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Prophylactic treatment of *Lacticaseibacillus paracasei* K56 ameliorates asthma by regulating short-chain fatty acid production and Treg differentiation

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ABSTRACT

Many studies have shown that the development of allergic asthma is associated with intestinal microbiota dysbiosis. Based on the gut-lung axis theory, probiotic intervention may be a potential strategy for respiratory diseases. *Lacticaseibacillus paracasei* K56 was reported to regulate gut microbiota in mice. However, its effect on allergic asthma has not been reported. In this study, we investigated the effect of the K56 on ovalbumin-induced asthma and its possible mechanisms. Our results showed that K56 reduced asthma symptoms and inflammatory cell infiltration in the lung of asthmatic mice. And K56 regulated the differentiation of helper T cells in the lung and intestine. Results from 16S rRNA gene sequencing showed that K56 prophylaxis significantly elevated the richness of *Akkermansia* and *Burkholderia*. Meanwhile, K56 abrogated the decrease in the levels of short-chain fatty acids (SCFAs) in the feces. In addition, a rebound in the mRNA expression of the SCFA receptors was observed after K56 prophylaxis. Notably, the regulatory T cell (Treg) frequencies and the *FFAR3* levels were positively correlated. These results suggest that K56 could attenuate asthma, possibly by modulating the intestinal microbiota and regulating Treg differentiation through SCFA metabolized. Our study showed that K56 may be used as a probiotic to prevent pulmonary inflammation and dysbiosis of the intestinal microbiota in allergic asthma.

1. Introduction

Allergic asthma is a common chronic inflammatory respiratory disease with high morbidity worldwide and an acute asthma attack can be fatal, leaving heavy burdens for families and consuming enormous amounts of economic and medical resources. The clinical manifestations are recurrent wheezing, shortness of breath, cough, etc., and difficulty in breathing may occur in severe attacks[1-2]. In recent years, the incidence of asthma has been increasing year by year worldwide. Clinical control of asthma currently uses glucocorticoids or β2 agonists for symptomatic relief[3]. However, long-term use of glucocorticoids may cause some adverse effects, including osteoporosis, hypertension, obesity, type 2 diabetes, gastrointestinal ulcers/bleeds, fractures, and cataracts, and develop drug resistance[4-5]. Therefore, finding effective ways to prevent asthma attacks or control symptoms is urgent.

Many studies have shown that intestinal microbiota plays a vital role in the pathogenesis of asthma[6-7]. The hygiene hypothesis and the concept of the gut-lung axis proposed that disruption of the gut microbiome in early life may be a predictor of the development of asthma. Nowadays, some evidence supports that the incidence of allergic asthma is closely related to the imbalance of gut microbiota[8-9]. Microorganisms that colonize the intestinal tract in

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large numbers can produce metabolites that activate the host immune response and recruit immune cell infiltration locally. Immune cells can circulate through the blood system and act distally in the lung[9]. Therefore, regulating the composition and community structure of gut microbiota can effectively relieve asthma. Recent studies have found that oral administration of *Lactobacillus* may alleviate asthma symptoms. For example, treatment with *Lactobacillus rhamnosus* can reduce airway hyperreactivity and the levels of helper T (Th) 2 cytokines in the bronchoalveolar lavage fluid (BALF) and serum, as well as suppress the pro-inflammatory response of Th17 cells in asthmatic mice[10-12]. Clinical studies have also confirmed that correcting gut microbiota disorder can attenuate allergic disease[13]. Therefore, it remains critical to investigate the microbiota of asthmatic patients and to develop new therapeutic approaches.

Probiotics are active microorganisms that can prevent or treat diseases by taking them orally. Various studies have shown that probiotics work by restoring the balance of gut microbiota and regulating its metabolites. *Lactobacillus* is one of the most widespread live bacteria and numerous experiments have shown that such species can help prevent allergic and respiratory diseases. Many of them can regulate the composition of the intestinal microbial community and the levels of its metabolites, especially the short-chain fatty acids (SCFAs). SCFAs can prevent the development and adhesion of potential pathogenic bacteria and enhance the integrity of epithelial cells, thus improving host system immunity[14]. Studies have shown that SCFA acting as a mediator of the gut-lung axis can promote the differentiation of regulatory T cells (Tregs), and reduce excessive inflammatory responses in gastrointestinal and airway diseases[15-16].

Lacticaseibacillus paracasei subsp. *paracasei* K56 was isolated from the intestine of a healthy infant and can be safely used in the food industry, and many experiments have shown that it has neither oral acute toxicity nor antibiotic resistance[17]. Nowadays drinking yoghurt, ice cream, or probiotic sachets containing K56 are available in China. Regarding its probiotic function, K56 was reported to increase the relative abundance of Proteobacteria, Actinobacteria, and Patescibacteria in the gut of obese mice[18]. In addition, studies have shown that obesity is likely a factor in increased asthma risk, and personalized dietary intervention may improve respiratory symptoms and signs and therapeutic response[19]. Therefore, we explored the possibility of K56 as an edible probiotic that exerts preventive and therapeutic effects on ovalbumin (OVA)-induced allergic asthmatic mice via the gut-lung axis. Moreover, the immunomodulatory mechanism of K56 in the intestine and lungs of asthmatic mice was investigated. Our study also provides reliable experimental guidance for the application of probiotics in the clinical treatment of allergic asthma.

2. Materials and methods

2.1 Preparation of K56

L. paracasei subsp. *paracasei* K56 was provided by Yili Co., Ltd. (Hohhot, China) in powder form, with the specifications of 1.5×10^{11} CFU/g. The bacterial powder was dissolved in 0.9% NaCl solution to prepare a 5×10^{9} CFU/mL concentration of K56 solution.

2.2 Establishment of OVA-induced asthmatic mouse model

Female BALB/c mice aged 8 weeks were purchased from SPF (Beijing) Biotechnology Co., Ltd. (SCXK (Jing) 2019-0010) (Beijing, China). All mice were housed in specific pathogen-free conditions. Room temperature was controlled at 22−28 °C, the humidity was controlled at 40%−60%, and a 12 h/12 h light/dark cycle was maintained. Mice had free access to an irradiated and sterilized maintenance feed as well as distilled water. After 1 week of acclimatization, mice were randomly divided into 3 or 4 groups (6 or 10 mice per group): control, OVA, OVA + K56 prophylactic, and OVA + K56 treatment. Except for the control group, mice in the other 3 groups were sensitized intraperitoneally on day 0 and day 14 with 0.2 mL of sensitizing solution containing 20 μg OVA (Sigma-Aldrich, USA) and 1/3 volume of Imject™ Alum (Thermo Fisher Scientific, USA). Then, the mice were challenged by aerosolized 1% OVA from day 21 to day 29. At the same time, mice in the control group were treated with an equivalent dose of phosphate buffered saline (PBS). Mice in the OVA + K56 prophylactic group were given K56 solution from day 1 to day 29 during the whole experiment, and mice in the OVA + K56 treatment group were given K56 solution from day 21 to day 29 during the nebulization. All animals were sacrificed on day 28. The animal care and protocol were approved by the Experimental Animal Ethics Committee of Beijing University of Chinese Medicine (BUCM-4-2021090106-3162).

2.3 Behavioral test

The asthmatic behaviors of the mice were observed and scored within 10 min from the start of nebulization. No scratching was scored as 0; 1−3 scratches were scored as 1; 4−6 scratches were scored as 2; and 7 or more scratches were scored as 3. Mice with no asthma symptoms are scored 0; mice with mild asthma symptoms are scored 3; mice with typical asthma symptoms such as shortness of breath, significantly faster breathing rate, or agitation are scored 6; mice with severe respiratory distress such as rapid head nodding and abdominal breathing are scored 9.

2.4 Assessment of pulmonary function

One day after the last challenge, airway hyperresponsiveness was measured using FlexiVent (SCIREQ, Montreal, QC, Canada) according to the manufacturer's instructions. Mice were anesthetized with 0.1 g/kg pentobarbital sodium and the trachea was exposed. Mice were then connected to a computer-controlled animal ventilator via a tracheal intubation tube. Acetylcholine at 0, 3.125, 6.25, 12.5, 25, and 50 mg/mL was used to stimulate the test, and the changes in the index were observed in mice. The following measures of respiratory mechanics were calculated: Respiratory system resistance (Rrs), respiratory system elasticity resistance (Ers), and respiratory system compliance (Crs). Data were recorded continuously and analyzed offline.

2.5 Histopathological evaluation

One day after the last challenge, mice were sacrificed and the lungs and colons were collected. The lung tissues and colons were washed in PBS. The tissues were then fixed in 10% formaldehyde at

room temperature, dehydrated in a graded concentration of ethanol, and then embedded in paraffin. Tissue sections of 4 μm were stained with hematoxylin and eosin (H&E) to evaluate the histopathological changes in the lung and the colon. Digital images of pulmonary morphology at 200 magnification were obtained using a light microscope and graded according to the reference[20].

2.6 Preparation of BALF and inflammatory cells isolation

Mice were sacrificed 24 h after the last challenge. The chest cavity was opened to expose the trachea and tracheal intubation was performed. The lungs were slowly injected with 0.8 mL of 0.5% bovine serum albumin (BSA) solution and subsequently recovered, and this was repeated three times. The collected BALF was then centrifuged to precipitate the cells. The first BALF supernatant was stored at −80 °C for later use.

2.7 Cell isolation from lung and intestinal tissues in mice

Single-cell suspensions were obtained from the lung and colon according to the previous study[21]. Fresh lung tissues were minced and incubated in 5 mL RPMI-1640 (Biological Industries, Israel) with 1 mg/mL collagenase type IV (Worthington, USA) and 5 μg/mL DNase I (Roche, Switzerland) for 40 min, then filtered through 70 μm cell strainers. After the removal of adherent connective tissue and Peyer's patches, the colon was clipped and washed twice by shaking in 20 mL D Hank's buffer (Solarbio, Beijing) containing 5 mmol/L ethylene diamine tetraacetic acid (EDTA) (Invitrogen, USA) and 1 mmol/L dithiothreitol (DTT) (Inalco, USA). Next, 10 mL RPMI-1640 (Biological Industries, Israel) with 2 mg/mL collagenase type III (Worthington, USA) and 5 μg/mL DNase I was used to digest colon tissues. The digested tissues were filtered through 70 μm cell strainers and separated with 40% Percoll. Single-cell suspensions of the lungs and colons were used for subsequent flow cytometry staining.

2.8 Flow cytometry analysis

BALF cells and single-cell suspensions isolated from lungs and colons were analyzed by flow cytometry. The cells were incubated in a complete medium with cell stimulation (Ebioscience, USA) at 37 °C for 4 h. After purified anti-mouse CD16/32 blocking, staining was performed with fluorescent-conjugated antibodies. Cells were then stained with PB anti-mouse CD45, PE-Cy7 anti-mouse CD11b, PE anti-mouse Siglec-F, APC anti-mouse Ly6G, AF700 anti-mouse F4/80, fluorescein 5-isothiocyanate (FITC) anti-mouse CD3, APC-Cy7 anti-mouse CD3, APC-Cy7 anti-mouse CD4, AF700 anti-mouse CD4, FITC anti-mouse interferon-γ, APC anti-mouse (interleukin, IL)-4, PE anti-mouse IL-17A, PerCP-cy5.5 anti-mouse CD25, and APC anti-mouse Foxp3. All these antibodies were purchased from Biolegend Ltd., U.S.A. The stained cells were detected by CytoFLEX and analyzed by FlowJo software.

2.9 OVA-specific immunoglobulin E (IgE) detection in BALF and serum by ELISA

The blood of mice was collected 24 h after the last challenge. Blood was left to stand for 2 h at room temperature, then centrifuged at 3 000 r/min, and serum was collected. OVA-specific IgE levels in serums and BALF were detected by ELISA MAX™ Mouse OVA Specific IgE ELISA Kit (439807, BioLegend, USA) according to the instructions.

2.10 RNA isolation, cDNA synthesis, and real-time polymerase chain reaction (PCR)

Total RNA was extracted from the colon using SteadyPure Universal RNA Extraction Kit (Accurate Biology, China). Then the RNA was reverse transcribed to cDNA using the Evo M-MLV RT Premix (Accurate Biology, China). The relative gene expression was quantified by Q-PCR using SYBR® Green Premix Pro *Taq* HS qPCR Kit (Accurate Biology, China) in a QuantStudio6 Flex system (Life Technologies, USA). The mRNA expressions of targeted genes were normalized using a housekeeping control (*GAPDH*) and calculated by the $2^{-\Delta\Delta Ct}$ method. The primer sequences used in this study are shown in Table 1.

2.11 DNA extraction, 16S rRNA gene amplification and sequencing, and raw data analysis

Fecal DNA was extracted using the QIAamp DNA Stool Mini Kit (Qiagen Ltd., Germany) following the manufacturer's protocol. The V3-V4 region of the 16S rRNA gene was amplified using universal primers 338F (5′-ACTCCTACGGGAGGCAGCAG-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′). After purification and quantification, the PCR products were pooled into equimolar amounts and sequenced on the Illumina MiSeq sequencer to generate paired-end reads of 300 bp.

Raw FASTQ files were de-multiplexed and quality-filtered using QIIME (version 1.9). In brief, low-quality sequences with a length of < 220 nt or > 500 nt, an average quality score of < 20, and sequences containing > 3 nitrogenous bases were removed. The remaining high-quality reads were then clustered into operational taxonomic units (OTUs) at a 97% similarity cutoff using UPARSE (version 7.1) and chimeric sequences were removed using UCHIME. Taxonomy assignment of OTUs was conducted using the RDP classifier against the SILVA 16S rRNA gene database (Release 132) with a confidence threshold of 0.70.

Table 1
Real-time PCR primer sequences.

Gene	Forward (5′-3′)	Reverse (5′-3′)
FFAR2	TGCTACGAGAACTTCACCCAA	CACACGAAGCGCCAATAACAG
FFAR3	TTCTGAGCGTGGCCTATCCA	AGACTACACTGACCAGACCAG
PPARγ	GGAAGACCACTCGCATTCCTT	GTAATCAGCAACCATTGGGTCA
GAPDH	GCACCACCAACTGCTTAG	GGATGCAGGGATGATGTTC

Alpha diversity was determined via the observed species index calculated by QIIME (version 1.9). Principal coordinates analysis (PCoA) was carried out based on Bray-Curtis distance using QIIME (version 1.9) to evaluate beta diversities. Besides, permutational multivariate analysis of variance (PERMANOVA, with 1 000 Monte Carlo permutations) was conducted based on the above distance matrices to compare the similarity of community structures between groups using the adonis function available in the "vegan" package of R (version 4.2.1). The differentially abundant bacterial taxa among groups were identified using linear discriminant analysis (LDA) effect size (LEfSe) analysis. Only taxa with an average relative abundance greater than 0.01% were considered.

2.12 Determination of SCFAs in the colon

The concentrations of SCFAs of colonic contents (acetate, propionate, butyrate, valerate, caproate, isobutyrate, and isovalerate) were detected with a gas chromatography-mass spectrometry (GC-MS) method as previously described[22-23]. Briefly, 20 mg of colon contents sample was added in a 2 mL centrifuge tube with 1 mL of 0.5% phosphoric acid solution (Hushi, Shanghai, China). Then, a small steel ball was added to the vortex for 10 min by a ball mill (MM400, Retsch, Germany). The mixture was treated with a 5 min ultrasonication in the ice bath and then centrifuged at 12 000 r/min for 10 min at 4 °C. Added 500 μL of methyl tert-butyl ether solvent containing internal standard to 100 μL of supernatant, centrifuged for 3 min, then treated the mixture with ultrasonication and centrifuged in the same conditions as above. Finally, 200 μL of supernatant was injected into an Agilent GC-MS (7890B-7000D, Agilent, USA).

2.13 Statistical analysis

Data were analyzed using SPSS (version 22.0, SPSS Inc., USA). Parametric data were analyzed using unpaired one-way ANOVA with Tukey's post hoc test. Nonparametric data were analyzed using the Kruskal-Wallis test. Data were presented as means and standard error of the mean (SEM). Correlation analysis was performed using the Spearman method. P values less than 0.05 were considered statistically significant.

3. Results

3.1 The K56 significantly relieved asthma attacks induced by OVA

To investigate the effect of K56 on asthma, we used the asthmatic mouse induced by the OVA challenge, and the K56 intervention was divided into two groups. K56 prophylactic administration was shown as OVA + P-K56, whereas K56 therapeutic administration was shown as OVA + T-K56. As shown in Fig. 1A, the body weight

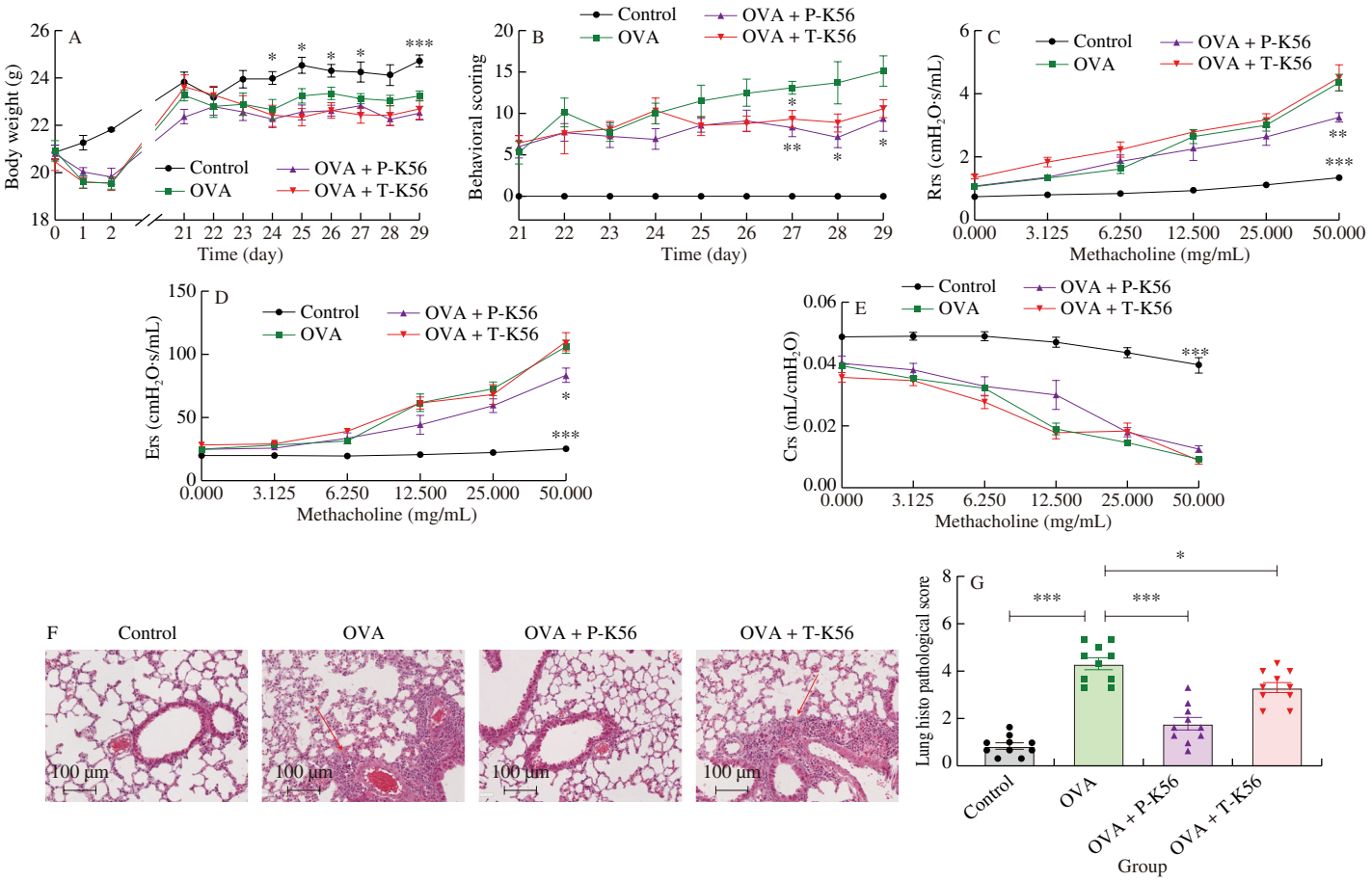

Fig. 1 K56 significantly relieved asthma attacks induced by OVA. (A) Effects of K56 on body weight; (B) Behavioral scoring during the OVA challenge; (C-E) Assessment of pulmonary function: (C) Rrs; (D) Ers; (E) Crs; (F) H&E staining of the lung (200 ×), red arrow means the inflammatory site; (G) Histopathological scores of the lung. Data were shown as means ± SEM, $n = 10$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, compared to the OVA group. Parametric data were analyzed using unpaired one-way ANOVA with Tukey's post hoc test. Nonparametric data were analyzed using the Kruskal-Wallis test.

of mice in the OVA group decreased compared to the control group, and both P-K56 and T-K56 groups did not rescue the decreased body weight (Fig. 1A). During the OVA challenge, we recorded and scored the behaviors of mice. The results showed that the number of nasal scratching and asthmatic behaviors significantly increased with the duration of nebulization, while K56 significantly alleviated the behavior score of mice on day 27, 28, and 29 (Fig. 1B).

Subsequently, to further verify the effect of K56 on the pulmonary function of mice, the airway hyperresponsiveness of mice was examined. The results showed that both total Rrs and Ers were increased in asthmatic mice compared to the control mice with improving methacholine concentration. K56 prophylaxis effectively alleviated the increase of Rrs and Ers in asthmatic mice (Figs. 1C-D). As for Crs, asthmatic mice exhibited a significant decrease in Crs, while K56 showed partial improvement in Crs at 12.5 mg/mL methacholine concentration (Fig. 1E).

To further verify the effect of K56 on lung inflammation in asthmatic mice, lung tissue was stained with H&E. Data revealed that asthma attack resulted in a substantial infiltration of inflammatory cells in the lungs and damage to the lung septa, as well as a significant increase in histopathological scores (Figs. 1F-G). However, K56 administration resulted in significant relief of inflammatory infiltrates and septal damage in the lungs, along with a significant reduction in inflammation scores. Surprisingly, the effect of prophylactic administration of K56 was better than that of therapeutic administration. These results suggested that the K56 mitigated loss of pulmonary function and lung histopathology in asthmatic mice.

3.2 *Prophylactic administration of K56 significantly reduced airway inflammatory cell infiltration*

Considering the precluding of K56 on inflammatory infiltrates of lung tissues in asthmatic mice, we further analyzed the phenotype of these inflammatory cells in BALF by flow cytometry. The results showed a significant increase in total cell count in the BALF of asthmatic mice compared to the control mice, accompanied by a significant dominance in eosinophils and macrophages (Figs. 2A-C). K56 administration tended to reduce these cells to varying degrees. However, only prophylactic administration effectively reduced the number of eosinophils and macrophages. It was also noteworthy that the number of $CD4^+$ T cells was significantly increased in the BALF of asthmatic mice compared to the control group, and it was significantly reduced in both K56 administrations (Fig. 2D). We subsequently measured the levels of OVA-specific IgE in BALF and serum by ELISA. The results showed a significant augment in OVA-specific IgE levels in asthmatic mice compared to the control mice, both in BALF and serum. Moreover, prophylactic administration of K56 corrected this change (Figs. 2E-F). These results suggested that K56 ameliorates airway inflammation in asthmatic mice, and the preventive effect was superior to the therapeutic administration.

3.3 *K56 prophylactic administration affected $CD4^+$ T cells in the lung of asthmatic mice*

Since the above results showed more significant efficacy with prophylactic administration, we further investigated the mechanism of asthma relief with K56 using the same approach as the previous prophylactic administration. To explore the effect of K56 on the lung $CD4^+$ T cell population, we conducted a flow cytometry analysis of single-cell suspensions from the lung. The results revealed a remarkable increase in total cells, Th2, Th17 cells, and Treg increased in asthmatic mice compared to the control (Figs. 3A, C-E), and an apparent decrease in Th1 cells (Fig. 3B). Compared with the asthmatic mice, K56 prophylactic administration significantly reduced the proportion of total cells and increased the proportion of Treg in the lung. At the same time, K56 prophylactic administration corrected the

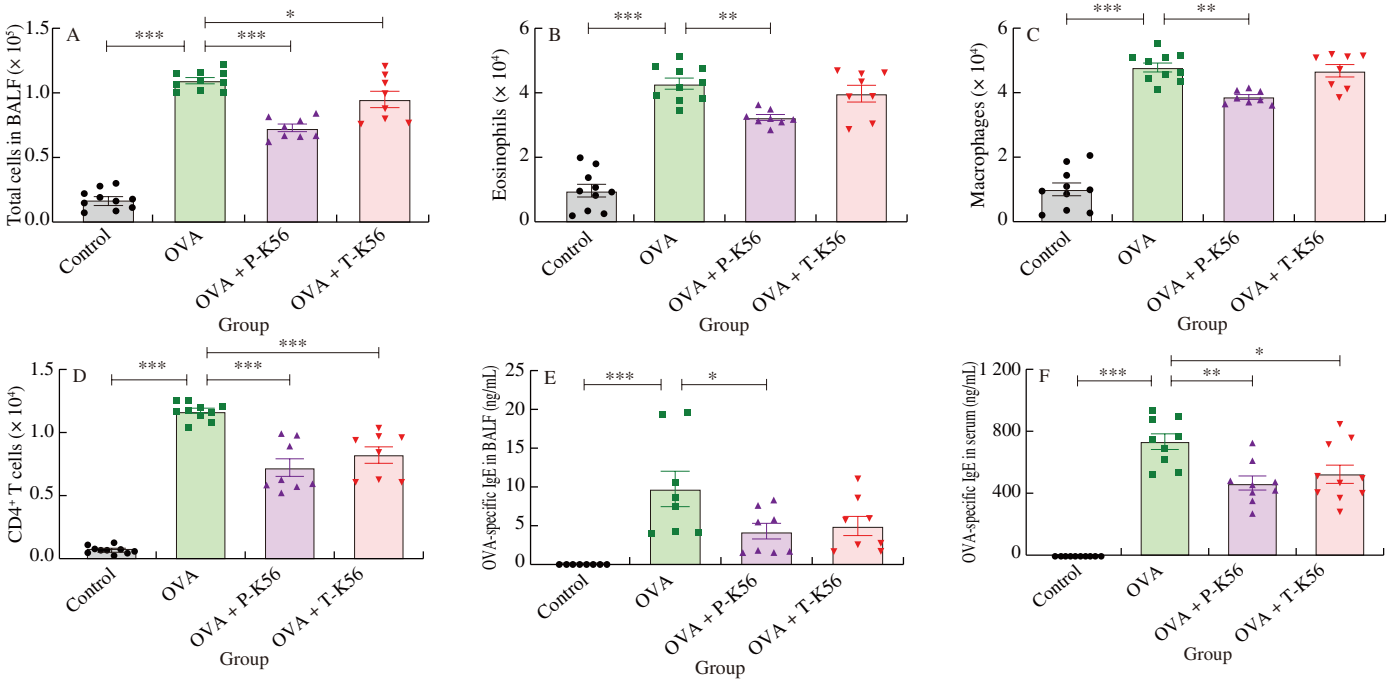

Fig. 2 Prophylactic administration of K56 significantly reduced airway inflammatory cell infiltration. (A) Total cells count in BALF; (B-D) The count of eosinophils (B), macrophages (**C**), and $CD4^+$ T cells (D) in BALF; (E-F) OVA-specific IgE in BALF and serum. Data were shown as means ± SEM, $n = 8–10$, $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

lung Th17/Treg imbalance in asthmatic mice (Fig. 3F). These results suggested that K56 prophylactic administration affected $CD4^+$ T cell differentiation in the lungs of asthmatic mice and restored lung Th17/Treg balance.

3.4 Prophylactic administration of K56 increased colon Treg in asthmatic mice

Considering oral administration of K56 as a food-grade probiotic isolated from the intestine of a healthy infant, we hypothesized that the K56 entering the intestine would first affect intestinal $CD4^+$ T cells, which would subsequently affect lung $CD4^+$ T cells in asthmatic mice. The colon length of asthmatic mice was reduced compared to control mice, and K56 prophylactic administration had an ameliorating effect on this condition (Fig. 4A). Subsequently, we performed flow cytometry assays on the $CD4^+$ T cell population from lamina propria of the colonic mucosa. The results revealed that although having no significant effect on the numbers of total cells, Th1, Th2, and Th17 cells (Figs. 4B-E), K56 prophylactic administration elevated the proportion of Treg (Fig. 4F). These results suggested that the alleviating effect of the K56 on asthma might be correlated with its promotion of intestinal Treg differentiation.

3.5 K56 prophylactic administration can affect gut microbiota in asthmatic mice

Previous studies have indicated that the gut microbiota can exert regulatory effects on Treg differentiation[24]. Therefore, we performed a 16S rRNA gene sequencing on murine feces to further investigate how the K56 affects the intestinal microbiota as well as the microbial potential effect on Treg differentiation. It was observed that the K56 intervention slightly improved the lower microbial community richness in the gut of asthmatic mice, as reflected by a lower observed species index (Fig. 5A). PCoA plots further demonstrated the significant separation of gut microbiota among different groups. PCoA based on Bray-Curtis distances (Control vs. OVA: $R^2 = 0.206$, $P = 0.038$; OVA vs. OVA + K56: $R^2 = 0.087$, $P = 0.378$) confirmed that the microbiota community structures of mice were greatly affected after OVA induction. In contrast, after the K56 intervention, the community structures tended to approach that of control mice (Fig. 5B).

Moreover, a significant difference in the community composition of the mice intestinal microbiota at the phylum and genus levels was also seen among different groups. At the phylum level, Bacteroidetes and Firmicutes comprised two dominant phyla, with their total relative abundance exceeding 60%. The relative abundance of Bacteroidetes was elevated in asthmatic mice and reduced in K56-treated mice, while Firmicutes was the opposite (Fig. 5C). At the genus level, *Bacteroides* was the most abundant, with an increase in asthmatic mice and a decrease in K56 treatment mice (Fig. 5C). Subsequently, we conducted the LEfSe analysis to search for differentially abundant genera among 3 groups of mice. At the genus level, compared with the control group, OVA significantly enriched *Clostridium*. While compared with K56, OVA significantly enriched *Prevotella* and *Thermus*, but K56 significantly enriched *Christensenella* (Fig. 5D).

We then looked for which genus emerged after the K56 administration (Fig. 6). Apparently, the relative abundance of *Clostridium* and *Turicibacter* was significantly elevated, while the abundance of *Flexispira* decreased in asthmatic mice. At the same time, the abundance of *Bacteroides*, *Prevotella*, *Burkholderia*,

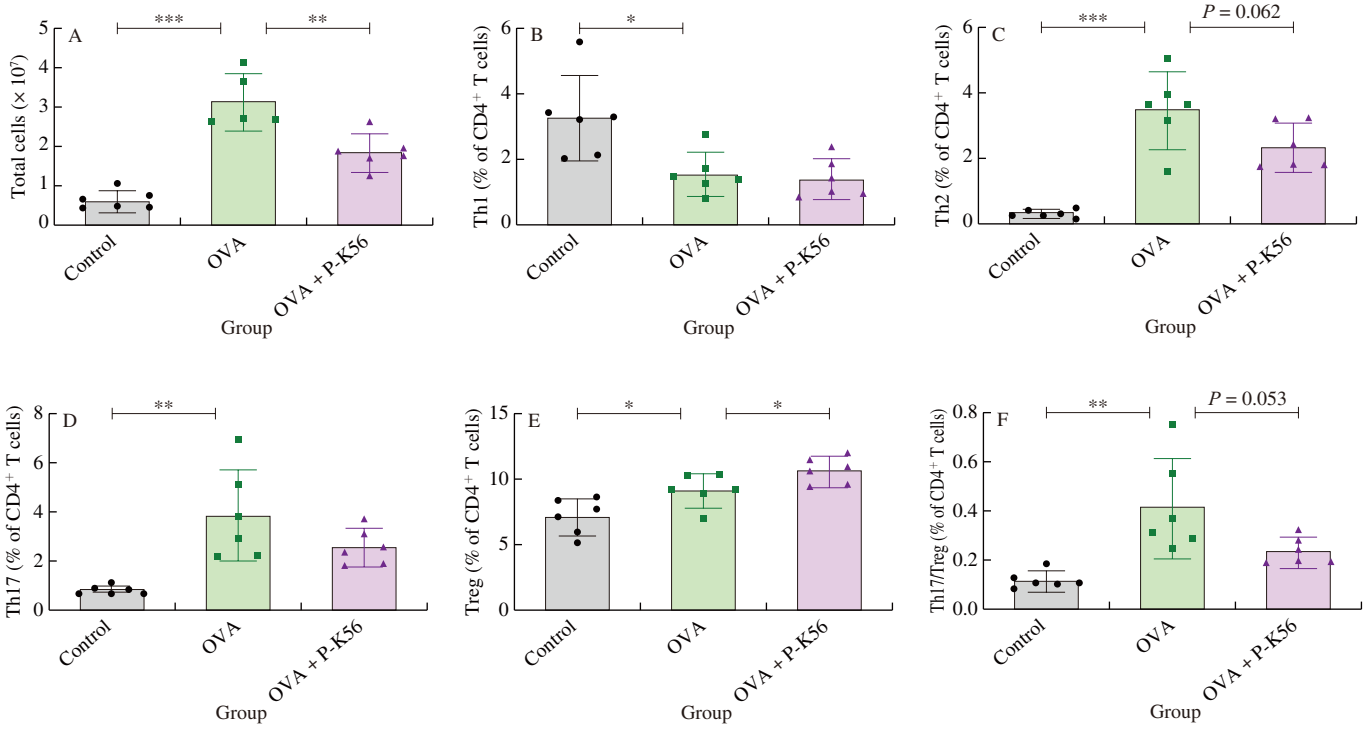

Fig. 3 Effect of K56 prophylactic administration on $CD4^+$ T cells in the lung of asthmatic mice. (A) Total cells count in the lung; (B-E) Proportion of Th1 cells (B), Th2 cells (C), Th17 cells (D), Tregs (E) in the lung $CD4^+$ T cells; (F) Th17/Treg of the lung $CD4^+$ T cells. Data were shown as means ± SEM, $n = 6$, $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

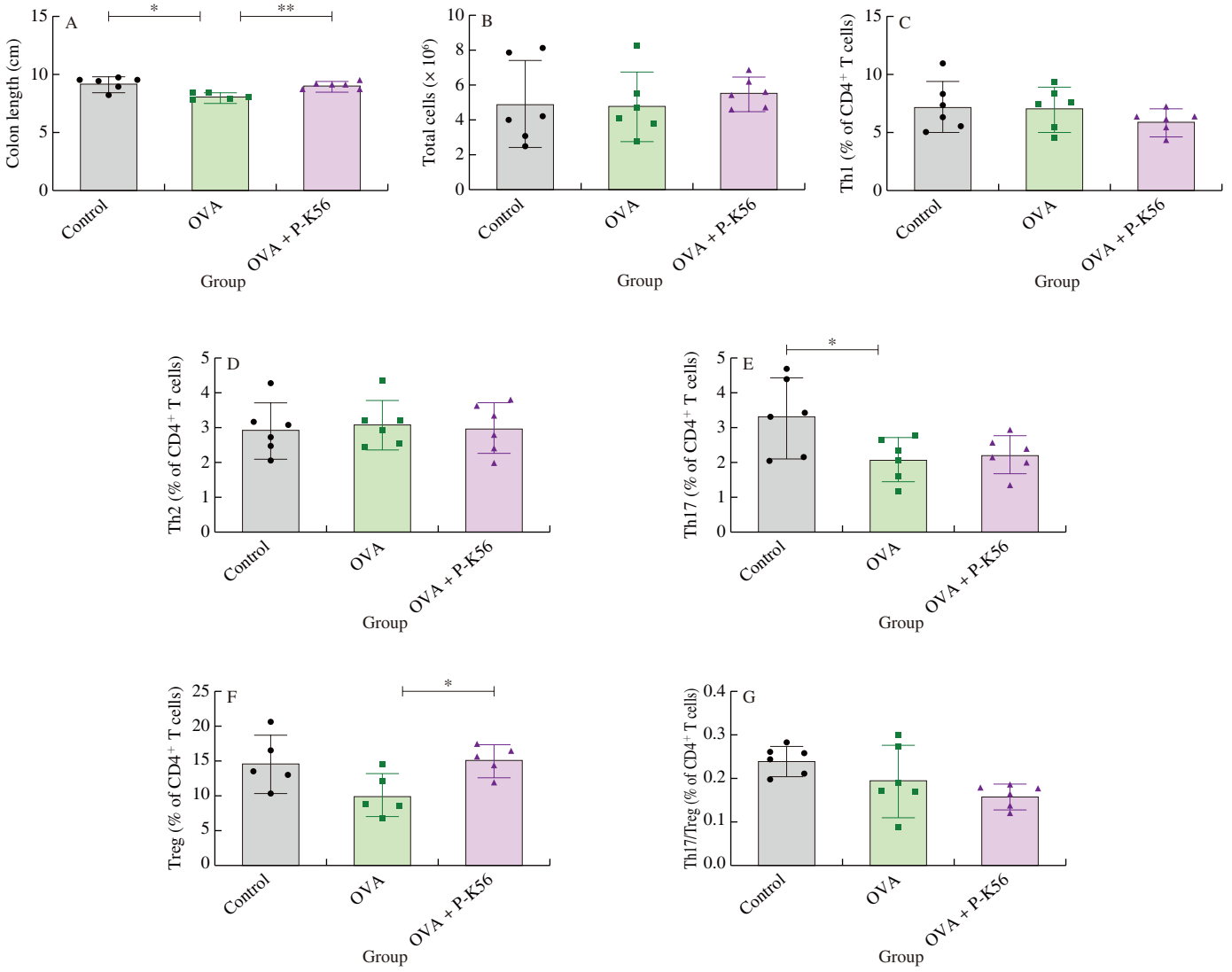

Fig. 4 Prophylactic administration of K56 increased colonic Treg in asthmatic mice. (A) Colon length of mice; (B) Total cells count in the colon; (C-F) Proportion of Th1 cells (C), Th2 cells (D), Th17 cells (E), Treg (F) in the colon $CD4^+$ T cells; (G) Th17/Treg of colon $CD4^+$ T cells. Data are shown as means ± SEM, $n = 6$, $^*P < 0.05$, $^{**}P < 0.01$.

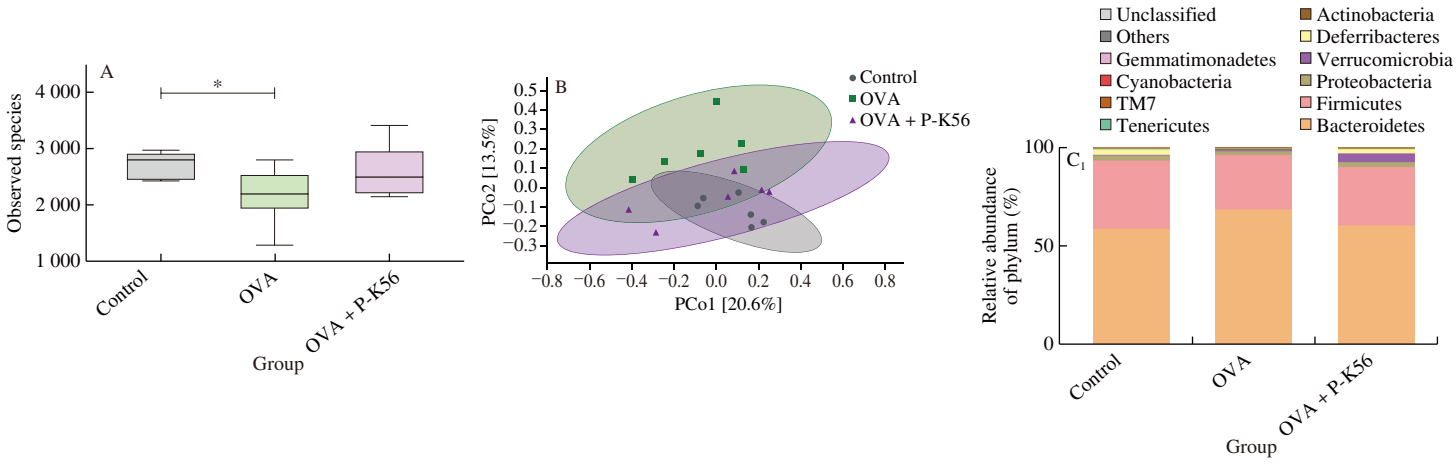

Fig. 5 Effects of K56 prophylactic administration on gut microbiota. (A) Observed species index; (B) PCoA based upon Bray-Curtis distances; (C) Abundant phyla and abundant genera among different groups of mice; (D) Differentially abundant genera identified by LEfSe with a LDA score (threshold ≥ 2). Data were shown as means ± SEM, $n = 6$, $^*P < 0.05$.

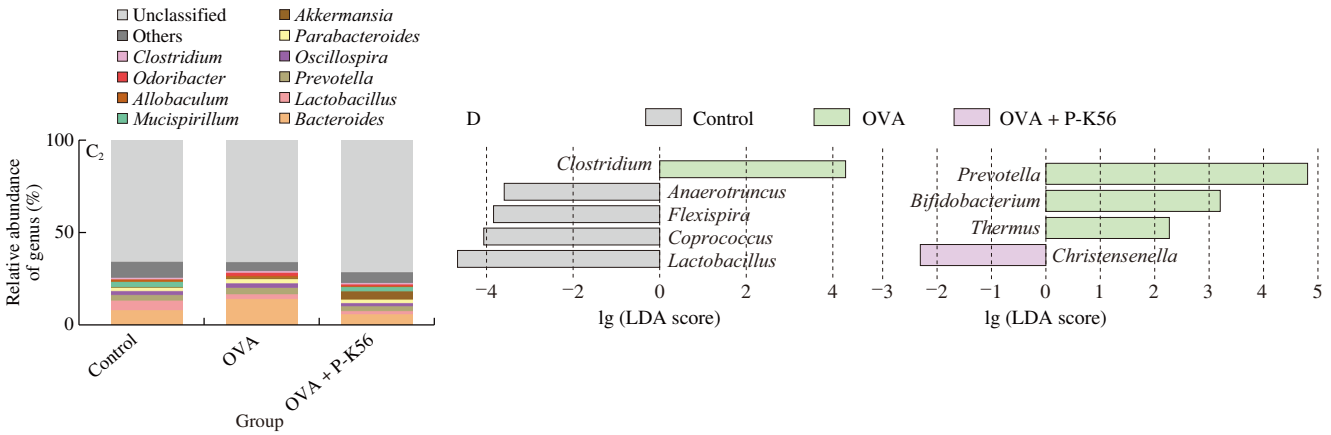

Fig. 5 (*Continued*)

Sutterella, and *Shigella* showed an increasing trend. However, K56 prophylaxis reversed the increase in the abundance of *Bacteroides*, *Prevotella*, *Clostridium*, *Turicibacter*, *Sutterella*, and *Shigella* in asthmatic mice, and significantly elevated the abundance of *Akkermansia* and *Burkholderia*. Overall, prophylactic administration of K56 significantly altered the composition of the gut microbiota community.

3.6 K56 prophylactic administration affected metabolites and SCFA receptors in asthmatic mice

The gut microbiota can produce SCFAs through anaerobic fermentation of dietary fibers, which can influence immune homeostasis by inducing Treg differentiation[25]. Therefore, we next investigated the levels of SCFAs of colonic contents in mice. Prophylactic administration of K56 reversed the significant decrease in the levels of butyrate, valerate, caproate, and isovalerate in the feces of mice due to asthma (Fig. 7A). At the same time, K56 could significantly improve isobutyrate levels (Fig. 7A). We next performed a correlation analysis between SCFAs and differential gut microbial genera (Fig. 7B). We found that K56 influenced SCFAs in close association with many intestinal microorganisms. Notably, *Burkholderia* was positively linked to butyrate while *Sutterella* was negatively associated with butyrate. In subsequent analyses, we found a significant positive correlation between butyrate and Treg (Fig. 7C). To further investigate how SCFAs affected Treg, we examined colonic SCFA receptors *FFAR2*, *FFAR3*, and *PPARγ* levels. The mRNA expression of *FFAR3* and *PPARγ* was significantly reduced in asthmatic mice compared to the control group, whereas K56 intervention elevated the mRNA expression of *FFAR3* and *PPARγ* (Fig. 7D). Additionally, further correlation analysis also showed that Treg in the colon were significantly and positively correlated with *FFAR3*, whereas they were not significantly correlated with *FFAR2* and *PPARγ* (Fig. 7E). These results suggested that K56

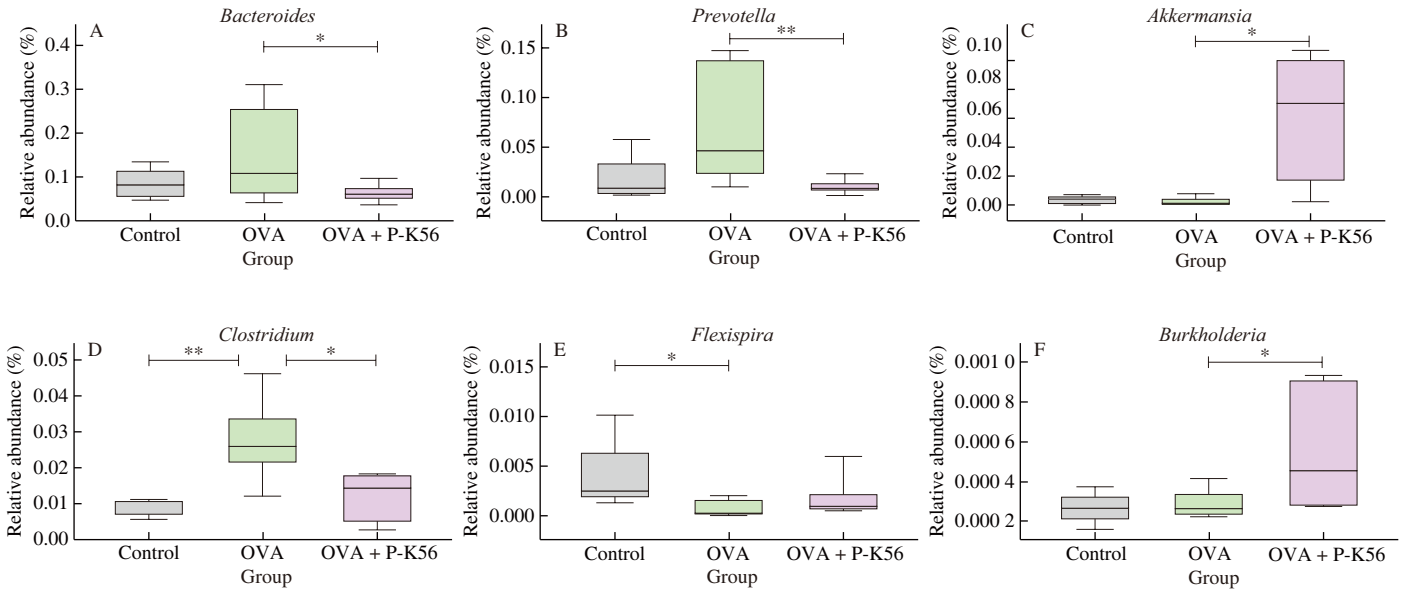

Fig. 6 Different genera emerged after the K56 administration. Relative abundance of *Bacteroides* (A), *Prevotella* (B), *Akkermansia* (C), *Clostridium* (D), *Flexispira* (E), *Burkholderia* (F), *Turibacter* (G), *Sutterella* (H), and *Shigella* (I). Data were shown as means ± SEM, $n = 6$, $^{*}P < 0.05$, $^{**}P < 0.01$.

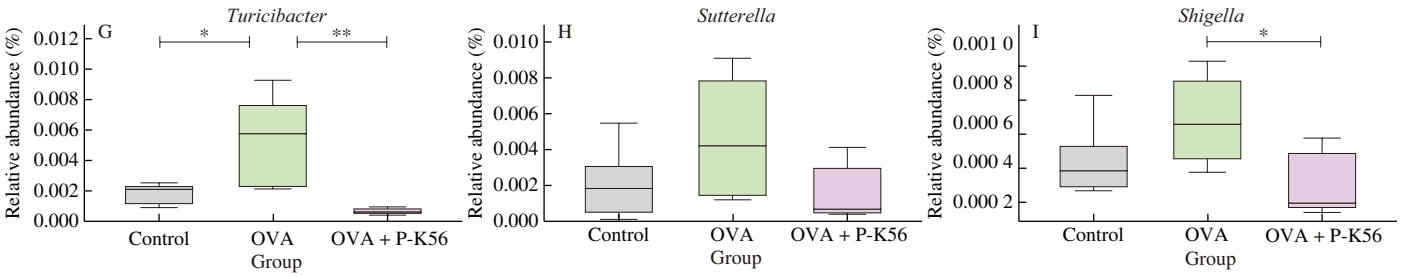

Fig. 6 (*Continued*)

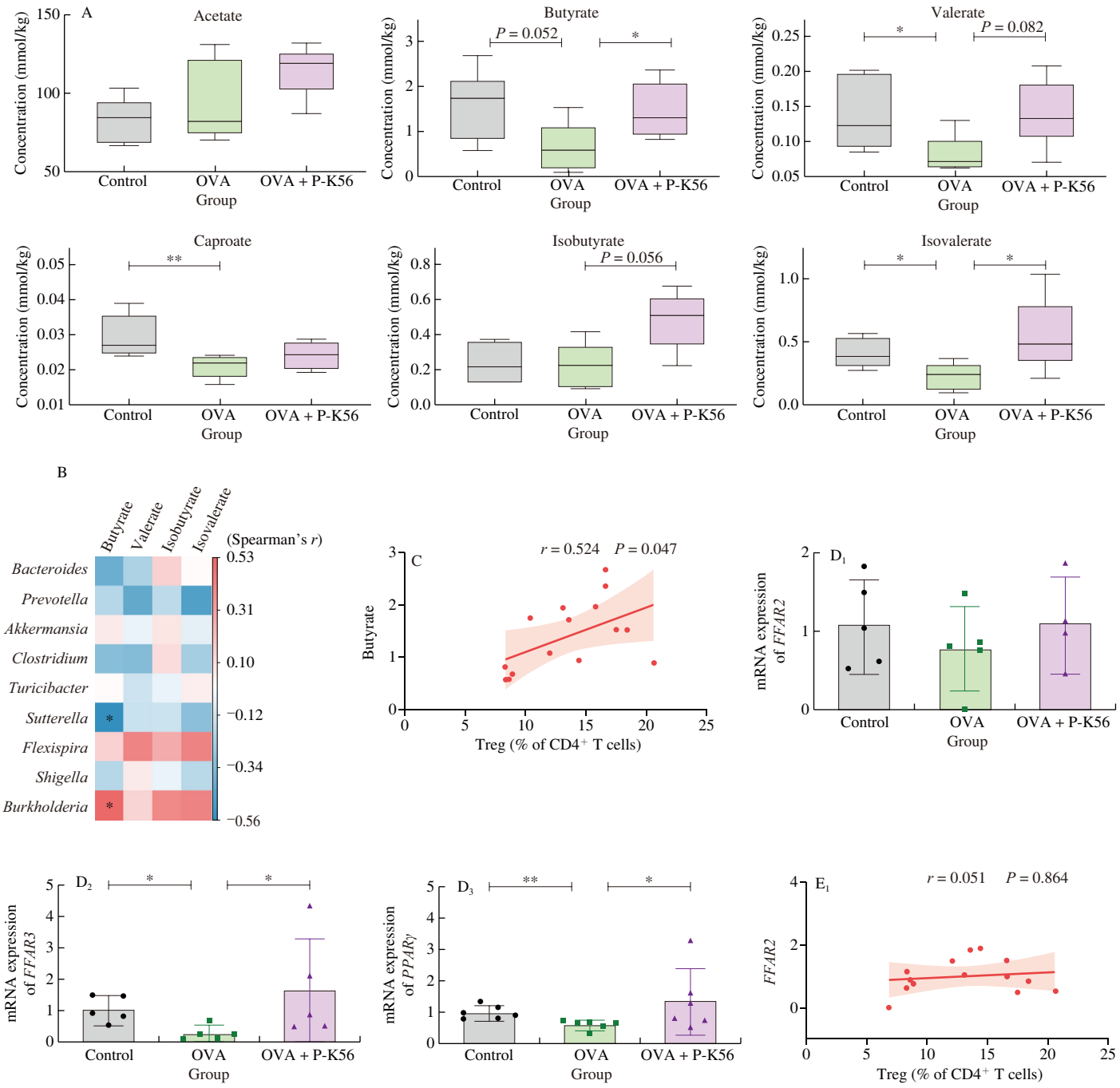

Fig. 7 Effects of K56 prophylactic administration on metabolites from gut microbiota. (A) The concentration of fecal SCFAs in the colon; (B) Spearman correlation between SCFAs and microbiota; (C) Correlation analysis between butyrate and Treg frequency in the colon. (D) Relative SCFA receptors mRNA expression of *FFAR2*, *FFAR3*, and *PPARγ*. (E) Correlation analysis between SCFA receptor mRNA expression and Treg frequency in the colon. Data were shown as means ± SEM, $n = 6$, $^{*}P < 0.05$, $^{**}P < 0.01$. Correlation analysis was performed using the Spearman method.

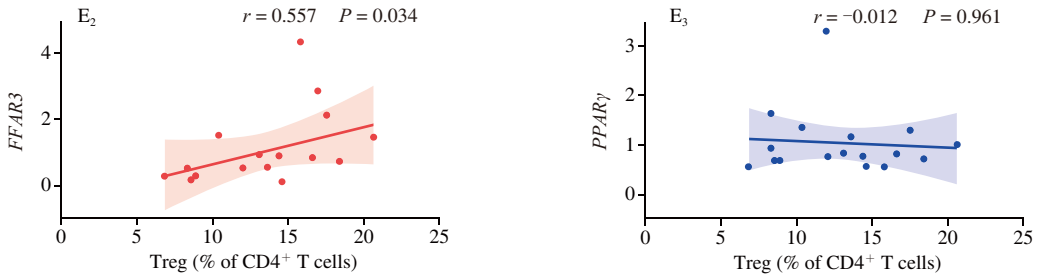

Fig. 7 (*Continued*)

could improve the levels of SCFAs, they subsequently activate the SCFA receptors, such as *FFAR3*, which might be involved in the differentiation of Treg.

4. Discussion

Allergic asthma is one of the most common chronic inflammatory disorders and affects more than 300 million people worldwide. The symptoms often first occur in childhood and may be associated with intestinal damage[26]. Uncontrolled inflammatory response plays a critical role in asthma attacks, mainly characterized by excessive proliferation of T lymphocytes and infiltration of eosinophils, promoting alveolar damage and airway remodeling[27]. Consistent with our findings, asthmatic mice show poorer pulmonary function and abnormal lung histopathology, along with increased total inflammatory cell count, macrophage, eosinophil infiltration, and IgE level in serum and BALF[28-29]. Research has shown that probiotics are a promising intervention for the prevention and treatment of various diseases[30]. Probiotics are living microorganisms that benefit the health of their hosts. It has been suggested that *L. paracasei* may play a protective role in asthmatic mice[31]. Therefore, we studied the role of probiotic K56 in asthma and its mechanism, which is safe for consumption. Surprisingly, K56 administration significantly improved the behavioral scores, airway hyperresponsiveness, lung pathology, elevated inflammatory cell infiltration, and IgE levels in asthmatic mice, indicating a significant effect of K56 on the relief of asthma symptoms.

Previous studies have shown that allergic asthma is an airway inflammatory disease driven primarily by a Th2 response. The over-augmentation of the Th2 response is manifested by significantly elevated IL-4, IL-5, and IL-13 expression *in vivo*, leading to various pathophysiological manifestations[32]. The immunological features of allergic asthma are the imbalance of Th2, Th17, and Treg, and Th17 responses are evoked but the function of Treg is impaired[33]. When an antigen stimulates the differentiation of naive $CD4^+$ T cells into Th2 cells, it also promotes their differentiation into Treg. Moreover, Treg has been shown to be a critical regulatory mechanism for maintaining immune tolerance to external antigen and preventing excessive Th2 responses[24,34]. However, for Treg, different studies have shown different increase or decrease results[27,35]. In our study, flow cytometry assays demonstrated that the K56 prophylactic intervention was accompanied by a decrease in Th2 cells and an increase in Treg levels in the lung, which in turn affects the Th17/Treg balance. Furthermore, K56 prophylactic administration alleviated the shortening of the colon and increased the proportion of Treg in the lamina propria of the colon.

The human microbiota is fundamental to guide the host's immune system development and balance. Dysbiosis, or microbial content alteration in the gut and respiratory tract, is associated with immune system dysfunction and lung disease development[25]. In addition, the intestinal microbiota plays a pivotal role in fighting infections, regulating the metabolic, endocrine, and immune systems, and influencing drug absorption and metabolism[36]. Previous studies demonstrating a connection between altered gut microbiota and airway illness provided evidence of gut-lung cross talk. The gut-lung axis, defined as the interrelationship between two organ systems in immune homeostasis and their physiological mechanisms, is strongly influenced by the functional state of the intestinal microbiota[37]. For example, dysbiosis of the intestinal microbiota mediates a dysregulated immune response in the lungs, supported by inflammatory mediators released by active T cells, which ultimately leads to increased mortality from respiratory tract infections[38]. Our research group has studied the effect of another probiotic, *Bifidobacterium lactis* BL-99, on colitis-related lung injury, further demonstrating microbial-mediated immune crosstalk between the gut and lung tissues, and our study also supported this[39].

In our study, the relative abundance of *Clostridium* and *Turicibacter* was significantly elevated, while the abundance of *Bacteroides*, *Prevotella*, *Burkholderia*, *Sutterella*, and *Shigella* showed an increasing trend, together with the abundance of *Lactobacillus* and *Flexispira* decreased in asthmatic mice. However, K56 reversed these alterations to varying degrees. Several related studies have shown that elevated *Sutterella* is found in the microbiome of asthmatic populations[40-41]. In addition, as a pathogenic bacterium, *Shigella* was also significantly enriched in the intestine of asthmatic mice and recovered after K56 administration. We also found a significant increase in the abundance of *Akkermansia* after K56 prophylactic administration, consistent with previous findings[42]. *Akkermansia* is a beneficial intestinal bacterium[43], and notably, reduced levels of *Akkermansia* have been observed in patients with inflammatory bowel diseases (mainly ulcerative colitis) and metabolic disorders, which suggests it may have potential anti-inflammatory properties[44]. A recent clinical study on the gut microbiota of children with allergic asthma showed that the number of *Akkermansia* in the gut microbiota of patients was significantly reduced compared to healthy controls. Furthermore, this bacterium may induce the secretion of the anti-inflammatory cytokine IL-10 and prevent the secretion of pro-inflammatory cytokines such as IL-12, thus exerting a reducing effect on inflammation[45]. Meanwhile, IL-10 is one of the important inducing factors for Treg differentiation[46]. The increased abundance of *Akkermansia* after K56 prophylaxis administration may contribute to IL-10 secretion and subsequently affect Treg differentiation, which

therefore exemplified alleviate asthma. These phenomena may shed light on the beneficial effects of K56 as a probiotic.

The impact of the microbiota on human health and disease is mediated through many metabolites, especially SCFAs, produced by resident microorganisms. SCFAs are anti-inflammatory chemicals with immunomodulatory functions and can induce anti-inflammatory cytokine expression and apoptosis of immune cells[47]. Furthermore, it has been shown that when microbial synthesis of SCFAs in the gut (primarily butyrate) increases, intestinal inflammation is subsequently reduced, and intestinal barrier integrity is repaired[48]. SCFAs can reduce the frequency and severity of lung infections[49-50]. SCFAs have the ability to modify the immune response on both a systemic and local level, which can enhance pathogen clearance and reduces tissue damage caused by elevated inflammation[51].

SCFAs are known to regulate host immune homeostasis, which is partly required for colonic Treg recruitment and differentiation[52]. SCFAs, especially butyrate, can influence the differentiation of Treg[51]. The dysfunction of Treg can lead to a breakdown of immune system regulation, allowing for an overwhelming Th2 response, which can develop into allergic asthma[34]. As a result of acetate and propionate treatment, Foxp3 and IL-10 expression in Tregs increases, along with an increase in colonic Treg numbers[15,53]. In our study, the results of the correlation analysis between SCFAs with Treg indicated that butyrate was correlated significantly with Treg. What's more, butyrate was also significantly elevated after the K56 administration. The anti-asthma activity of K56 might be related to increased Tregs and orchestrating SCFAs-producing microbes in the gut. Therefore, in our study, we think that butyrate might be one of the most important SCFAs associated with the K56 regulation of intestinal microbial metabolism in asthmatic mice.

FFAR3 (GPR41) and FFAR2 (GPR43) receptors can be activated by SCFAs, mainly by acetate, butyrate, and propionate. Meanwhile, it can reduce intestinal inflammation by activating or triggering the regulation of PPARγ expression[54]. It has been shown that FFAR3 can promote the differentiation of Treg, which in turn has an impact on the disease process[55]. This is consistent with our findings that K56 intervention significantly increased butyrate levels and mRNA expression levels of *FFAR3* and *PPARγ*, suggesting that K56 intervention can have an effect on Treg differentiation probably through the SCFAs-GPCRs pathway, thereby reducing inflammation. Considering that SCFAs were reported to increase Treg by inhibiting histone deacetylases (HDACs)[51], we made a Spearman's correlation analysis between Tregs and SCFA receptors, and *FFAR3* showed a remarkably positive correlation with Tregs. Furthermore, we found elevated intestinal Treg and lung Treg, which may be related to the ability of Treg to migrate to sites of inflammation to exert its effects[56-57].

Together, based on our data, we speculate that the preventive effect of K56 on asthma may influence the level of SCFAs and the expression of FFAR by affecting the structure of the intestinal microbiota, which in turn promotes the differentiation of Treg.

5. Conclusion

Our research showed that prophylaxis administration of K56 alleviated airway hyperresponsiveness and inflammatory infiltration.

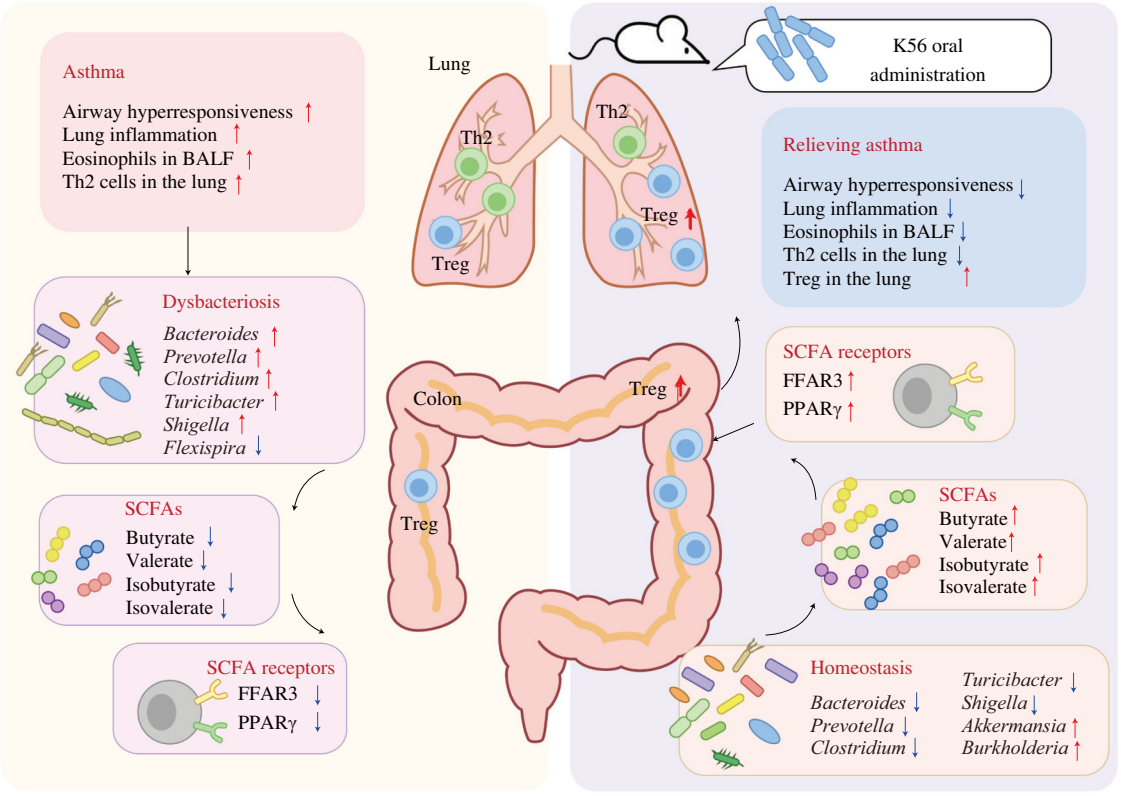

Fig. 8 Summary hypothesis diagram. *L. paracasei* K56 coordinates the structure of the intestinal microbial community and its metabolites SCFAs, further contributing to the expression of SCFA receptors and Treg differentiation, ultimately ameliorating airway inflammatory infiltration and airway hyperresponsiveness in asthmatic mice. The red arrows mean up-regulation, and the blue arrows mean down-regulation.

The mechanism behind this may be due to that K56 was able to alter the gut microbiota and the immune response (rescued the decreased gut Tregs and boosted lung Tregs) that restored the host homeostasis from the asthmatic situation (Fig. 8). Despite what we added to the field of gut-lung axis study, our research still has limitations. For example, the precise cellular localization of the SCFA receptor needs further validation, and how the SCFA receptor affects Treg differentiation and intestinal Treg harmonizes pulmonary immune homeostasis, remains further studied. At the same time, we will explore the targeted mechanisms of K56 on Tregs in the asthma model, via *FFAR3* agonists and antagonists *in vivo* and *in vitro* experiments, further confirming the correlation between receptors and immune cells.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgments

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Data Availability Statement

Sequencing data and analysis codes are available in NCBI-SRA (BioProject: PRJNA928391) https://dataview.ncbi.nlm.nih.gov/object/PRJNA884638?reviewer= i0lhfs1m67teos45ruvtrpq1ks.

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