

Exploration of the effect of mixed probiotics on microbiota of allergic asthma mice

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ABSTRACT

Co-relation between allergic asthma and microbiota varying with diet has been extensively investigated, implicating that oral supplement of alternative pharmaceuticals are potential for asthma control. Probiotics are of great concern due to its beneficial effects on the host, whereas the potential mechanisms and the optimal dose need to be further explored. In the present study, three different doses of mixed strains were given orally to mouse model of allergic asthma induced by ovalbumin (OVA). Continuous administration of mixed probiotics could alleviate OVA-induced allergic inflammation through reducing costimulatory molecules on the surface of dendritic cells (DCs) and increasing mucosal gut-primed Tregs induced by mesenteric CD103⁺DCs. Mixed probiotics-induced protective effect relates to gut microbiota instead of lung microbes. Microbial diversity and Bacteroidetes/Firmicutes (B/F) ratio are augmented upon probiotic strains. Interestingly, treatment with mixed strain lead to an increased levels of genus *Lactobacillus*. *Lactobacillus* genus-Operational Taxonomic Unit (OUT) analysis indicated that OTU9 may be the dominant strain from mixed probiotics providing protective effect. The low dose seems to be the best in the whole study. Our results provide new evidence for probiotic application in allergic diseases and support the idea that targeted gut microbiota will be an effective approach for allergic airway diseases.

1. Introduction

Allergic diseases has been reckoned as an important public health problem, with asthma affecting up to approximately 300 million population worldwide in which affected individuals may suffer from increased health-care costs, as well as reduced quality of life [1,2]. Asthma is typically characterized by chronic eosinophilic airway inflammation and airway remodeling, which are correlated with mucus hypersecretion of goblet cells [3].

The prevalence of asthma involves the interaction of immune system, genetic and environmental factors. The microbiota plays an essential

role in shaping the development of the human immune system throughout our lives, and particularly during the establishment of immune functions in early childhood [4,5]. Emerging evidence relates atopy and asthma to the composition and function of the human microbiome. It is generally considered that microbial imbalance both in gut and lung holds great relevance to the occurrence or severity of allergies and asthma [6–11]. Yet host microbiome are susceptible to be affected by various factors including lifestyle, disease, antibiotics or diet, etc. [12]. Similarly, microbiota is also different between the asthmatic and non-asthmatic. For example, the prevalence of *Bifidobacterium breve* (*B. breve*) was higher in samples from wheezing infants, while *B. longum*

Abbreviations: OVA, ovalbumin; AHR, airway hyperresponsiveness; BALF, bronchoalveolar lavage fluid; DCs, dendritic cells; Tregs, regulatory T cells; 16SrRNA, 16S ribosomal RNA; PAS, periodic acid-schiff; PLN, peribronchial lymph nodes; MLN, mesenteric lymph nodes; FBS, fetal bovine serum; FCS, fetal calf serum; OTU, Operational Taxonomic Unit; MCh, acetyl-β-methylcholine chloride; ELISA, enzyme-linked immunosorbent assay; H&E, hematoxylin/eosin; Ig, immunoglobulin; PCoA, principal coordinate analysis; LDA, linear discriminant analysis; LEfSe, linear discriminant analysis effect size; MHCII, histocompatibility complex class II; B/F, bacteroidetes/firmicutes; Der p, dermatophoides pteronyssinus; BLAST, Basic Local Alignment Search Tool; MFI, mean fluorescence intensity; mMCP-1, mouse mast cell protease 1.

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was more abundant in non-wheezing infants [13]. Infants at high risk of asthma displayed a transient decrease of *Lachnospira*, *Veillonella*, *Faecalibacterium* and *Rothia* spp. during the first 3 months of life, as well as diminished fecal acetate and augmented urinary microbial metabolites [14]. Nasal secretion from asthmatic children of 6–17 years of age present a distinct microbiota structure dominated by genus *Moraxella* correlated with increased asthma exacerbation risk and activation of eosinophils [15].

Co-relation between allergic asthma and microbiota varying with diet has been extensively investigated, implicating that oral supplement of alternative pharmaceuticals are potential for asthma control. Probiotics are defined as live microorganisms which confer a health benefit on the host when administered in adequate amounts [16]. In the present study, a mixture of probiotics (*Lactobacillus gasseri*, *Lactobacillus salivarius*, *Lactobacillus johnsonii*, *Lactobacillus paracasei*, *Lactobacillus reuteri*, *Bifidobacterium animals*) isolated from healthy Asiatic neonatal intestinal tract was administered orally to the mouse model of asthma with three different doses. Anti-allergic effect of mixed strain and microbiota both in gut and lung were compared among three different doses. Although previous clinical and animal studies have shown that the mixed probiotics comprising six strains possesses an anti-allergic potential, it remains poorly understood whether the mixed strains also have the same potent, and whether such therapeutic effect relating to changed microbiota and presenting dose dependence phenomenon.

2. Materials and methods

2.1. Animal experiment

Male Balb/c mice of 6–8 week were obtained from the Laboratory Animal Center, the Fourth Military Medical University (Xi'an, China). All animals took standard diet and water ad libitum. All the experimental procedures of animals were approved by the Ethics Committee of Fourth Military Medical University (20170402).

Mouse model of allergic asthma was established as described previously [17]. In brief, experimental mice were sensitized intraperitoneally using ovalbumin (OVA, 20 µg per mouse; Sigma, St. Louis, MO, USA) plus with 500 µg alum (days 0, 14, 28 and 42). Starting from day 21st, these animals were exposed to 1%OVA aerosols with an INQUA NEB plus (Omron Company limited, Dalian, Liaoning, China) for 30 min, three times per week (days 21–46). The control mice were only sensitized/challenged using PBS. Different doses of bacterial suspension (0.2 mL/mouse) were given orally from day 0 to day 46 (Fig. 1). Analysis were performed on day 47.

2.2. Preparation of mixed probiotics

The probiotic strains was kindly offered by Ningbo Lvkang Rehabilitation Technology Co Ltd (Ningbo, Zhejiang, China) as lyophilized powder including six bacterial strains (*Lactobacillus gasseri*, LK001;

Lactobacillus salivarius, LK002; *Lactobacillus johnsonii*, LK003; *Lactobacillus paracasei*, LK004; *Lactobacillus reuteri*, LK005; and *Bifidobacterium animals*, LK011) without any additive. Three different doses of bacterial suspension were prepared using PBS: the low (L-LK, 1×10^7 CFU/d), medium (M-LK, 1×10^8 CFU/d) and high doses (H-LK, 1×10^9 CFU/d), respectively.

2.3. Measurement of lung function

Airway hyperresponsiveness (AHR) was assessed by the total lung resistance (Rrs, cm H₂O.s/mL) using the FlexiVent system (SCIREQ, Montreal, Canada) on day 47, as previously mentioned [18]. Anaesthetized (pentobarbital sodium, 90 mg/kg) mice were subjected to trachea cannula (stainless steel cannula 18 G) and then exposed to increasing doses (1.5–100 mg/mL) of acetyl-β-methylcholine chloride (MCh, Sigma). Recorded values were averaged for each dose and used to perform dose-dependent curves for each group.

2.4. Analysis of bronchoalveolar fluid

Briefly, the airways of anaesthetized mice were lavaged three times using PBS (0.8 mL) via a tracheal intubation. The bronchoalveolar lavage fluid (BALF) was collected to determine the total number of inflammatory cell under light microscopy. After that, BALF were centrifuged (2500g, 5 min, 4 °C). Cell pellet were stained using Wright Giemsa stain. Standard morphological criteria were used to sort different leucocyte populations.

2.5. Lung histopathology

Isolated lung tissue was fixed with 4% paraformaldehyde for at least 24 h and then embedded in paraffin. Hematoxylin/eosin (H&E), periodic acid-schiff (PAS) and Masson's trichrome staining were performed respectively for inflammation, goblet cells hyperplasia and collagen fibres with standardized protocols. Slides were read for histologic assessment by professional personnel under light microscopy.

2.6. Flow cytometry analysis of immune cells

Isolated mesenteric lymph nodes (MLNs), peyer's patches (PPs), peribronchial lymph nodes (PLNs), small intestine lamina propria (siLP), and spleen were handled in RPMI 1640 medium containing 400 U of type I collagenase, 2% fetal bovine serum (FBS). ACK lysis buffer was applied to crack Erythrocytes. A 70-mm cell strainer was used to filter cells suspension. Single-cell suspensions were used for FACS analyses. Firstly, single-cells were stained by surface markers containing CD4-PerCP, CD11c-PerCP-Cy5.5, CD25-APC, CD103-PE, I-A/I-E-APC, CD86-PE, CCR9-FITC, CD40-PE (BioLegend, San Diego, CA, USA). If necessary, those cells were fixed and permeabilized using Fixation/Permeabilization solution (BioLegend) and then stained for intracellular

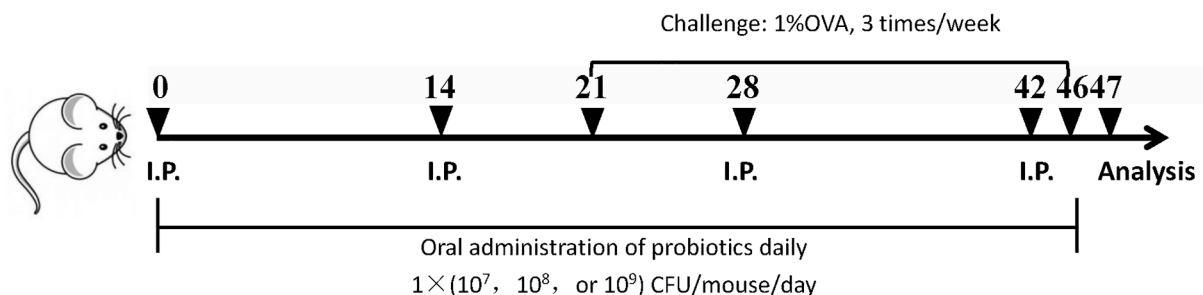


Fig. 1. Experimental procedure. Mice were sensitized with four intraperitoneal injections of ovalbumin (OVA, 20 µg per mouse) at 14-day intervals (days 0, 14, 28 and 42) and challenged with 1%OVA aerosols three times per week (days 21–46). Mice in the treatment group received bacterial suspension with three different doses or PBS orally from day 1 to day 46. Analyses were performed 24 h after the last aerosol.

markers Foxp3-PE. Data were acquired using BD FACSCalibur (BD Biosciences, San Jose, CA, USA) and analyzed by FlowJo 10.0.7 soft (Tree Star Inc., Ashland, OR).

2.7. *In vitro* co-culture assay

MLNs cells of OVA + L-LK and OVA mice were stained with CD11c-PerCP-Cy5.5 and CD103-PE and then further sorted by MoFlo™ XDP cell sorter (Beckman Coulter, Brea, CA, USA) for the expression of CD103⁺DCs to a purity of >95%. Naïve CD4⁺T cells were sorted from spleen of naive mice using a mouse naïve CD4⁺T Cell Isolation Kit (Miltenyi Biotech, Bergish-Gladbach, Germany). Then purified CD103⁺DCs (1×10^5) were cultured with naïve CD4⁺T cells (1×10^6) in the existence of OVA (50 µg/ml) and plate-bound anti-CD3 (1 µg/ml, BioLegend) for 72 h in RPMI 1640 supplemented with 10% FBS, 1% penicillin/streptomycin and 0.5% glutamine. After cultivation, precipitated cells were collected for Tregs analysis. Culture supernatants were harvested for levels of IL-4, IL-13.

2.8. ELISA testing

BALF, serum, and cell culture supernatants were collected for cytokines, immunoglobulins (Igs) and mast cell protease 1 (mMCP-1). Enzyme-linked immunosorbent assay (ELISA) Kits (Chondrex, Redmond, WA, USA; ReyBiotech, Norcross, GA, USA; eBioscience, San Diego, CA, USA) were used to assess the levels of BALF IL-4, IL-13, IL-17A, IL-10 and mMCP-1, serum OVA-specific IgE and IgG1, and supernatants IL-4 and IL-13. All experimental procedures involved were performed following the manufacturer's instructions.

2.9. Gut and lung microbiota analysis

Mice feces and lung tissue were collected under sterile conditions on day 47 and kept at −80 °C for further analysis. Nested PCR was used to amplify the V3–V4 region of the bacterial 16S rRNA gene. MiSeqPEX300bp platform was applied to sequence the above amplicons. USEARCH 8.0 was employed to extract clean data from raw data. Operational Taxonomic Unit (OTUs) were assorted on the basis of 97% similarity after chimeric sequences removed using UPARSE (version 7.1 <http://drive5.com/uparse/>). The RDP Classifier against the Silva (SSU123) 16S rRNA database was applied to analyze the phylogenetic affiliation of each 16S rRNA gene sequence using confidence threshold of 70%. Sample diversity metrics were assessed based on the non-parametric Shannon-Wiener diversity index and Simpson diversity index. Bray-curtis distance were computed in QIIME. Principal coordinate analysis (PCoA) plots was generated to visualize the dissimilarity using QIIME pipeline. 10,000 permutations (QIIME package) was used to test for the statistical significance between the groups using permutational MANOVA. Differential abundance of taxa was detected by the linear discriminant analysis (LDA) effect size (LEfSe). R (<http://www.R-project.org/>) was used to generated bar plots and PCoA plots.

2.10. Data and statistical analysis

Data were shown as means ± SEMs. SPSS software (Version 24.0; IBM, Armonk, NY, USA) were used for data analysis. Statistical significance was compared by one-way analysis of variance for parametric and Mann-Whitney U for non-parametric tests. A p value of <0.05 was indicated statistically significant.

3. Results

3.1. LK reduced OVA-induced allergic airway inflammation

Mouse model of allergic asthma was established and LK was administrated orally to OVA-treated mice through the experiment (3

times/week) to confirm whether it could down-regulate allergic inflammatory response (Fig. 1). Lung resistance improved by LK-treatment (50 and 100 mg/ml MCh), particularly in the low dose (Fig. 2a). OVA-specific serum IgE and IgG1 also decreased by LK-treatment (Fig. 2b). Similarly, inflammatory cell infiltration of lung showed a dramatic decrease, which was due to a relative reduction of eosinophils and neutrophils (Fig. 2c). Quantities of lung inflammatory cytokines including IL-4, IL-13, and IL-17A were significantly reduced upon LK-intervention, which present an opposite trend to anti-inflammatory cytokine IL-10 (Fig. 2d). DCs isolated from PLNs of LK-treatment mice manifested a less activated phenotype, which was characterized by decreased surface expression of the co-stimulatory molecules CD40, CD86, and major histocompatibility complex class II (MHCII) (Fig. 2e). LK-intervention mice displayed reduced mast cell activation in comparison with those untreated (Fig. 2f).

3.2. LK suppressed airway remodeling features of asthmatic mice

To examine the effect of LK-intervention on airway remodeling, lung tissues of mice were isolated for histopathological analysis. In the lung sections of asthmatic mice, increased peribronchial and perivascular cellular infiltration, mucus hypersecretion, and collagen deposition were observed as compared to the control. Treatment with LK was effective in reducing histopathological changes, typically the low dose, of sensitized and challenged mice in comparison with the untreated. Hence, histological findings demonstrate that LK-treatment could improve airway remodeling (Fig. 3).

3.3. LK expands tolerogenic CD103⁺DCs in the MLN.

CD103⁺DCs have been described widely due to their highly specific tolerogenic function [19,20]. To further figure out whether LK-induced anti-inflammatory and anti-allergic mechanisms relates to such tolerogenic DCs. MLNs, PPs and spleen were isolated for flow cytometric analysis. Reduced frequency of CD103⁺DCs (Fig. 4a) and increased expression of MHCII (Fig. 4d) on these DCs in the MLNs were observed in the asthmatic group as compared to the control. While treatment with LK, especially the low dose, led to dramatically increased CD103⁺DCs and decreased MHCII in the MLN. However, there were no change in the expression of CD103⁺DCs and MHCII in PPs or spleen (Fig. 4b, c, and e, f). These findings suggest that LK-induced anti-allergic response is associated with the accumulation of tolerogenic MLN CD103⁺DCs.

3.4. LK accumulates Foxp3⁺Tregs expression induced by CD103⁺DCs

CD103⁺DCs exist in the small intestine at high frequency and migrate to MLN to initiate and maintain immune tolerance by induction of regulatory T cells [21,22]. Therefore, LK-induced protective effect on Foxp3⁺Treg populations was examined. Firstly, we assessed the frequency Treg cells in siLP and BALF. We observed that both gut and lung displayed lower expression of Foxp3⁺Treg in the asthmatic group than the control group (Fig. 5b). Furthermore, LK-treatment, typically the low dose, resulted in significantly increased Tregs in the airway and intestinal expressing the gut-homing chemokine receptor CCR9 as compared to those untreated (Fig. 5b, c), which suggested that these Foxp3⁺Tregs probably originated from intestine. Then, In order to evaluate the capability of stimulated MLN CD103⁺DCs inducing naïve CD4⁺Tcells into Treg cells, purified mesenteric CD103⁺DCs from the asthmatic or L-LK-intervention mice was co-cultured with naïve CD4⁺T from spleen of naïve mice. MLN CD103⁺DCs isolated from OVA + L-LK group showed good potential to promote Treg differentiation, as demonstrated by the increased proportion of Foxp3⁺Treg in the cultured cells (Fig. 5d). Co-cultured supernatants were used for Th2 cytokines including IL-4 and IL-13, both of which displayed an obvious decrease as compared to the allergic mice (Fig. 5e). Taking together, these results indicate that LK-induced MLN CD103⁺DCs contribute to accumulate

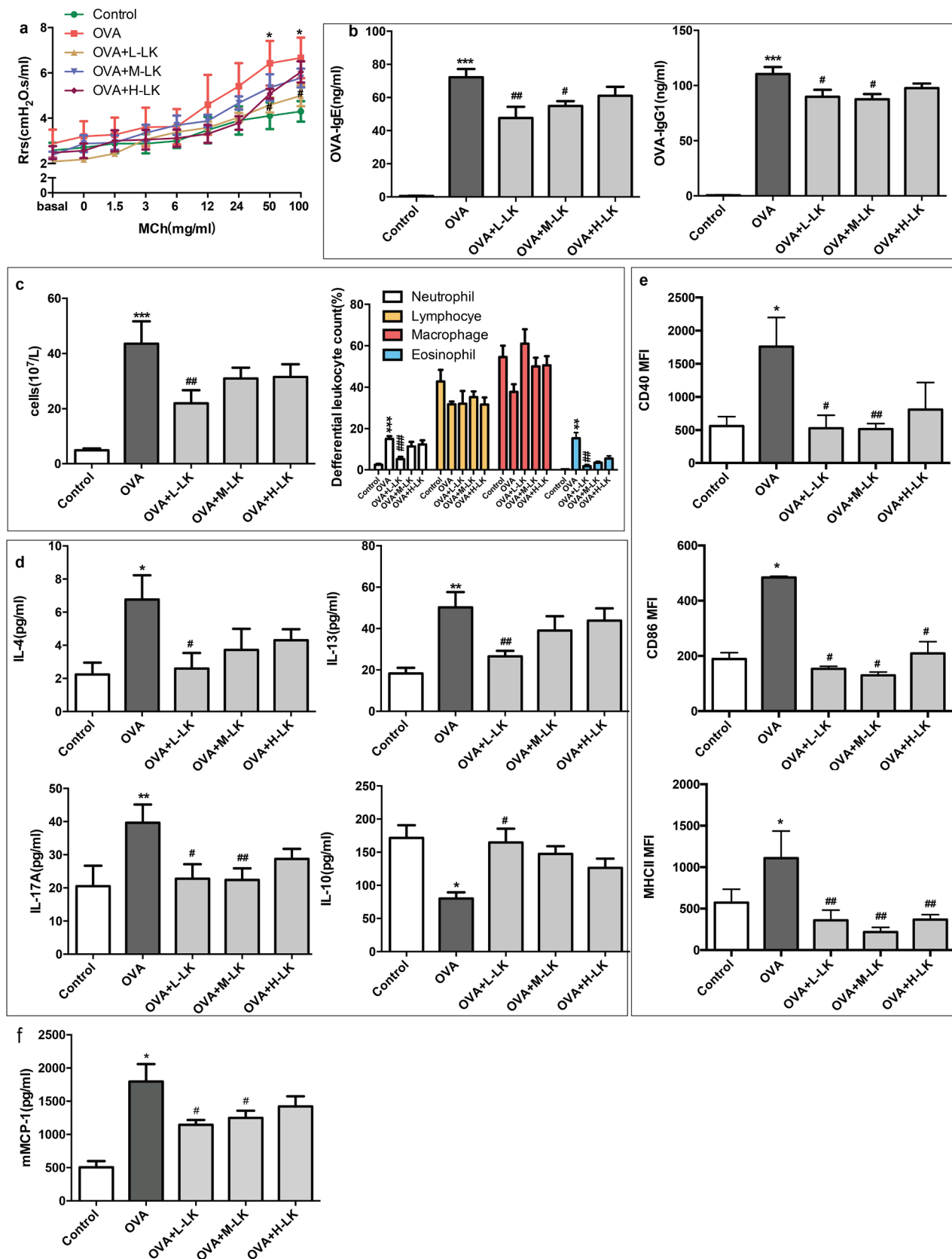


Fig. 2. LK reduces OVA-induced allergic airway inflammation. Serum and BALF were collected on day 47. **a.** AHR (n = 6). **b.** Serum levels of OVA-IgE (n = 5–7). **c.** Cellular infiltration of BALF (n = 5–6). **d.** Concentrations of cytokines in BALF, (n = 5–8). **e.** MFI expression of the co-stimulatory molecules on CD11c⁺ DCs in PLN. **f.** BALF levels of mMCP-1. Data are presented as mean ± SEM. **p* < 0.05, ***p* < 0.01, ****p* < 0.001 versus the control group, and #*p* < 0.05, ##*p* < 0.01, ###*p* < 0.001 versus the OVA group.

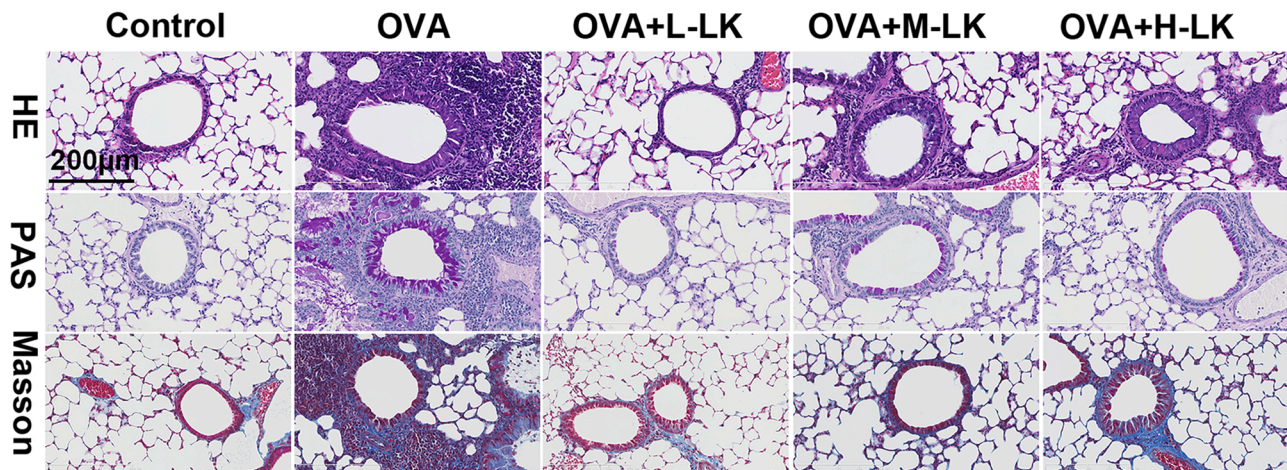


Fig. 3. LK improves airway remodeling of asthmatic mice. On day 47, lung slides of mice were used for histopathological analysis to assess the effect of mixed probiotics on airway remodeling. H&E for inflammation, PAS for goblet cells hyperplasia, and Masson's trichrome staining collagen fibres were performed with standardized protocols ($n = 6$), the scale bar is 200 μm . Slides were read for histologic assessment by professional personnel under light microscopy.

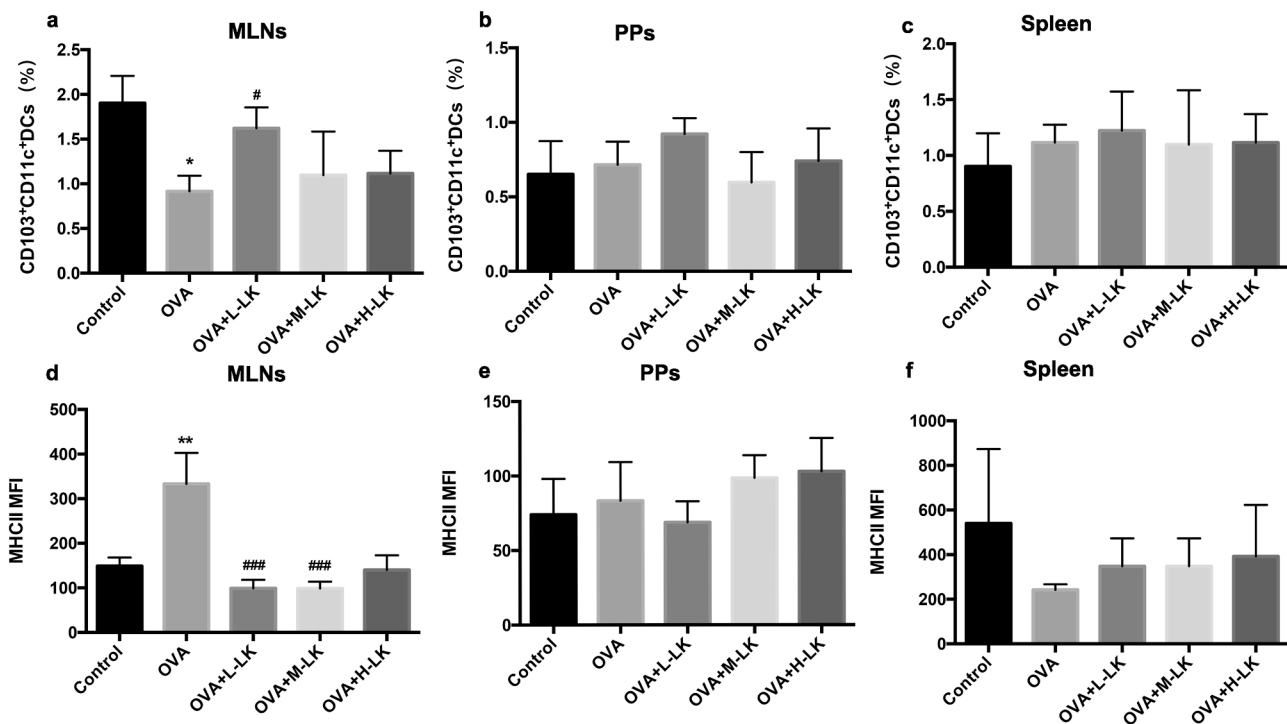


Fig. 4. LK expands MLN CD103⁺CD11c⁺DCs. MLNs (a), PPs (b) and spleen (c) were dissected on day 47. a–c. The frequency of CD103⁺CD11c⁺DCs cells in MLNs (a), PPs (b) and spleen (c) among lymphocytes ($n = 4-6$). d–f. MFI expression of the co-stimulatory molecules MHCII in MLNs (d), PPs (e) and spleen (f) on CD11c⁺DCs. Data are presented as mean $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus the control group, and # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ versus the OVA group.

regulatory Treg cells and thus suppress OVA-induced allergic inflammation.

3.5. LK modulates gut microbiota composition in OVA-sensitized mice

It is evidenced that microbiota has an essential influence over immune cell homeostasis [23] and susceptibility to allergic inflammation [24,25]. Therefore, we analyzed LK-induced effect on intestinal and lung microbiota. Both Shannon (Fig. 6a) and Simpson (Fig. 6b) diversity index were calculated to measure differences of taxonomic diversity between different groups. By contrast, taxonomic diversity was obviously increased upon LK administration compared to the asthmatic mice. Similarities of all samples was evaluated by PCoA and ecologic

distances which is calculated based on the bray curtis distances. Obvious separation was observed between L-LK-treatment and asthmatic or between asthmatic and control group, while paired LK-treatment and control group were much closer to each other than to the any two group (Fig. 6c). Considering the bray curtis distance on the basis of the first dimension (PC1) of PCoA (Fig. 6d), microbial composition was more and more similar to the control group from the high to low dose group, also the low is distinctly distinguished from that of the asthmatic group.

Differential abundance of taxa was detected to compare the relative contribution of different taxa using LEfSE. We identified a total of 52 different taxa with significant abundance differences at different levels among the five groups, of which 3 significantly abundant taxa at phylum level and 21 at genus were noted (Fig. 7a, b). Three discriminative

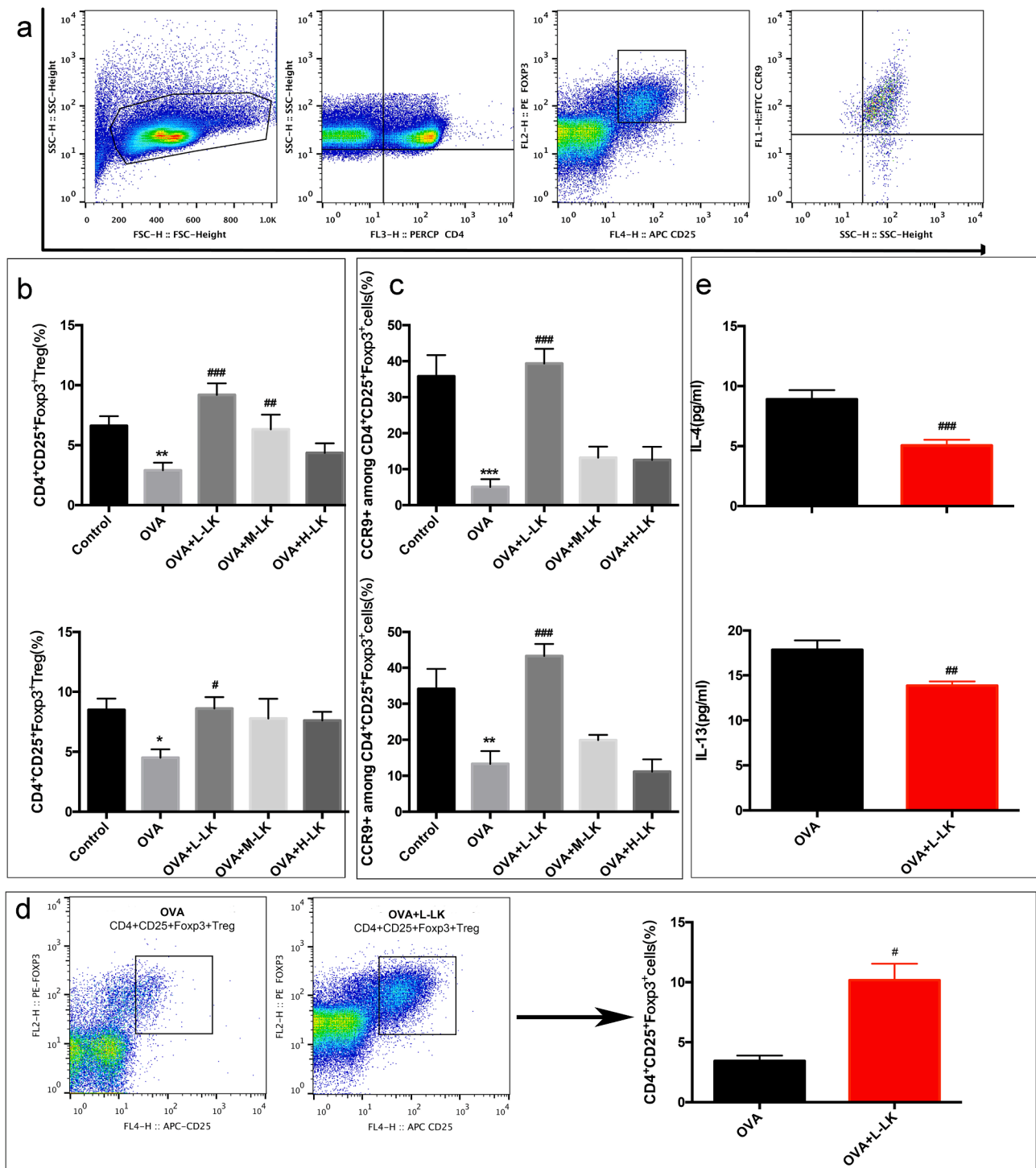


Fig. 5. LK stimulates mucosal Tregs expression induced by MLN CD103⁺DCs. BALF and siLP were collected for Tregs on day 47. Regulatory T cells were identified as CD4⁺ CD25⁺Foxp3⁺ Treg cells. **a.** The gating strategy of Tregs. **b.** The frequency of CD4⁺ CD25⁺Foxp3⁺ Treg cells among CD4⁺T cells in BALF (up, n = 5–7), and siLP (down, n = 5–6). **c.** The frequency of CCR9-expressing cells among the CD4⁺CD25⁺Foxp3⁺Treg population in BALF (up, n = 5–7), and siLP (down, n = 5–6). **d–e.** MLN CD103⁺DCs were cocultured with naïve CD4⁺T cells purified from the spleen of naïve mice in the presence of OVA (50 µg/ml) for 72 h. Cells were analyzed by flow cytometry for Treg cells (**d**, n = 4–8). Culture supernatants were harvested for levels of IL-4, IL-13 (**e**, n = 4–8). Data are presented as means ± SEM. **p* < 0.05, ***p* < 0.01, ****p* < 0.001 versus the control group, and #*p* < 0.05, ##*p* < 0.01, ###*p* < 0.001 versus the OVA group.

phylum was further analyzed among the five groups using the nonparametric Mann-Whitney test and we found that Bacteroidetes decreased obviously while Firmicutes increased in OVA-exposed mice, which can be reversed by oral administration of L-LK (Fig. 7c). The Bacteroidetes/Firmicutes (B/F) ratio was also significantly increased by the L-LK-treatment (Fig. 7d). Among the twenty-one discriminative

genera (Fig. 8), *Ruminococcus2*, *Alloprevotella*, *Parabacteroides*, *Lachnospiraceae_incertae_sedis*, *Oscillibacter* and *Barnesiella* were reduced significantly in the asthmatic, of which *Ruminococcus2* and *Barnesiella* were increased by L-LK and M-LK-treatment. Interestingly, *Lactobacillus* presented a significant decrease in LK-intervention group compared with that of the asthmatic, particularly in the low dose (Fig. 8).

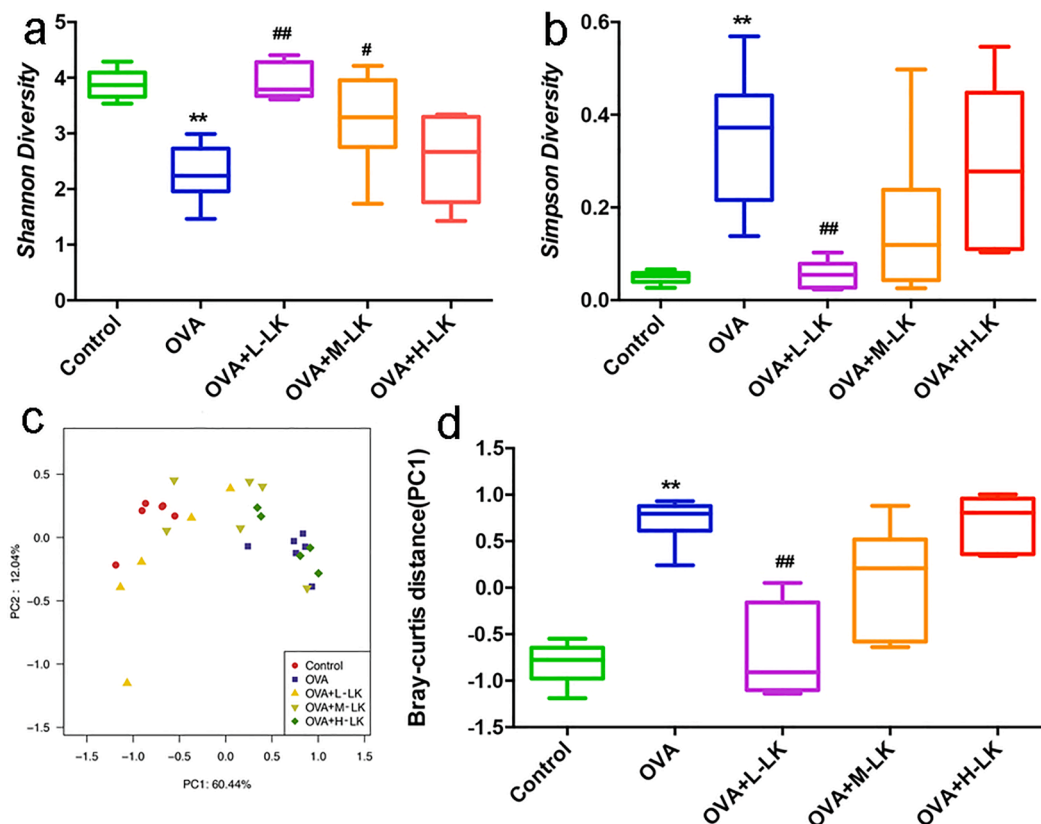


Fig. 6. LK increases bacterial diversity of gut microbiota. a, b. Alpha-diversity was calculated with Shannon index (a) and Simpson index (b). c. PCoA plot based on the Bray-Curtis distance (nonparametric Mann-Whitney test). d. Mean Bray-Curtis distance between each group based on the first dimension (PC1) of PCoA (nonparametric Mann-Whitney test). n = 5–6. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus the control group, and # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ versus the OVA group.

Key OTU Heatmap was constructed using random forests technique to make it clear how correctly the relative abundance of each genus could classify different groups of samples (Fig. 9a). We have identified 6 *Lactobacillus* OTUs (OTU1, OTU3, OTU9, OTU133, OTU401, and OTU481) in fecal samples, of which the latter three (OTU133, OTU401, and OTU481) are not displayed in the Heatmap due to the relative low OTU numbers. Further analysis showed that the number of OTU1 and OTU481 increased significantly in OVA-exposed mice while reduced in the LK-treatment group and grew gradually from the low dose group to the high (Fig. 9b), which suggested that these two OTUs may be the potential screening tools for differentiating the asthmatic from control. Conversely, OTU9 presented an obviously decreased trend which can be increased by LK supplement except for the low dose (Fig. 9b), which indicates that OTU9 may be the bacterial strain we administered orally. However, there were no alteration in OTU3 among the five groups. OTU 133 and OTU 401 have been detected only in the medium, or the low and high dose group. To confirm what we suspect about genus *Lactobacillus*, *Lactobacillus*-OTU sequence detected in our experiment were compared with that of nucleotide databases using the Basic Local Alignment Search Tool (BLAST). Interestingly, 99% similarity have been found between OTU3 and *Lactobacillus reuteri* DSM 20016, OTU9 and *Lactobacillus gasseri* ATCC 33323, and OTU401 and *Lactobacillus paracasei* ATCC 334 chromosome or *Lactobacillus paracasei* subsp. *paracasei* JCM 8130 DNA, which indicated that these three OTUs may derive from the mixed probiotics, particularly the OTU9 which may be the dominant strain providing protective effect on OVA-induced allergic airway inflammation (Supplementary file 1). However, there was no change about lung microbiota among the five groups in our study (Supplementary file 2).

4. Discussion

In this study, we investigate the protective effect of the mixed probiotics on OVA-induced allergic airway inflammation. The mixed strain has been demonstrated to inhibit allergic asthma through accumulating mucosal Foxp⁺Tregs coaxed by MLN CD103⁺DCs. Experiment in vitro also indicates that CD103⁺DCs could induce regulatory T cells differentiation and suppress Th2 allergic response. Furthermore, alteration of gut microbiota rather than lung microbiota relates to allergic asthma which can be alleviated by mixed probiotics via modulating intestinal flora, especially the low dose.

Interestingly, the low dose (1×10^7 /d CFU) of mixed probiotics has been proven to be more effective than the other two (1×10^8 CFU/d, and 1×10^9 CFU/d) in our experiment. Although oral treatment with probiotics has been found beneficial to attenuate allergic diseases [26–28], there is no clear standards between bacterial number and probiotic effects. For example, for *Dermatophoides pteronyssinus* (Der p)-induced mouse model of allergic asthma, the low dose (1×10^7 CFU/d) of *Lactobacillus gasseri* may be better to alleviate allergic airway inflammation through PPAPy activation in dendritic cells than the high (1×10^9 CFU/d) [29]. Another study found that *Lactobacillus gasseri* at 4×10^6 CFU/d is most effective for Der p-induced allergic asthma [30]. However, *Lactobacillus gasseri* SBT2055 at 1×10^9 CFU/d was investigated to be effective in preventing *Porphyromonas gingivalis*-accelerated periodontal diseases [31]. Notably, the dose effects of probiotics remain poorly understood, which may be associated with treatment duration, disease and allergen types, and strain specificity, etc. Consequently, it is not exact that the higher the dose and the better the effect, the optimal dose of prevention or treatment still needs to be further explored.

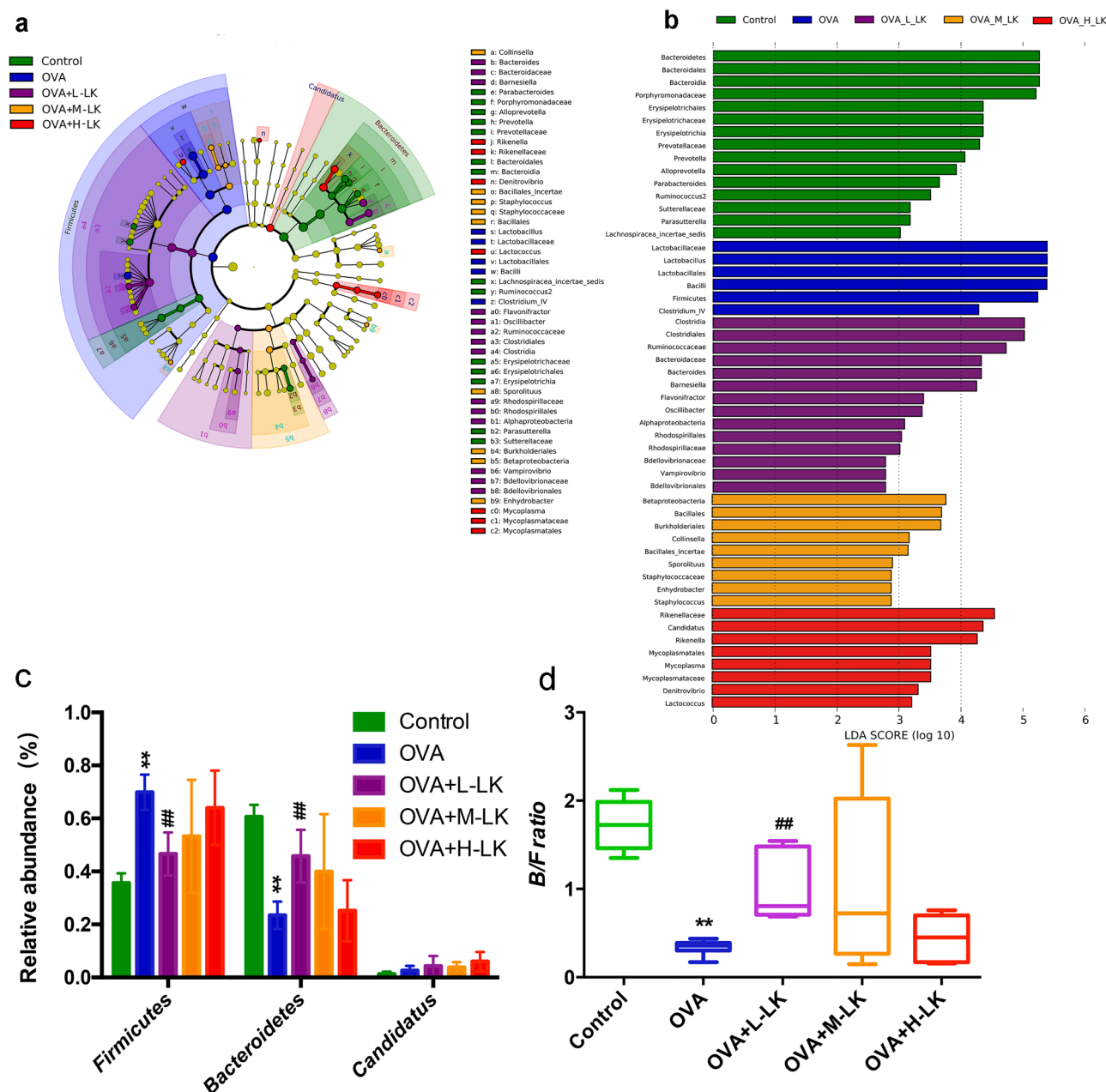


Fig. 7. Differentially abundant taxa among different groups. **a**, **b**. Differential taxa identified by LEfSe (**a**) with LDA values (**b**). **c**. Taxa enriched at phylum level are identified by LEfSe. **d**. *Bacteroidetes/Firmicutes* (B/F) ratio. $n = 5-6$. p values were calculated using the nonparametric Mann-Whitney test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ versus the control group, and # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ versus the OVA group.

DCs are the primary antigen-presenting cells responsible for the antigen-specific activation of naive T cells [32]. CD103⁺DCs have been described widely because of their highly specific tolerogenic function [19,20]. A combination of signals from microbes results in CD103⁺DCs migrating to MLN from gut to initiate and maintain immune tolerance by induction of regulatory T cells [21,22]. Previous study found that signals from the gut flora and its metabolites of SPF mice can imprint long-lasting tolerogenic properties on the MLN stromal cells which are able to stimulate the accumulation of Tregs even 20 weeks after transplantation, while stromal cells from germ-free mice are unable to promote the production of Treg cells [33]. This regulatory mechanism plays a key role in the immunoregulatory action of many probiotics. This idea is supported by some animal studies in which allergic response can be down-regulated through the induction of Treg cells by administration of probiotic strains like *Lactobacillus rhamnosus*, *B. lactis*, and *B. breve*

[34–37]. Some bacterial species could promote peripheral Treg cell differentiation and suppress Th2 response by generating metabolites during dietary fibre fermentation, especially the short-chain fatty acids (SCFAs) including acetate, butyrate and propionate [38]. SCFAs exposure contributes to increased activity of retinal dehydrogenase 2 (RALDH2) in mesenteric CD103⁺DCs, RALDH2 facilitate the transformation of dietary Vitamin A into retinoic acid, which promotes the production of Treg cells [39]. In the present study, oral administration of LK led to increased frequency of Fox3⁺Treg cells induced by MLN CD103⁺CD11c⁺-expression DCs. High expression of MHCII on DCs in the treatment mice suggests that LK suppressed immune activation induced by allergic response, which was demonstrated by increased Treg cells and reduced Th2 cytokines in the in vitro experiment. An increase of CCR9⁺Tregs indicates that massive migration of gut-primed Tregs to the lung may involve the mechanism, which was supported by

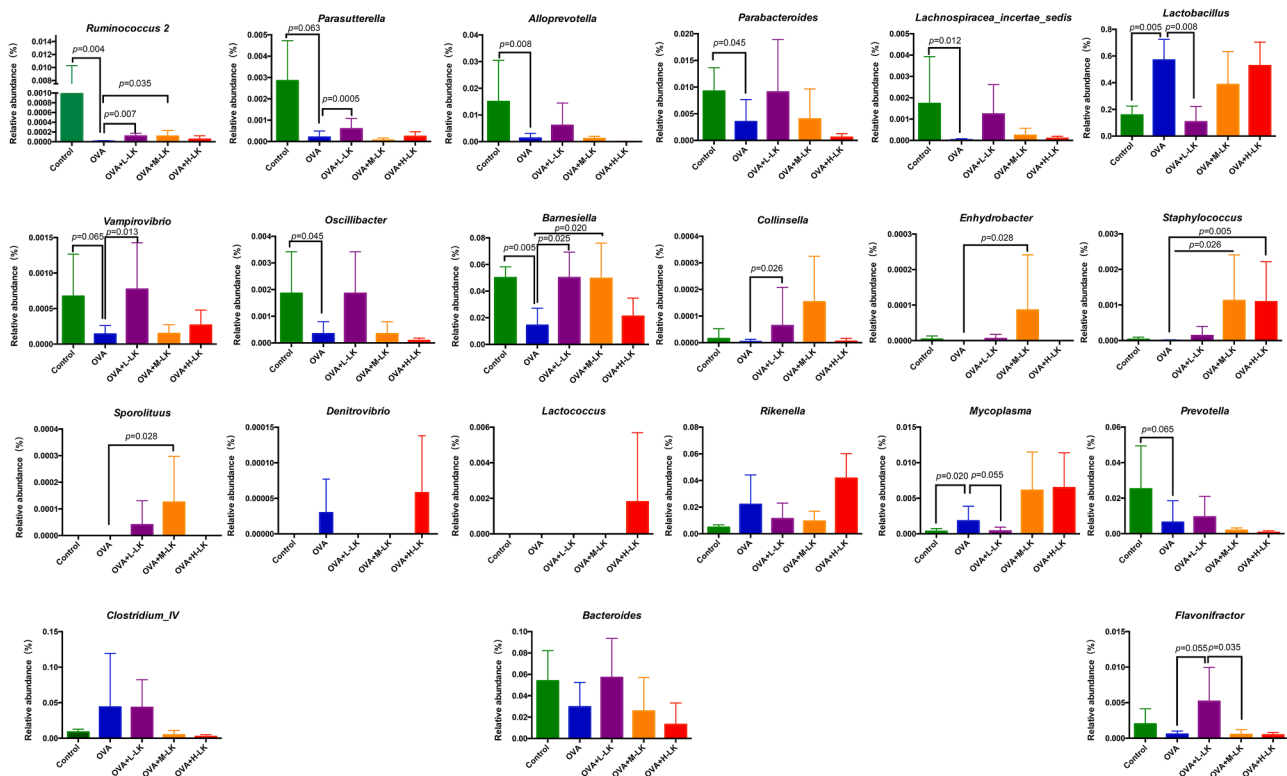


Fig. 8. Different taxa at genus among different groups. Twenty-one discriminative genera were presented as Fig. 8. $n = 5-6$. p values were calculated using the nonparametric Mann-Whitney test. $P < 0.05$ was considered statistically significant.

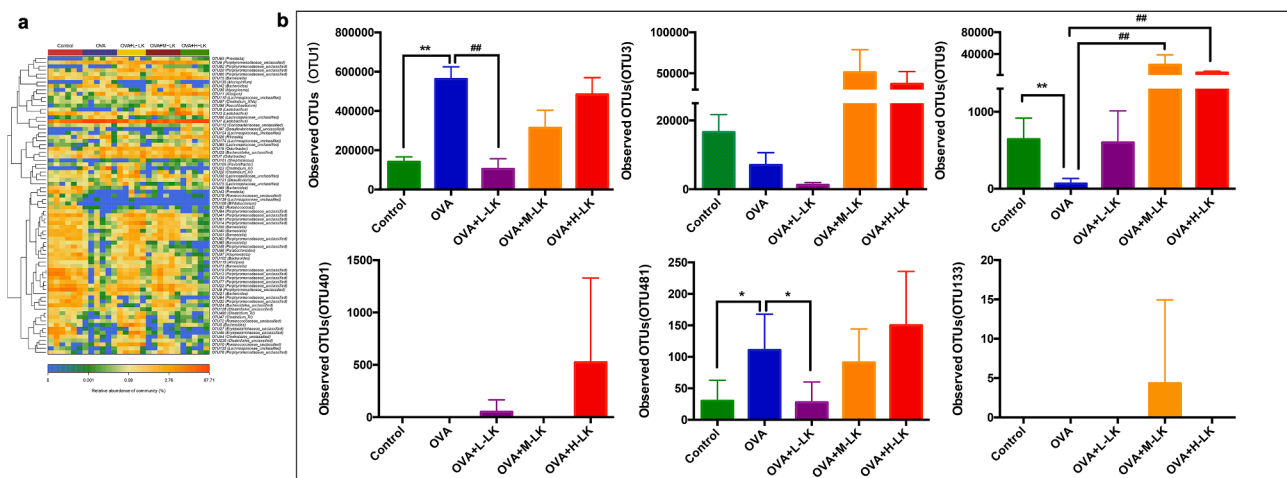


Fig. 9. Analysis of discriminative genus at OTU level. a. Heatmap of OTU table. b. Observed OTUs numbers from genus *Lactobacillus*. $n = 5-6$. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ versus the control group, and $\#p < 0.05$, $\#\#p < 0.01$, $\#\#\#p < 0.001$ versus the OVA group.

He et al' idea that the lymph and/or bloodstream may act as a link between the gut and the affected site on the lung [40].

Commensal microbiota compromises many microorganisms which cover the whole mucosal surfaces of host, but the majority reside in the intestinal tract. Host-microbes symbiosis play a key role in maintaining health and immune homeostasis. Changes of intestinal flora has been considered to be one of the critical indicators of allergic diseases [41–44]. Emerging scientific evidence from either human or animal studies has indicated that increasing asthma prevalence is closely related to microbiota. In our study, gut microbiota was different between the control and asthmatic mice, which was indicated by reduced Shannon and Simpson index. Probiotics has been demonstrated to have

immunomodulatory effective on allergic asthma. Thus, we detected whether probiotics-induced protective effect was associated with gut microbiota in our study. In our present experiment, LK-intervention contributes to reverse gut flora disturbance similar to that of the control mice, particular the *Barnesiella* and *Ruminococcus2*, which were enriched in the control but significantly decreased in the asthmatic mice. Previous studies show that intestinal microbiota containing *Barnesiella* species is associated with reduced gut inflammation, which not only cures Vancomycin-resistant *enterococcus faecium* colonization but also facilitate Cyclophosphamide-induced immunomodulatory effects of cancer [45,46]. Another research reported that decline of propionate-producing *Barnesiella* led to increased allergic inflammation [47],

which is similar to our study. *Ruminococcus2* has been rarely implicated in allergic diseases, but it increased significantly along with reduced allergic airway inflammation induced by LK treatment. Nevertheless, what makes us puzzled is that genus *Lactobacillus* was raised in the OVA-intervention mice while reduced by oral administration of mixed probiotics which is made up of five different *Lactobacillus* and *Bifidobacterium* animals. Genus-OUT levels analysis showed that all six OTUs of *Lactobacillus* doesn't display a similar changing trend, which may help us unfold the puzzle. The possible reason is that not all of genus *Lactobacillus* are beneficial for organism. Maybe the increased number of harmful OTU1 and OTU481 in OVA-exposed mice was counteracted by a nearly equal amount of the beneficial strain OTU9, which ultimately result in decreased *Lactobacillus*. It is reported that allergy diseases such as asthma often company with imbalance of gut flora which is characterized with more phylum Firmicutes and less phylum Bacteroidetes [48,49]. Similar to the present study, decreased B/F ratio was observed in the asthmatic model, which can be increased by LK-treatment. These findings showed that the intestine microbiota have an important impact on allergopathology development, which are in line with the previous research. As well, LK is effective to alleviate OVA-induced allergic inflammation via regulating gut microbial communities.

Lung microbiota has also been proved to be closely related to allergic asthma [9,10,50]. Surprisingly, there is no change of lung microbiota among the five groups in our study. Isolated lung lobes was used to microbiota sequencing in our study. Maybe that microbes in partial lung lobes cannot completely represent the flora of the whole lung, which is one limitation of our experiment. Also, collecting BALF or the whole lung tissue for further study is undoubtedly necessary.

5. Conclusions

In summary, these findings demonstrate that the mixed probiotics has remarkably immunomodulatory effects on OVA-induced allergic inflammation, which is associated with gut microbiota instead of lung microbes. The mechanism may also involves accumulation of gut-primed Foxp3⁺Treg induced by MLN CD103⁺DCs, which may migrate to the lung through the lymph and/or bloodstream. Furthermore, the higher dose does not necessarily generate the better effect, maybe the suitable is the best. Totally, our results provide new evidence for probiotic application in allergic diseases and support the idea that targeted gut microbiota will be an effective approach for allergic airway diseases.

CRedit authorship contribution statement

Juan Zhang, Jingyi Ma, Hui Su and Xin Sun participated in the conception and design of the study. Juan Zhang, Jingyi Ma and Qihong Li performed laboratory work. Juan Zhang and Jingyi Ma are responsible for data analysis and writing-original draft. Hui Su and Xin Sun also performed the supervision and writing-review&editing.

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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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.cellimm.2021.104399>.

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