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Long-chain polyunsaturated fatty acids improve airway pathological features and gut microbial imbalances in BALB/c mice with ovalbumin-induced asthma

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ABSTRACT

Long-chain polyunsaturated fatty acids (LCPUFAs) and the gut microbiota individually exert notable effects on asthma. However, it remains unknown whether LCPUFAs modulate the gut microbial composition. Twenty-four male BALB/c mice were randomly assigned to control (saline), asthma (ovalbumin (OVA)), and LCPUFAs groups (OVA plus LCPUFAs). The enhanced pause value was observed to decrease upon the administration of LCPUFAs in mice challenged with methacholine (12.5, 25, and 50 g/mL). LCPUFAs treatment decreased the number of eosinophils in BALF compared to that in non-treated asthmatic mice. The interleukin 5 levels in BALF and the airway PAS scores decreased after the asthmatic mice were treated with LCPUFAs. The treatment markedly reversed the imbalances in the gut microbial composition, as evidenced by the beta diversity analysis, and decreased the relative abundance of *Akkermansia*. These findings suggested that LCPUFAs modulated the gut microbial composition and improved airway pathological features in mice with OVA-induced asthma.

1. Introduction

Asthma is a common chronic inflammatory disease of the lower airways. It is characterized by T helper type 2 (Th2)-mediated responses along with interleukin (IL)-4, IL-5, and IL-13 overexpression (Global Initiative for Asthma, 2019). This leads to the exacerbation of the hallmarks of allergic responses, such as immunoglobulin (Ig) E levels, eosinophil recruitment, mucus production from goblet cells, and smooth muscle contraction (Paul and Zhu, 2010).

Epidemiological findings have shown that the consumption of a diet rich in long-chain polyunsaturated fatty acids (LCPUFAs) is related to a low prevalence of asthma and good asthma control (Barros et al., 2008; Burns et al., 2007). Eicosapentaenoic acid (EPA, 20:5 n–3) and docosahexaenoic acid (DHA, 22:6 n–3) are two important acids of LCPUFAs. Supplementation with LCPUFAs (55% EPA and 37% DHA) in the third trimester of pregnancy led to a reduction of 7% in the absolute risk of persistent wheeze or asthma and lower respiratory tract infections in one-third of the offspring (Bisgaard et al., 2016).

Endogenous anti-inflammatory lipids derived from lipid synthesis and metabolic processes contribute to the development of lung inflammation (Duffney et al., 2018). Lipoxins (LXs) can inhibit polymorphonuclear leukocyte transmigration and elicitation of pro-inflammatory responses of innate immune effectors (Haworth & Levy, 2007). Resolvins are primarily anti-inflammatory lipids derived from omega-3 fatty acids, these include resolvin D series (RvDs) and protectins derived from DHA and resolvin E series (RvEs) derived from EPA (Serhan, Dalli, Colas, Winkler, & Chiang, 2015). RvE1-mediated decreasing of IL-23, IL-6 and IL-17, as well as increasing of IFN-γ and lipoxin A4 may contribute to improve the allergic airway inflammation (Haworth, Cernadas, Yang, Serhan, & Levy, 2008). Moreover, EPA and DHA can reduce inflammation in asthma through the nuclear factor kappa B (NF-κB) signal (Delerive, Fruchart, & Staels, 2001; Novak,

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Babcock, Jho, Helton, & Espat, 2003).

Lipid metabolism plays an important role in the synthesis of compounds that form structural elements during virus replication (Heaton & Randall, 2011). Coronavirus disease (COVID-19) has affected over 100 million individuals globally. Diabetes mellitus, severe obesity, and hypertension, which are almost always accompanied by hypersemia, increase the risks of infection and mortality in patients with COVID-19 (Muniyappa & Gubbi, 2020; Wu and McGoogan, 2020). The levels of total protein, albumin, ApoA1, HDL cholesterol, and total cholesterol were reduced in patients with COVID-19 (Kočar, Režen, & Rozman, 2021). Furthermore, significant abnormalities in lipid metabolism were also observed in the red blood cells (RBCs) of patients with COVID-19, which could be attributed to the structure and functions of RBCs (Thomas et al., 2020). Sphingolipids exhibit anti-inflammatory, neuroprotective, and anti-coagulatory effects (Hannun & Obeid, 2018). A sphingomimetic drug (FTY720, Fingolimod) is currently under assessment in a clinical trial (NCT04280588 and NCT04276688—ClinicalTrials.gov) for the treatment of COVID-19 (approved by FDA). This drug was selected based on its immunomodulatory, anti-inflammatory, and anti-thrombotic effects (Giovannoni et al., 2020; Huwiler & Zangemeister-Wittke, 2018; Lythgoe & Middleton, 2020). Therefore, lipid metabolic pathways may be one of the useful targets for the treatment of COVID-19.

The human gut is colonized by a wide variety of microorganisms (≥1000 types), the collective genomes of which are ~100 times larger than the human genome (Bäckhed et al., 2004). The total microbial population in human adults is considered to exceed the total number of somatic and germ cells by at least one order of magnitude (Xu & Gordon, 2003). The gut microbiome is an important modulator of immune responses, and its dysbiosis is linked to the development of immune-mediated diseases, such as asthma (Human Microbiome Project Consortium, 2012; Russell et al., 2012; Zhang et al., 2020). The microbial composition reportedly exhibits a significant correlation with the pathology of asthma (Barcik, Boutin, Sokolowska, & Finlay, 2020). In this study, we investigated whether treatment with LCPUFAs improved airway pathology and altered gut microbial dysbiosis in BALB/c mice with ovalbumin (OVA)-induced asthma.

2. Materials and methods

2.1. Animals

Twenty-four adult male 6-week-old BALB/c mice (21 ± 1.74 g) were obtained from Beijing Vital River Laboratory Animal Technology (Animal Quality Certificate Number: 114007003; Animal Use Permit Number: SYXK (Henan) 2016-0008) in Beijing, China. The animals were maintained under specific pathogen-free conditions (24 ± 1 °C, 12-h light/dark cycle) with free access to water and chow.

2.2. OVA-challenge mouse model and experimental design

We randomly divided the mice into three groups of eight mice each: control group (saline), asthma group (OVA only), and LCPUFAs group (OVA+LCPUFAs). One mouse in the control group died due to suffocation after gavage before the experimental endpoint was reached. The asthma mouse model was developed according to a previously described method (Sugawa et al., 2008). In the asthma and LCPUFAs groups, the mice were sensitized by treatment with OVA (100 μg; intraperitoneal injection; grade III; Sigma-Aldrich, Saint Louis, MO, USA) in 0.2 mL of alum adjuvant (Thermo Scientific, Waltham, MA, USA) administered once per week on days 0, 7, and 14. Next, the mice were challenged with OVA (50 μg, inhalation) twice per day from days 21 to 28. Saline was administered at the same volume and frequency in the control group. From days 21 to 28, the mice in the LCPUFAs group were gavaged with LCPUFAs (1000 mg/kg/d; 50% EPA and 50% DHA) (Mochimaru et al., 2018). The LCPUFAs were replaced with saline for mice in the control and asthma groups.

2.3. Measurement of airway hyperresponsiveness (AHR)

AHR was assessed at the indicated time points using methacholine (Mch)-induced obstruction of pulmonary airflow in conscious mice. The obstruction of pulmonary airflow was measured based on enhanced pause (Penh) in a whole-body plethysmograph (Buxco Electronics, Troy, NY, USA), as described previously (Hansen, Berry, DeKruyff, & Umetsu, 1999). Briefly, the mice were administered saline by ultrasonic atomization for 2 min, following which the baseline Penh values were recorded for 2 min. This was followed by increasing the inhaled doses (3.125–50 mg/mL) of Mch by ultrasonic atomization and monitoring of Penh.

2.4. Bronchoalveolar lavage fluid (BALF) collection

The mice were sacrificed by subjection to pentobarbital overdose. The BALF samples were collected by flushing the lungs with 0.7 mL of PBS via the tracheal cannulae. The BALF samples were centrifuged (100*g* for 10 min at 24 °C) to pellet the cells. The supernatant was stored at –80 °C for enzyme-linked immunosorbent assay (ELISA). The pelleted cells were resuspended in 500 μL of PBS to determine the cell count using Wright's staining.

2.5. Elisa

The levels of IL-4, IL-5, IL-13, and thymic stromal lymphopoietin (TSLP) in the BALF samples were measured using an ELISA kit (Bioswamp, Wuhan, China) according to the manufacturer's instructions.

2.6. Preparation of lung tissue specimens

The left lung lobes were fixed in paraformaldehyde (0.1 mM). Next, paraffin-embedded sections with a thickness of 5 μm were cut for hematoxylin and eosin (H&E) staining, periodic acid-Schiff (PAS) staining, and Masson's staining.

2.7. Immunohistochemical (IHC) analysis

IHC analysis was performed using 5 μm-thick paraffin-embedded lung tissue sections. After deparaffinization and rehydration, the slides were placed in citric acid buffer and heated for antigen retrieval, following which the slides were blocked with 5% bovine serum albumin for 1 h at room temperature. Next, the tissue sections were treated with anti-rabbit antibodies against alpha smooth muscle actin (α-SMA) (1:200, Cell Signaling Technology, Beverly, MA, USA), E-cadherin (1:200, Cell Signaling Technology), and transforming growth factor beta 1 (TGF-β1) (1:200, Cell Signaling Technology) (primary antibodies) in a wet box overnight at 4 °C. The slides were then treated with horseradish peroxidase-conjugated goat anti-rabbit secondary antibodies and observed under a light microscope. Semi-quantitative analysis was performed using Image Pro Plus v6.0 (Media Cybernetics, San Diego, CA, USA).

2.8. MiSeq sequencing

Fecal and cecal samples were collected from the mice and frozen immediately in liquid nitrogen. DNA was extracted from the fecal samples using the QIAamp Fast DNA Stool Mini kit (Qiagen, Stanford, VA, USA) according to the manufacturer's instructions. Next, for DNA detection, the samples were electrophoresed using 0.8% agarose gel. The V3-V4 region of the bacterial 16S rDNA gene was amplified using PCR with the primers 341 F, 5′–CCTAYGGGRBGCASCAG–3′ and 806 R, 5′–GGACTACHVGGGTWTCTAAT–3′. The reactions were performed in a total volume of 25 μL, which contained 10 ng of the DNA template, 1×

PCR buffer, $MgCl_2$ (1.5 mM), dNTPs (0.4 μM), forward primer (1.0 μM), reverse primer (1.0 μM), and KOD-Plus-Neo enzyme (0.5 U; Toyobo, Osaka, Japan). The thermocycling conditions were as follows: 1 min at 94 °C, followed by 30 cycles of 20 s at 94 °C, 30 s at 54 °C, and 30 s at 72 °C, and 5 min at 72 °C. The experiments were repeated three times for each sample.

The PCR amplicons were separated by electrophoresis using 2% agarose gel. Next, the section of the gel containing the DNA was removed and DNA purification was performed using the QIAquick Gel Extraction kit (Qiagen) according to the manufacturer's instructions. DNA was quantified using a Qubit™ 2.0 Fluorometer (Thermo Scientific), following which the amplicons were pooled in equimolar quantities. A library was constructed using the TruSeq™ DNA PCR-Free Sample Prep kit (Illumina, San Diego, CA, USA). The constructed library was quantified and sequenced using the PE300 mode within a MiSeq sequencing system (Illumina, MiSeq Reagent Kit v3).

2.9. Bioinformatics analysis of the microbiota

Based on Usearch (http://drive5.com/uparse/), the UPARSE algorithm was used to perform operational taxonomic unit (OTU) clustering at a consistency level of 97%. The sequence with the highest frequency in each OTU was selected as the representative sequence of the OTU. Annotation analysis was performed using the UCLUST algorithm and SILVA database. We used FastTree to construct a phylogenetic tree. Each sample was homogenized and resampled using the least data in the sample. Each taxonomical level (phylum, class, order, family, and genus) was analyzed separately. Inhouse Perl scripts were used to analyze alpha (within samples) and beta (among samples) diversities. Unweighted UniFrac for principal coordinates analysis (PCoA) and the

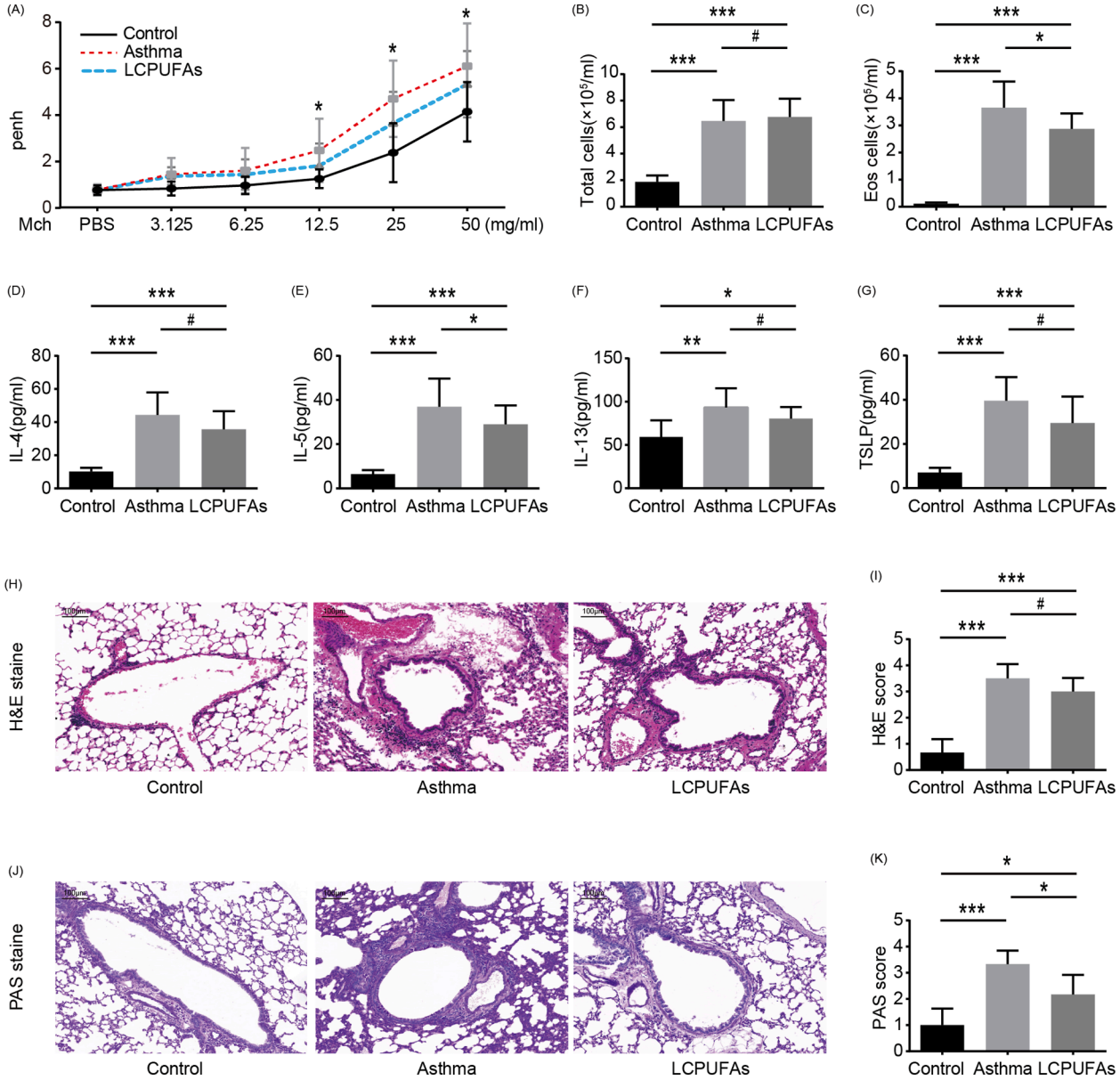

Fig. 1. LCPUFAs prevent OVA-induced AHR and airway inflammation in mice. (A) Assessment of AHR following allergen sensitization and challenge. (B and C) Total number of cells and eosinophils in BALF samples. (D, E, F, and G) Analysis of pro-inflammatory cytokine expression in BALF using ELISA. (H, I, J, and K) H&E and PAS staining shows peribronchial inflammatory infiltration and airway hypersecretion. Columns and error bars represent mean ± SD. AHR: airway hyperresponsiveness; Mch: methacholine; BALF: bronchoalveolar lavage fluid; Eos: eosinophils; IL-4: interleukin 4; IL-5: interleukin 5; IL-13: interleukin 13; TSLP: thymic stromal lymphopoietin; LCPUFAs: long-chain polyunsaturated fatty acids; H&E: hematoxylin and eosin; PAS: periodic acid-Schiff. $^{\#}P > 0.05$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

unweighted pair group method with arithmetic mean clustering trees were used to assess the variations among experimental groups (beta diversity).

2.10. Accession number

The names of the repository/repositories and the accession numbers can be found below: https://www.ncbi.nlm.nih.gov/, PRJNA657739.

2.11. Statistical analysis

Data from the three groups are presented in terms of mean ± standard error of mean. Normality was assessed using the Shapiro–Wilk test. For parametric variables, the differences in Penh and levels of pro-inflammatory cytokines were assessed using one-way ANOVA followed by Newman–Keuls post hoc tests. The Bonferroni test was applied as a correction for multiple comparisons. SPSS 17.0 (IBM, Armonk, NY, USA) and Prism v5.0 (GraphPad, San Diego, CA, USA) were used for statistical analyses. $P < 0.05$ was considered significant.

3. Results

3.1. LCPUFAs prevent OVA-induced AHR and airway inflammation in mice

The Penh of asthmatic mice was higher than that of control mice. However, it decreased significantly ($P < 0.05$) after LCPUFAs treatment of asthmatic mice stimulated with Mch at high concentrations (12.5, 25, and 50 g/mL) (Fig. 1A). The total number of cells in the BALF samples of asthmatic mice was significantly higher than that in control mice ($P < 0.05$) (Fig. 1B). Treatment with LCPUFAs significantly reduced the

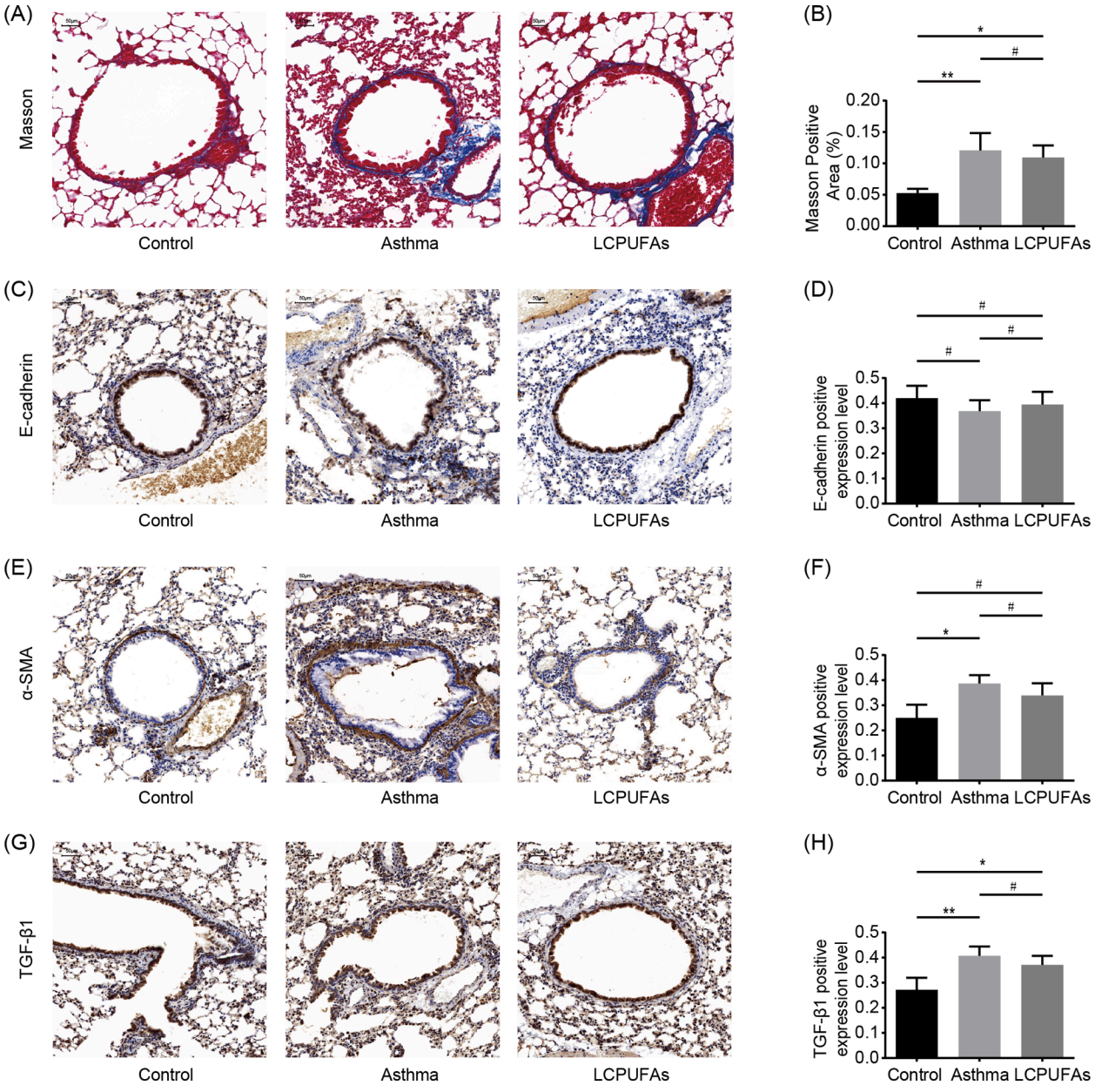

Fig. 2. LCPUFAs attenuate OVA-induced airway remodeling in mice. (A and B) Masson's staining shows the presence of collagenous fibers in the airway. (C-H) Lung tissue samples with staining for E-cadherin, α-SMA, and TGF-β1 detection were analyzed using immunohistochemistry. Columns and error bars represent mean ± SD. LCPUFAs: long-chain polyunsaturated fatty acids; α-SMA: alpha smooth muscle actin; TGF-β1: transforming growth factor beta 1. $^{\#}P > 0.05$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

numbers of eosinophils in BALF compared to that in asthmatic mice ($P < 0.05$) (Fig. 1C). The IL-4, IL-5, IL-13, and TSLP levels were significantly higher in asthmatic mice than in control mice ($P < 0.05$), LCPUFAs treatment significantly reversed the level of IL-5 (Fig. 1D, E, F, and G). After LCPUFAs treatment, the H&E score decreased compared to that in asthmatic mice; however, the change was not significant ($P > 0.05$) (Fig. 1H and I). Compared to that of asthmatic mice, the PAS score of LCPUFAs-treated mice decreased significantly ($P < 0.05$) (Fig. 1J and K).

3.2. LCPUFAs attenuate OVA-induced airway remodeling in mice

As indicated by the results of Masson's staining, the positively stained areas in the basement membrane increased significantly in asthmatic mice than in control mice ($P < 0.05$), LCPUFAs treatment reversed these changes, although the differences were not significant ($P > 0.05$) (Fig. 2A and B). The IHC results indicated that the relative α-SMA and TGF-β1 expression increased significantly ($P < 0.05$) and E-cadherin expression decreased in the airways of asthmatic mice ($P > 0.05$). Treatment with LCPUFAs reversed these changes, but the differences were not significant ($P > 0.05$) (Fig. 2C-H).

3.3. LCPUFAs modulate the structure and diversity of the gut microbiota

The relative abundances of the microbial species identified in each group were indicated using rank-abundance curves. A wide range of the curve on the horizontal axis indicated high species abundance, a smooth curve indicated even species distribution, and a steep curve indicated uneven species distribution. As shown in Fig. 3A, B, and C, the latter tended to be flat, indicating that the sample abundance and uniformity were reasonable. The plateau of the rarefaction curves indicated that the sampling depth in each stool sample was sufficient for identifying new microbial species and determining their overall richness (Fig. 3D). The total number of OTUs increased from 2,249 to 3,479 in asthmatic mice

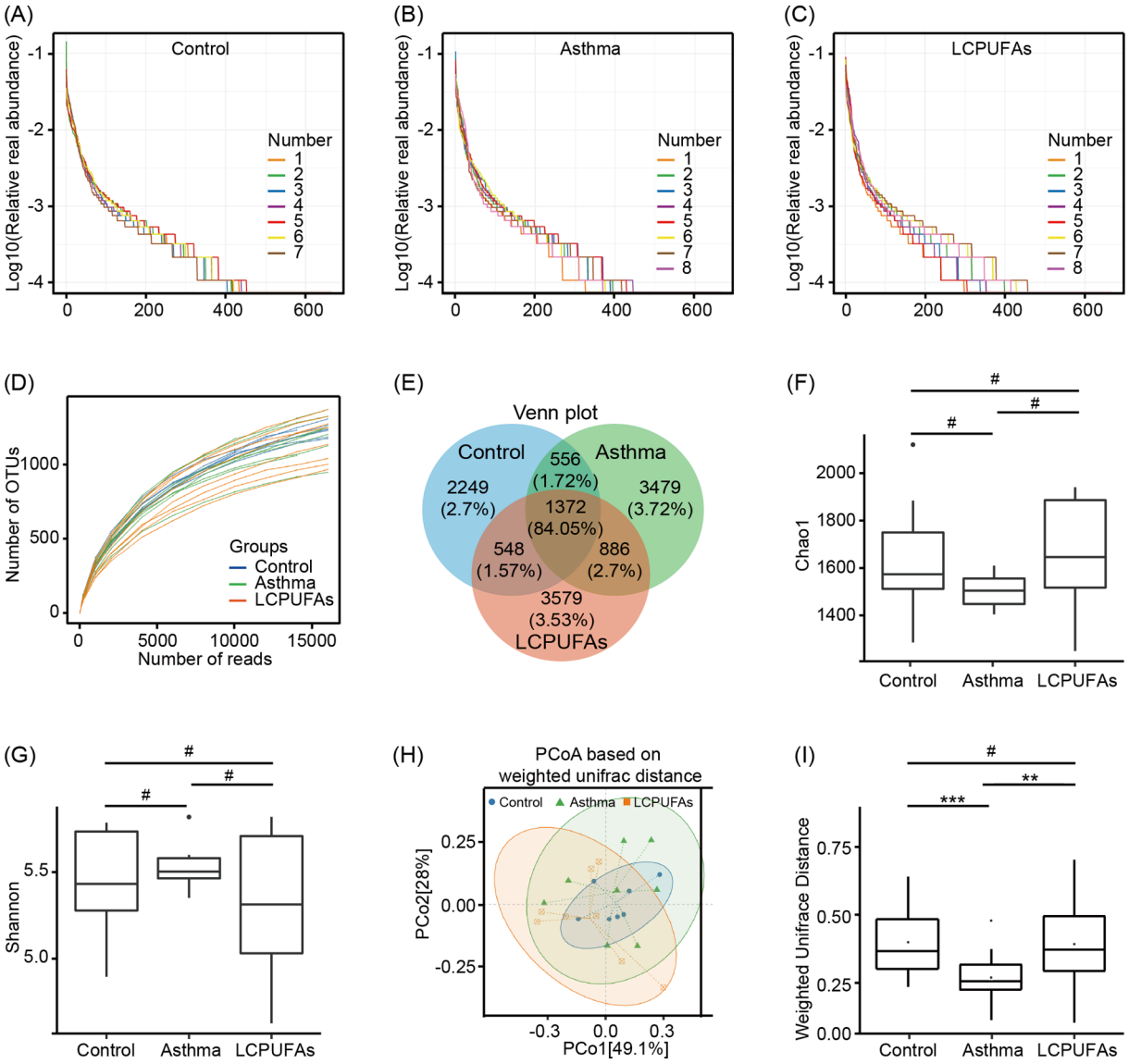

Fig. 3. LCPUFAs modulate the gut microbial structure and diversity. (A, B, and C) Rank-abundance analysis. (D) Rarefaction curve: the abscissa represents the number of randomly selected sequencing strips, the ordinate represents the alpha diversity index calculated based on the number of sequencing strips, and the curves of different colors represent different samples. (E) Venn plots shows the OTU abundance in the three groups. (F and G) The Chao1 Index and Shannon–Wiener Index represent the alpha diversity. (H and I) Beta diversity analysis using PCoA based on the weighted UniFrac distance shows the differences in the gut microbial composition among the three groups. LCPUFAs: long-chain polyunsaturated fatty acids; OTU: operational taxonomic unit; PCoA: principal coordinates analysis. $^{\#}P > 0.05$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

compared to that in control mice, and the total number of OTUs increased to 3,579 in mice treated with LCPUFAs (Fig. 3E). Alpha diversity analysis revealed that the differences in the Chao1 Diversity Index and Shannon Diversity Index among the three groups were not significant ($P > 0.05$) (Fig. 3F and G). Analysis of the beta diversity using PCoA based on weighted UniFrac distance and permutational multivariate analysis of variance revealed that the differences in the gut microbial composition of asthmatic mice decreased significantly, and treatment with LCPUFAs reversed this ($P < 0.05$) (Fig. 3H and I).

3.4. Specific changes in the gut microbiota after treatment with LCPUFAs

The relative abundances of the predominant taxa from the three groups were determined using sequencing. A detailed overview of the gut bacterial composition of the three groups was illustrated at the phylum level. Bacteria from phylum Firmicutes were most abundant in the three groups, the relative abundance in asthmatic mice increased significantly after LCPUFAs treatment ($P < 0.05$) (Fig. 4A and C). Compared to that in control mice, the relative abundance of Verrucomicrobia in asthmatic mice increased significantly ($P < 0.05$), LCPUFAs treatment reversed this significantly in asthmatic mice ($P < 0.01$) (Fig. 4A and D). The reduction in the relative abundance of *Akkermansia* was most significant in asthmatic mice, and LCPUFAs treatment reversed this change significantly ($P < 0.05$) (Fig. 4B and E).

3.5. Species analysis and prediction of community function

LEfSe analysis (also known as Linear discriminant analysis Effect Size) and cladograms were used to identify important species with significantly different abundances in the different groups. *Bacteroides stercoris* was enriched significantly in control mice. After LCPUFAs treatment, bacteria from phylum Firmicutes showed significant enrichment ($P < 0.05$) (Fig. 5A and B). *Akkermansia glycaniphila* was enriched significantly in control mice at the phylum, class, order, family, and genus levels ($P < 0.05$) (Fig. 5A and B). The prediction of pathway enrichment for intestinal flora performed using the Kyoto Encyclopedia of Genes and Genomes (KEGG) database differed considerably for the three groups (Fig. 5C). According to the KEGG database analysis, the pathways enriched significantly in asthmatic mice were "glycan biosynthesis and metabolism", "lipopolysaccharide biosynthesis proteins", and "lipopolysaccharide biosynthesis". After LCPUFAs treatment, the genes associated with the "phosphotransferase system", "replication and repair in chromosome", and "glycolysis gluconeogenesis" were enriched significantly. Gut bacteria that interacted with each other were identified by analyzing the co-abundance networks using Cytoscape. Asthmatic mice had a high abundance of Akkermansia, Clostridium_sensu_stricto_15, and Candidatus_saccharimonas, and the bacterial groups present showed a positive correlation with those present in mice from the control and LCPUFAs groups (Fig. 5D).

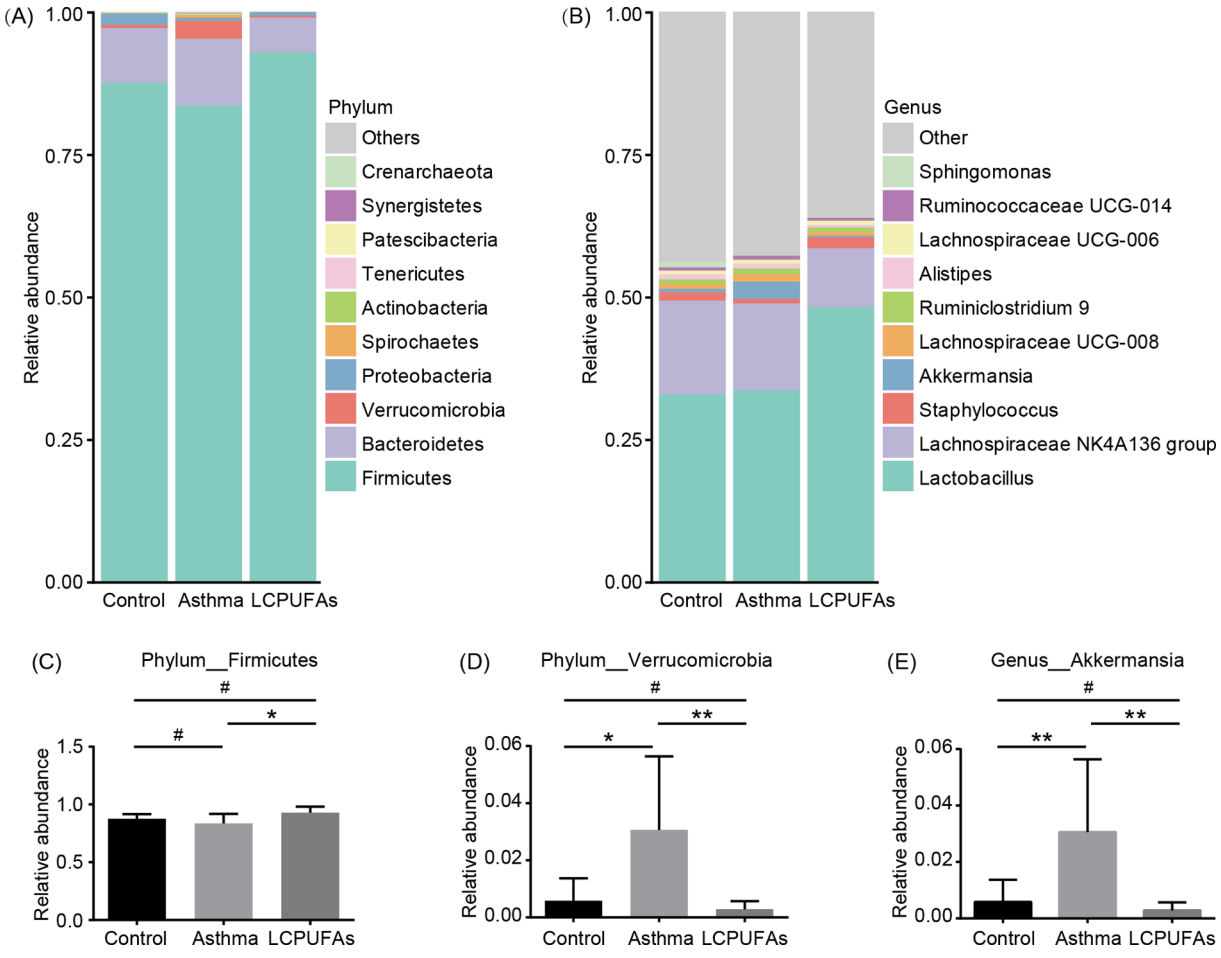

Fig. 4. LCPUFAs modulate the gut microbial composition. (A and B) Taxonomic distribution (phylum and class levels) and the relative abundance of bacteria detected in fecal samples. (C) Relative abundance of bacteria from the phylum Firmicutes. (D) Relative abundance of bacteria from the phylum Verrucomicrobia. (E) Relative abundance of bacteria from the genus *Akkermansia*. Columns and error bars represent mean ± SD. LCPUFAs: long-chain polyunsaturated fatty acids. $^{\#}P > 0.05$, $^{*}P < 0.05$, $\&^{**}P < 0.01$, $^{***}P < 0.001$.

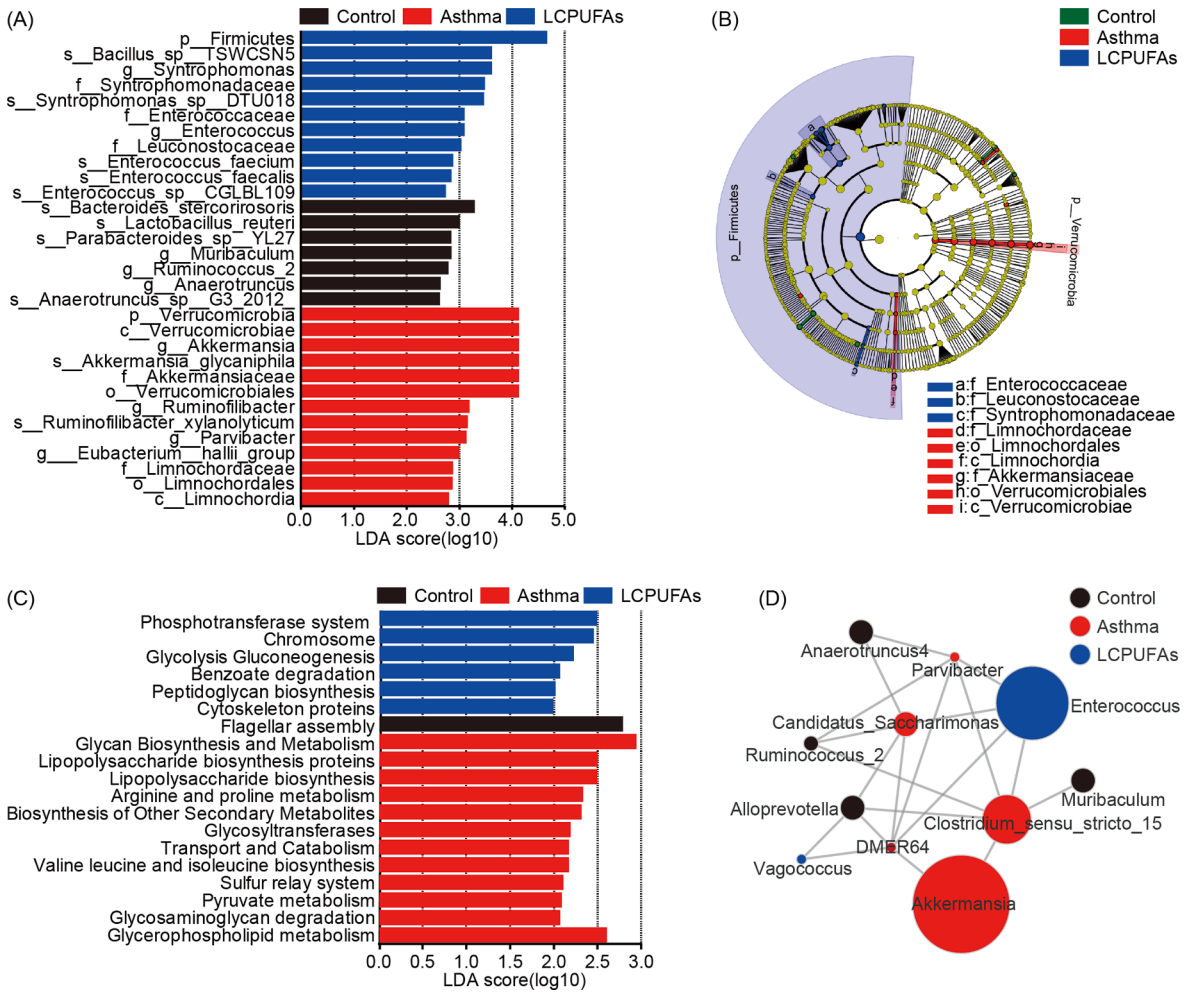

Fig. 5. Species analysis and prediction of community function. (A and B) LEfSe analysis and cladograms were used to identify important species with significantly different abundances in different groups. (C) Representative enriched pathways based on the Kyoto Encyclopedia of Genes and Genomes database classified into two functional categories using PICRUSt. (D) Analysis of the co-abundance networks of genera in the three groups. The size of each circle indicates the relative abundance of each genus. Colors indicate different groupings, the edge size denotes the relative abundance of different bacterial groups, and the node thickness represents the correlation coefficient. LCPUFAs: long-chain polyunsaturated fatty acids; LDA: linear discriminant analysis.

4. Discussion

LCPUFAs are components of cell membranes and regulate several important cellular functions (Schmitz & Ecker, 2008). LCPUFAs were shown to effectively alleviate the symptoms of inflammatory diseases, including asthma (Bisgaard et al., 2016). The ratio between n–6 and n–3 LCPUFAs in tissues and cells was reported to affect AHR and airway inflammation, and a reduction in n–6 LCPUFAs improved signs of inflammation (Bilal et al., 2011). EPA and DHA are n–3 LCPUFAs present abundantly in fish oil. Supplementation with n–3 LCPUFAs was shown to reduce the prevalence of asthma and the risk of persistent wheeze or asthma (Barros et al., 2008; Bisgaard et al., 2016; Burns et al., 2007; Schwartz & Weiss, 1994). Prenatal supplementation with LCPUFAs decreased the levels of the Th2 cytokines IL-4 and IL-13, and therefore, reduced the risk of asthma and allergic diseases in adolescents (Sordillo et al., 2019).

However, in recent years, controversies related to the efficacy of EPA and DHA supplementation in chronic inflammation have increased, and studies have shown that supplementation with n–3 LCPUFAs does not always relieve airway inflammation and asthma symptoms in vivo and in vitro (Brannan et al., 2015; Schuster et al., 2014; Weylandt et al., 2015; Woods, Thien, & Abramson, 2002). Moreover, Li et al. demonstrated that n–3 LCPUFAs intake exhibits an inverse longitudinal association with the incidence of asthma in young American adults, and DHA treatment led to a more significant inverse association than EPA treatment (Li et al., 2013). Mochimaru et al. showed that EPA alleviated eosinophilic inflammation in airways upon its conversion into 12-hydroxy-17,18-epoxyeicosatetraenoic acid (Mochimaru et al., 2018). Fussbroich et al. observed a significant increase in PGE_3 and TxB_3 levels after EPA supplementation in mice with HDM-induced asthma, which involved the participation of pro-inflammatory lipid mediators; meanwhile, supplementation with LCPUFAs (50% EPA and 50% DHA) improved airway inflammation by inhibiting the adverse effects of EPA (Fussbroich et al., 2020). These findings were consistent with ours, which indicated that treatment with LCPUFAs (50% EPA and 50% DHA) in the OVA-induced mice asthmatic model reversed AHR and suppressed Th2 cell-mediated inflammation, such as that induced via IL-4, IL-5, IL-13, and TSLP; however, the changes were not significant. Concurrently, PAS staining revealed that LCPUFAs decreased mucus production from the airway epithelium.

LCPUFAs can attenuate harmful responses in the occurrence of inflammation and oxidative stress via binding to the ligands, PPARs, resulting in the changing of lipid and glucose metabolism and the transcription of factor NF-κB (Echeverría, Ortiz, Valenzuela, & Videla, 2016; Poynter & Daynes, 1998). In addition, supplementation with LCPUFAs was reported to enhance the levels of RvE1, RvE2, RvD1, and RvD2, and possibly, of PDs, to block the inflammatory reaction in the liver of mice administered a high-fat diet (Echeverría et al., 2019;

Serhan & Petasis, 2011). Inflammation is generally accompanied by oxidative stress. Asthma is a chronic inflammatory disease that is also related to oxidative stress (Andrianjafimasy et al., 2017). Soto-Alarcón et al. reported that LCPUFAs supplementation increased glutathione-S-transferase and superoxide dismutase activities and activated nuclear factor erythroid 2-related factor 2 (Nrf2) signaling, which reduced the levels of oxidative stress (Soto-Alarcón et al., 2019). Nrf2 is an important regulator of the antioxidant response element. The activation of Nrf2 signaling can help prevent injury induced by oxidative stress, such as that observed in asthma (Liu, Gao, & Ci, 2019).

Airway remodeling is another important pathological feature of asthma, particularly severe asthma (Kaminska et al., 2009). The major pathological features of airway remodeling include airway wall thickening, fibrosis in the subepithelial regions, interstitial fibrosis close to the airways, myocyte hypertrophy and hyperplasia, myofibroblast hyperplasia, and mucous metaplasia (Zhu et al., 2001). Bargut et al. found that pretreatment with fish oil for 8 weeks reduced OVA-induced peribronchiolar matrix deposition in asthmatic mice (Bargut et al., 2013). Abreu et al. reported that intratracheally administered EPA-pretreated mesenchymal stromal cells reduced bronchoconstriction, alveolar collapse, total cell counts (in BALF, bone marrow, and lymph nodes), and airway collagen fiber content in mice with HDM-induced asthma (Abreu et al., 2018). In our study, Masson's staining revealed decreased fibrosis in the subepithelial regions in asthmatic mice treated with LCPUFAs; however, the change was not significant. Concurrently, IHC analysis revealed that the restoration of the expression levels of the epithelial-mesenchymal transition biomarkers E-cadherin, α-SMA, and TGF-β1 after treatment with LCPUFAs was not significant. This could be attributed to the fact that the duration LCPUFAs treatment was considerably short.

The human gut has a large surface area (approximately 150–200 m^2), with 105–1011 bacteria present per mL of luminal content (Barcik et al., 2020; Sender, Fuchs, & Milo, 2016). Gut bacteria perform several important functions, such as vitamin production, ion absorption, local and systemic innate immune responses, and fermentation of food, all of which are factors associated with good health (Segal et al., 2016). Alterations in the gut microbiota have been linked to the pathogenesis of various diseases (Schirmer et al., 2016; Stokholm et al., 2018; Ver Heul, Planer, & Kau, 2019; Zhu et al., 2016).

The gut microbiota plays an important role in asthma (Barcik et al., 2020; Huang et al., 2017). In our study, Firmicutes, Bacteroidetes, and Proteobacteria were the most prevalent phyla in control mice. The relative abundances of Firmicutes and Proteobacteria decreased and that of Verrucomicrobia increased significantly, with Verrucomicrobia being the phylum with the third-highest prevalence in asthmatic mice. In a human birth cohort study, an increase in the relative abundance of *Streptococcus* and *Bacteroides* and reduction in the relative abundances of *Bifidobacterium* and *Ruminococcus* in fecal samples during the first 100 days after birth were associated with the development of atopic wheeze (Arrieta et al., 2018). *Akkermansia* has been shown to stimulate the proliferation of anti-inflammatory regulatory T cells, which interact with the host immune system (Shin et al., 2014). A study conducted in the United States of America showed that a low relative abundance of *Bifidobacteria*, *Akkermansia*, and *Faecalibacterium* and a high abundance of *Candida* and *Rhodotorula* might promote $CD4^+$ T cell dysfunction, which is linked to a high risk of atopy and asthma development (Fujimura et al., 2016). However, in our study, we observed that the relative abundance of *Akkermansia* increased considerably in mice with OVA-induced asthma. Additionally, Michalovich at al. reported that the relative abundance of *Akkermansia* in the gut microbiota decreased in both obese and non-obese patients with severe asthma (Michalovich et al., 2019).

Diet is an important factor that affects the gut microbial composition (Kau, Ahern, Griffin, Goodman, & Gordon, 2011). Thorburn et al. showed that mice fed a high-fiber diet exhibited distinct gut microbial patterns, and the gut microbiota influenced AHR in an asthmatic mice model (Thorburn et al., 2015). In our study, we found that LCPUFAs treatment significantly reversed the changes in microbial composition observed in asthmatic mice, as indicated in the beta-diversity analysis. Bacteria from the phyla Firmicutes and Verrucomicrobia were most abundant in the three groups of mice. Firmicutes can produce butyrate as a metabolic end product, which reduces eosinophilic airway inflammation and increases the potential for harvesting energy from diet (Berthon, Macdonald-Wicks, Gibson, & Wood, 2013; den Besten et al., 2013). After LCPUFAs treatment, the abundance of bacteria from the phylum Firmicutes decreased significantly. In our study, LCPUFAs treatment significantly reversed the increase in the abundance of *Akkermansia* in asthmatic mice. Sonoyamak et al. observed that fatty acids and their metabolites potentially regulate the abundance of *Akkermansia* species (Sonoyama et al., 2009).

Enriched pathways from the KEGG database were predicted using PICRUSt. Genes associated with "glycan biosynthesis and metabolism", "lipopolysaccharide biosynthesis proteins", and "lipopolysaccharide biosynthesis" were notably enriched. This indicated that dysbiosis in mice with OVA-induced asthma leads to functional changes in the gut microbiota potentially related to the upregulation of sugar and lipid anabolism. LCPUFAs treatment altered this condition significantly by enriching genes associated with "phosphotransferase system", "replication and repair in chromosome", and "glycolysis gluconeogenesis".

Although the clinical applicability of LCPUFAs in asthma remains debatable, some evidences suggest that LCPUFAs supplementation could prevent food allergy (Hoppenbrouwers, Cvejić Hogervorst, Garssen, Wichers, & Willemsen, 2019), modulate inflammation, and improve certain obesity-associated comorbidities in pediatric metabolic syndrome (Amatruda et al., 2019). The consumption of fish and LCPUFAs was shown to be associated with a reduced risk of colorectal cancer in a large European cohort (Aglago et al., 2020). Supplementation with LCPUFAs might improve insulin resistance, hypertension, and dyslipidemia in obesity-associated metabolic syndrome by decreasing the plasma triglyceride content (Lorente-Cebrián et al., 2013). A systematic review and meta-analysis revealed that LCPUFAs supplementation led to improvements in metabolic risk factors, alanine transaminase and γ-glutamyl transaminase levels, liver fat content, and steatosis score in patients with nonalcoholic fatty liver disease (Musa-Veloso et al., 2018).

In conclusion, LCPUFAs treatment was shown to modulate the gut microbiota composition and to improve AHR and airway pathological features in mice with OVA-induced asthma. This study provides novel insights into the regulation of the gut microbiota in mice with asthma using LCPUFAs. However, the precise effects of LCPUFAs on asthma remain controversial; particularly, the type, dosage, and duration of LCPUFAs administration should be investigated further.

5. Ethics statements

The study protocol was approved by the ethics committee of the First Affiliated Hospital of Zhengzhou University (2019-KY-009). All experimental procedures were conducted in accordance with Animal Research: Reporting of In Vivo Experiments and the Guidelines of Intentional Standards of Animals Care and the Ethics and Laboratory Animals Use Committee of the First Affiliated Hospital of Zhengzhou University.

CRediT authorship contribution statement

Tianci Jiang: Conceptualization, Methodology, Investigation, Data curation, Formal analysis, Software, Writing - original draft, Writing - review & editing. **Pengfei Li:** Conceptualization, Methodology, Validation, Writing - original draft. **Junwei Zhao:** Methodology, Investigation, Validation, Writing - original draft. **Lingling Dai:** Investigation, Visualization, Validation. **Di Sun:** Investigation, Methodology, Visualization. **Meng Liu:** Investigation. **Lin An:** Investigation, Methodology, Visualization. **Liuqun Jia:** Investigation. **Xiaogang Jing:** Methodology.

Huan Wang: Methodology. **Shujun Wu:** Methodology. **Yu Wang:** Methodology. **Zhe Cheng:** Conceptualization, Methodology, Writing - review & editing, Supervision, Resources, Funding acquisition, Project administration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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