing the β diversity of BALF microbiota (P = 0.001). (**F**) PCoA plot showing the β diversity of gut microbiota (P = 0.001). PCoA of all samples using weighted UniFrac distance. PCoA, principal coordinates analysis. (n = 5 per group, *P ≤ 0.05 among all the groups; **P ≤ 0.01 among all the groups; #P ≤ 0.05 vs the OVA group).

Abbreviations: CON, control mice; OVA, OVA-induced asthmatic mice; BUD, OVA-induced asthmatic mice with budesonide treatment; ALL, respiratory samples of OVA-induced asthmatic mice with low-dose azithromycin treatment; AHL, respiratory samples of OVA-induced asthmatic mice with high-dose azithromycin treatment; ABL, respiratory samples of OVA-induced asthmatic mice with low-dose azithromycin and budesonide treatment; ALF, gut samples of OVA-induced asthmatic mice with low-dose azithromycin treatment; AHF, gut samples of OVA-induced asthmatic mice with high-dose azithromycin treatment; ABF, gut samples of OVA-induced asthmatic mice with low-dose azithromycin and budesonide treatment.

different abundances of bacterial communities using LEfSe (Figure 5C). The LEfSe analysis indicated that among the respiratory microbiota, Bacteroidales were present in control mice, while Pseudomonas and Rhizobiales were predominant in OVA-challenged mice, Burkholderia was abundant in OVA-challenged mice treated with high-dose azithromycin, Halomonas and Oceanospirillales in OVA-challenged mice treated with low-dose azithromycin, and Chloroplast and Streptophyta in OVA-challenged mice treated with both low-dose azithromycin and budesonide.

In contrast to the respiratory microbiota, Bacteroidetes, Firmicutes, and Proteobacteria were the dominant phyla in the gut microbiota across all groups. Proteobacteria were more abundant in OVA-challenged asthmatic mice than in control mice, and this abundance significantly decreased in OVA-challenged asthmatic mice treated with azithromycin and budesonide (Figure 5D). At the genus level, the three most abundant genera in the gut microbiota were Bacteroides, Lactobacillus, and S24-7. Compared to control mice, Bacteroidetes and Lactobacillus were reduced in OVA-challenged asthmatic mice, whereas their levels significantly improved in OVA-challenged asthmatic mice treated with azithromycin and budesonide (Figure 5E). LEfSe analysis of the gut microbiota (Figure 5F) showed that Lactobacillus was present in

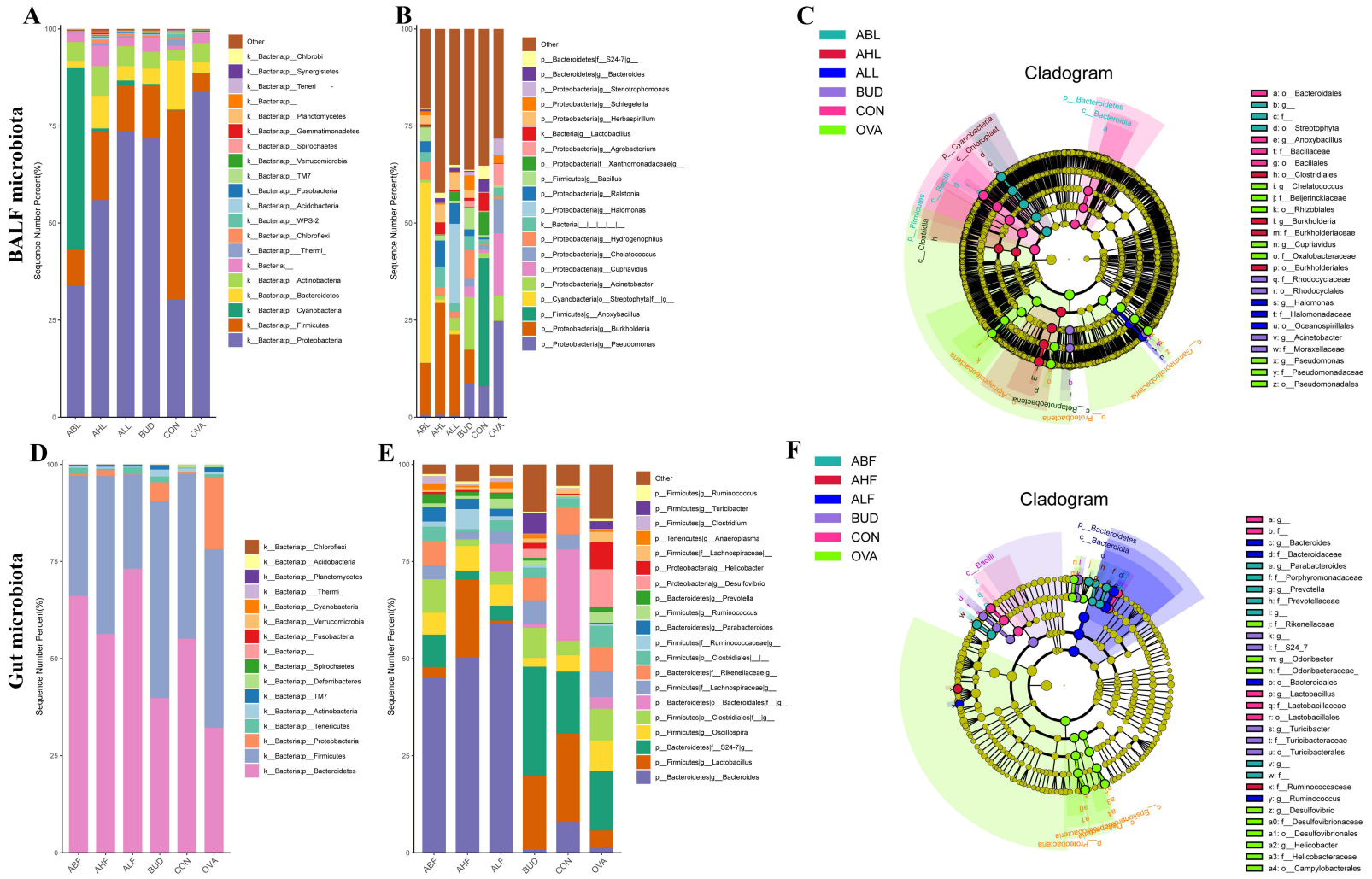

Figure 5 Effects of azithromycin on the composition of the respiratory and gut microbiota in ovalbumin (OVA)-challenged asthmatic mice. (**A**) Composition of bronchoalveolar lavage fluid (BALF) microbiota at the phylum level. (**B**) Composition of BALF microbiota at the genus level. (**C**) Different abundances of bacterial communities in the BALF samples, as indicated in LEfSe analysis. The circles represent phylogenetic levels from phylum (innermost circle) to genera (outermost circle). LDA scores > 4.5. (**D**) Composition of the gut microbiota at the phylum level. (**E**) Composition of the gut microbiota at the genus level. (**F**) Different abundances of bacterial communities in the gut samples, as indicated in LEfSe analysis. LDA scores > 4.0. (n = 5 per group, adjusted p values ≤ 0.05).

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control mice, Desulfovibrio and Helicobacter in OVA-challenged mice, Bacteroides in OVA-challenged mice treated with low-dose azithromycin, Ruminococcaceae in OVA-challenged mice treated with high-dose azithromycin, and Parabacteroides and Prevotella in OVA-challenged mice treated with low-dose azithromycin and budesonide. These results indicate that azithromycin significantly influences the composition of the respiratory and gut microbiota in asthmatic mice.

In OVA-Challenged Asthmatic Mice, Vancomycin and Neomycin Aggravated Airway Inflammation, While Azithromycin Mitigated It

To investigate the effects of different antibiotics on airway inflammation in OVA-induced asthmatic mice, lungs were stained with H&E and PAS (Figure 6A). Compared with control mice, OVA-challenged mice exhibited pronounced inflammatory cell infiltration around the small airways, bronchial wall thickening, and constriction. Vancomycin and neomycin treatment further aggravated airway inflammation in OVA-challenged mice. FMT alleviated airway inflammation in OVA-challenged asthmatic mice, regardless of vancomycin and neomycin intervention. Treatment with azithromycin and budesonide significantly alleviated airway inflammation, with high and low doses of azithromycin being equally effective in OVA-challenged mice. The combination of low-dose azithromycin and budesonide yielded the greatest reduction in airway inflammation in OVA-challenged asthmatic mice. PAS staining revealed an increased number of mucus-secreting goblet cells in OVA-challenged mice compared to control mice. Both high and low doses of azithromycin were less effective than budesonide at reducing airway mucus secretion in OVA-challenged mice, with the PAS staining trends in other groups aligning with those observed in H&E staining (Figure 6B).

Leukocyte counts in BALF samples were assessed using Giemsa staining (Figure 6C). Compared to control mice, the total leukocyte count, eosinophil count, and neutrophil count in the BALF of OVA-challenged mice were significantly elevated, and these counts increased further following vancomycin and neomycin intervention. FMT reduced leukocyte counts in OVA-induced asthmatic mice, irrespective of vancomycin and neomycin intervention. Treatment with azithromycin and budesonide significantly decreased total leukocyte counts, as well as eosinophil and neutrophil counts in the BALF of OVA-challenged mice. Azithromycin was less effective than budesonide at reducing total leukocyte and eosinophil counts, whereas it was more effective than budesonide at reducing neutrophil counts in the BALF of OVA-challenged mice.

These results demonstrate that in OVA-challenged asthmatic mice, treatment with vancomycin and neomycin exacerbated airway inflammation, whereas azithromycin treatment alleviated it.

Vancomycin and Neomycin Increased the Level of Inflammatory Cytokines in OVA-Challenged Mice, While Azithromycin Significantly Decreased Them

Total IgE, OVA-specific IgE, IL-4, IL-6, and IL-17A levels in BALF and total IgE in serum were measured by ELISA (Figure 7). Compared to control mice, OVA-challenged mice exhibited significantly higher levels of total IgE (Figure 7A and B), OVA-specific IgE (Figure 7C), IL-4 (Figure 7D), IL-6 (Figure 7E), and IL-17A (Figure 7F), with these effects further amplified by vancomycin and neomycin interventions. FMT significantly reduced levels of IgE and inflammatory cytokines, regardless of vancomycin and neomycin intervention. Similar to OVA-challenged mice treated with budesonide, the levels of total IgE, OVA-specific IgE, IL-4, IL-6, and IL-17A in BALF and total IgE in serum of OVA-challenged mice treated with azithromycin were significantly decreased. However, azithromycin was less effective than budesonide at reducing total IgE and OVA-specific IgE levels. Compared to budesonide, high-dose azithromycin showed no significant difference in the reduction of IL-4, IL-6, and IL-17A levels ($p > 0.05$). The combination of low-dose azithromycin and budesonide was superior to budesonide alone in reducing IL-6 and IL-17A levels.

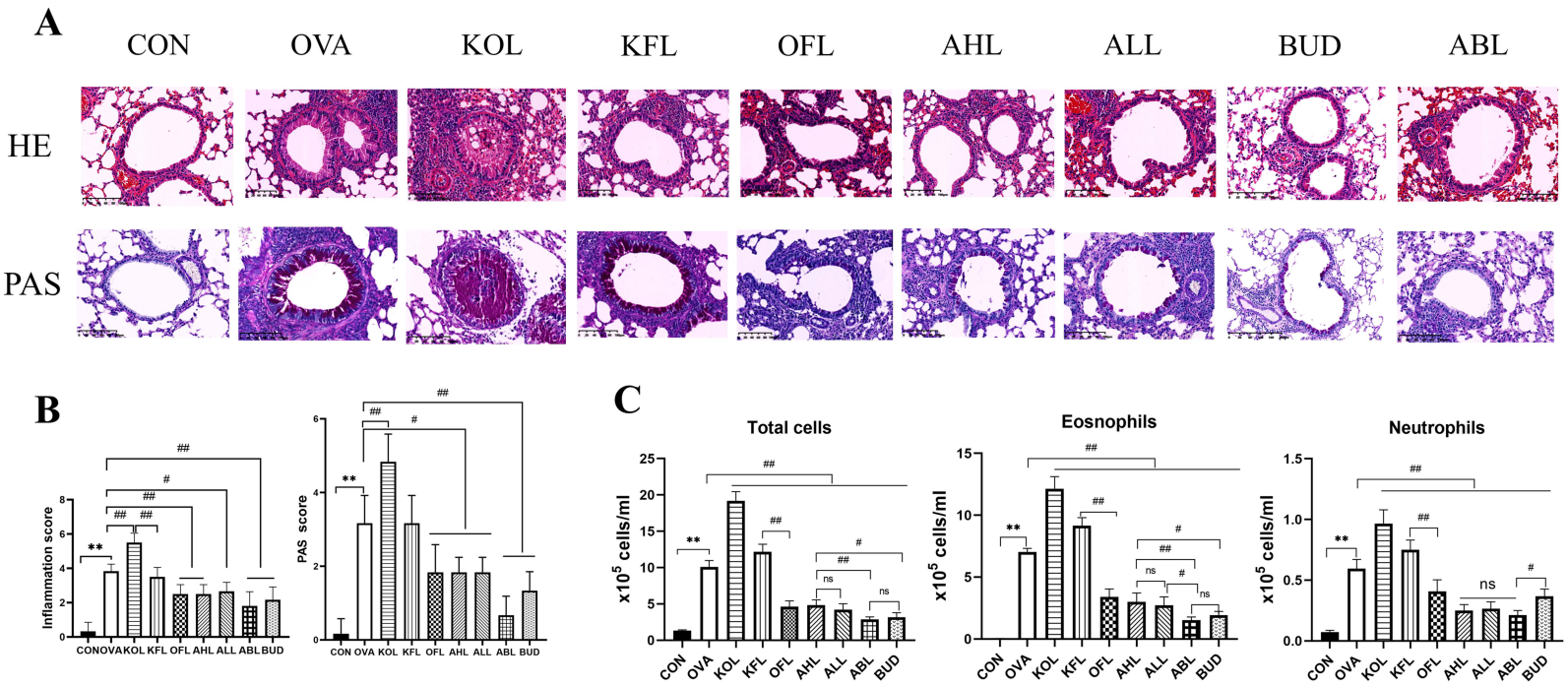

Figure 6 Effect of vancomycin and neomycin and azithromycin on airway inflammation in ovalbumin (OVA)-challenged asthmatic mice. (**A**) Representative haematoxylin and eosin (H&E) staining performed to observe airway inflammation in asthmatic mice (200×). Periodic acid-Schiff (PAS) staining indicating the mucus-producing goblet cells around the small airways (200×). (**B**) Inflammation scores are based on H&E staining. PAS scores indicating the mucus-producing goblet cells around the small airways (**C**). Leukocyte cell type counts in BALF samples. The BALF was centrifuged, and the precipitated cells were counted after staining using Wright-Giemsa. Data are expressed as mean ± standard deviation (SD). (n = 6, **P ≤ 0.01 vs the OVA group; #P ≤ 0.05 between the two groups, ##P ≤ 0.01 between the two groups, ns: no difference.).

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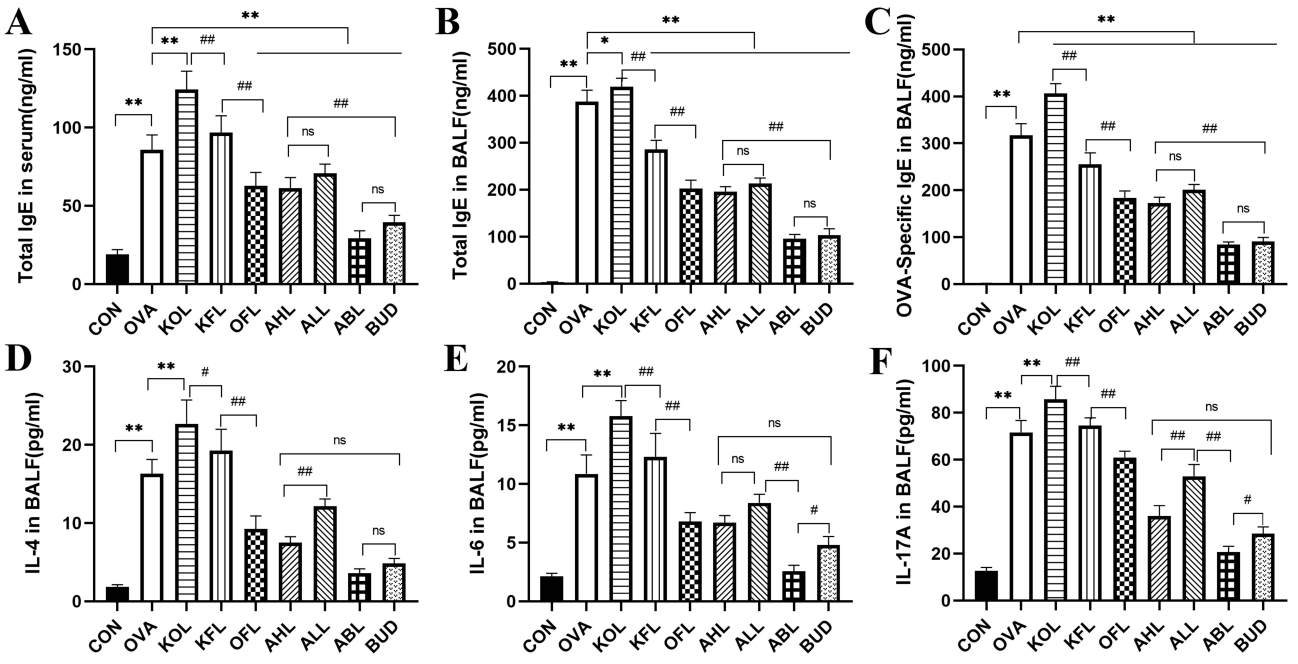

Figure 7 Effects of vancomycin and neomycin and azithromycin on IgE and inflammatory cytokines of asthmatic mice. Serum samples from different groups were collected to detect the levels of (**A**) total IgE. BALF samples from different groups were collected to detect the levels of (**B**) total IgE, (**C**) ovalbumin (OVA)-specific IgE, (**D**) IL-4, (**E**) IL-6, and (**F**) IL-17A using ELISA. Data are expressed as mean ± standard deviation (SD) and were tested using one-way ANOVA. (n = 6, *P ≤ 0.05 vs the OVA group; **P ≤ 0.01, vs the OVA group; $^{\#}$P ≤ 0.05, between the two groups; $^{\#\#}$P ≤ 0.01 between the two groups, ns: no difference).

Discussion

In the present study, we explored the effect of the gut-lung microbiome axis on airway inflammation in OVA-challenged asthmatic mice, revealing that azithromycin can reduce airway inflammation in asthma and may be related to the modulation of the gut-lung microbiome axis.

Firstly, by comparing the gut and respiratory microbiota of control mice and OVA-challenged asthmatic mice, we found that the β-diversity and microbial composition of the gut and respiratory microbiota of asthmatic mice were significantly different from those of control mice. Most previous studies have confirmed changes in the diversity of the gut and respiratory microbiota in patients with asthma and animal models.[26–28] The role of the gut microbiome in asthma has been studied by microbiome depletion in mice.[29] Most extreme microbiome studies employ germ-free (GF) animals that are devoid of microorganisms.[30,31] In this study, vancomycin and neomycin were used to induce dysbiosis of the gut microbiota in asthmatic mice. Many previous studies[32,33] have used vancomycin alone or in combination with other antibiotics, including neomycin and metronidazole, to establish models of gut commensal microbiota disorders. The gut microbiota diversity and microbial composition of asthmatic mice treated with vancomycin and neomycin were significantly altered, as was the respiratory microbiota. Russell. et al[34,35] found that exposure to vancomycin during early life altered the mouse gut microbiome and reduced short-chain fatty acids, leading to disrupted immune homeostasis and increased susceptibility to allergic lung inflammation. Moumen et al[36] found that maternal vancomycin treatment during pregnancy was associated with an increased severity of asthma in offspring in a dose-dependent manner. Previous studies[37,38] have also found alterations in the lung microbiome of mice exposed to vancomycin during early life.

The two groups of asthmatic mice were administered FMT before atomisation stimulation. The diversity and composition of the gut microbiota of asthmatic mice was greatly improved, and the diversity and composition of the respiratory microbiota were also significantly improved. Randomised controlled studies have shown that FMT is effective in treating ulcerative colitis, irritable bowel syndrome, and hepatic encephalopathy.[39] The manipulation of the gut microbiota through probiotics and FMT to combat asthma has become a popular research topic.[40] Thus, FMT may be a potential therapy for asthma in the future.[41]

Our study showed that airway inflammation in OVA-challenged asthmatic mice was further aggravated by vancomycin and neomycin treatment. In OVA-challenged asthmatic mice, FMT can alleviate airway inflammation in lung tissues, reduce the level of total IgE in serum, reduce the leukocyte cell type counts and IgE and cytokines levels in BALF regardless of the intervention of vancomycin and neomycin. Thus, the gut-lung microbial axis plays an important role in the pathogenesis of airway inflammation in asthma.

The current study and most previous studies[42,43] suggest that azithromycin can reduce airway inflammation in asthma. In the present study, we compared the effects of azithromycin and budesonide on airway inflammation in asthmatic mice. Both azithromycin and budesonide significantly alleviated airway inflammation in the lung tissues of OVA-challenged mice. Azithromycin was less effective than budesonide in reducing the total number of leukocytes and eosinophil counts, whereas azithromycin was more effective than budesonide in reducing neutrophil counts in the BALF of OVA-challenged mice. However, azithromycin was less effective than budesonide in reducing IgE levels in the BALF and serum. Compared to budesonide, high-dose azithromycin showed no significant difference in the reduction of IL-4, IL-6, and IL-17A levels in the BALF. It has been reported that the dosage of azithromycin for the control of asthma exacerbation is 500 or 250 mg three times a week, taken orally as tablets.[5,44] We compared the effects of high- and low-dose dose azithromycin on airway inflammation in OVA-challenged mice. For OVA-challenged mice treated with azithromycin, high and low doses were equally effective in improving airway inflammation in lung tissues and decreasing leukocyte cell type counts and IgE and cytokine levels in the BALF. Surprisingly, low-dose azithromycin combined with budesonide was superior to budesonide alone in reducing airway inflammation, especially neutrophil counts and IL-6 and IL-17A levels in the BALF of asthmatic mice. These results suggested that azithromycin combined with budesonide was more effective against non-eosinophilic asthma. Low-dose azithromycin is used as an add-on treatment for severe asthma[44] in adults, and azithromycin has long been used in patients with persistent symptoms of asthma, despite standard ICS/LABA treatment, to reduce asthma exacerbations and improve quality of life.[45]

Subsequently, we investigated the effects of azithromycin on respiratory and gut microbiota and found that it can affect the β-diversity of the respiratory and gut microbiota in OVA-challenged mice. Therefore, we investigated the effects of azithromycin on the composition of respiratory and gut microbiota. At the phylum level, azithromycin significantly reduced the abundance of Proteobacteria in the gut microbiota, but had little effect on the respiratory microbiota, whereas the combination of low-dose azithromycin and budesonide significantly reduced the abundance of Proteobacteria in the respiratory microbiota. At the same time, azithromycin can also increase the abundance of Firmicutes and Bacteroides in the gut microbiota. Slater et al[46] first found that azithromycin therapy was associated with decreased bacterial richness in the airways and altered the composition of airway microbiota, including Pseudomonas, Haemophilus, and Staphylococcus. However, Park et al[19] revealed that azithromycin had a greater effect on the gut microbiota and less of an effect on the respiratory microbiota in asthmatic mice. The differences in the results may be related to the different types of specimens, different modelling methods, and the dose and time of azithromycin administration. Proteobacteria have often been reported in previous studies to have significantly increased gut and respiratory microbiota in patients with asthma or animal models, especially in non-eosinophilic asthma.[2,47,48] At the genus level, the abundance of Pseudomonas was significantly reduced in the respiratory microbiota of the OVA-challenged asthmatic mice treated with azithromycin. Bacteroides and Prevotella were the dominant bacterial groups in the gut microbiota of asthmatic mice treated with azithromycin. Ferri et al[49] showed that Pseudomonas is an important risk factor for persistent and frequent asthma exacerbation. An increased abundance of Bacteroides in the gut microbiota is associated with a reduced risk of asthma.[50] Prevotella was found to be significantly more abundant in the gut microbiota of non-asthmatic individuals than in that of asthmatic patients.[51] Thus, azithromycin may reduce airway inflammation in asthmatic mice by regulating the gut-lung microbiome axis.

In conclusion, we found that OVA-challenged asthmatic mice with gut microbiota disorders also showed changes in respiratory microbiota, and airway inflammation was further aggravated. Following FMT to restore the gut microbiota, respiratory tract microbiota disorder was partially improved, and airway inflammation was significantly reduced. Thus, the gut-lung microbiome axis plays an important role in airway inflammation in asthma. Furthermore, this study demonstrated that azithromycin reduced airway inflammation in asthmatic mice, particularly non-eosinophilic inflammation, for which low-dose azithromycin combined with budesonide was more effective. Further studies on the effects of

azithromycin on the gut-lung microbiome axis indicated that azithromycin could significantly improve the diversity and microbial composition of the gut microbiota and exert certain effects on the respiratory microbiota. We hypothesize that azithromycin can reduce airway inflammation in asthma and may be related to modulation of the gut-lung microbiome axis.

Data Sharing Statement

The raw sequences of 45 female mice have been submitted to the NCBI Project under accession number PRJNA1176458. It should be noted that the raw sequences of the CON, OVA and BUD group in the BALF samples were used in the previous article (Front Pharmacol. 2022, 13: 911667) by our research team.

Ethics Statement

All programs and operations of the experiment were conducted in strict compliance with Shanghai Regulations on the Management of Experimental Animals. The experimental protocol was approved by the Center for Laboratory Animals, Shanghai Children's Hospital, Shanghai JiaoTong University, Shanghai, China (approval no. 2017Y003).

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Disclosure

The authors declare that this study was conducted in the absence of any commercial or financial relationships that could be construed as potential conflicts of interest.

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