

Research paper

Lactobacillus rhamnosus GG induced protective effect on allergic airway inflammation is associated with gut microbiota

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ABSTRACT

Great interest has been taken in the use of beneficial bacteria for allergic diseases recently, but the underlying mechanisms through which probiotics induces immune regulation or immune tolerance are poorly understood. We aimed to explore whether *Lactobacillus rhamnosus* GG (LGG)-induced beneficial effect relates to the change of microbiota. LGG was administered orally to mouse model of ovalbumin (OVA)-induced allergic airway inflammation. Our findings manifested that LGG-treatment contributes to protect against OVA-induced allergic airway inflammation by expanding mesenteric CD103⁺ DCs and accumulating mucosal Tregs. Moreover, protective effect induced by LGG is associated with gut microbiota instead of lung flora. Collectively, our findings indicate that LGG induced protective effect is associated with gut microbiota and provide a new evidence of probiotic application in the allergic airway inflammation.

1. Introduction

Allergic diseases affect up to 30% of the population worldwide, with asthma being one of the most common chronic diseases in which affected individuals may suffer considerable morbidity [1–4]. It is estimated that asthma affects 300 million individuals worldwide, most probably as a result of changing environment and reduced exposure to microbial antigens during infancy [5,6]. The classic asthma presentation is generally regarded as a T helper 2 (Th2) airway inflammation with mucus hypersecretion and variable degrees of airflow obstruction associated with airway hyperresponsiveness (AHR) to nonspecific stimuli, all of which finally leading to bronchial remodeling [7]. Although corticosteroids are effective at managing the disease, 10% of patients do not respond to the treatment, and they are associated with severe long-term side effects [8–11]. Accordingly, there is an increasing demand for proven alternatives to pharmaceutical products from both healthcare professionals and consumers.

Alteration of microbiota contributes to the development of allergies and asthma [12,13]. Changes of microbiota could result in differences to downstream immune responses or immune development, both of which can affect immune mechanisms in distal mucosal sites such as the lung and result in asthma. However, deficiencies in the immune system can also affect the microbiota composition, which can affect distal mucosal sites and have a role in the development of atopic conditions [14]. Probiotic bacteria have been shown to modify immune responses in vitro [15–17] and in animals [18,19], and are defined as “live microorganisms which confer a health benefit on the host when administered in adequate amounts”. Animal models revealed that supplementation with *Lactobacillus rhamnosus* GG (LGG) resulted in reduction in the major features of allergic airway inflammation in a murine model of experimental asthma [20]. Protective effects were also transferred to the offspring when mothers were supplemented with LGG before and during pregnancy and weaning [21]. Several human studies also demonstrated that LGG was highly effective to reduce the risk for

Abbreviations: LGG, *Lactobacillus rhamnosus* GG; OVA, ovalbumin; AHR, airway hyperresponsiveness; BALF, bronchoalveolar lavage fluid; Th, T helper; DCs, dendritic cells; Tregs, regulatory T cells; 16S rRNA, 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; SW, Shannon-Wiener; PCoA, principal coordinate analysis; LDA, linear discriminant analysis; LEfSe, linear discriminant analysis effect size; MHCII, histocompatibility complex class II; ANOVA, analysis of variance; PERMANOVA, permutational MANOVA; ROC, receiver operating characteristics; AUC, area under curve

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the development of allergic asthma [22,23]. Yet despite all that, some controversial conclusions are still drew, and there have been rare reports to demonstrate whether LGG-induced protective effect on allergic airway inflammation is associated with the alteration of microbiota. Here, we aimed to explore LGG-induced beneficial effect on allergic asthma and whether it relates to the change of microbiota.

2. Material and methods

2.1. Mice

Male BALB/c mice of 6–8 weeks were obtained from the Laboratory Animal Center of the Fourth Military Medical University and housed under conventional conditions. A standard extruded pellet diet and water were provided *ad libitum*. Experimental procedures were approved by the Ethics Committee for Animal Studies of the Fourth Military Medical University (20170403) and performed in accordance with their guidelines of the Institutional Animal Care and Use Committee.

2.2. Experimental design

Allergic airway inflammation was induced as described previously [24] with minor modifications. Briefly, sensitization was made by four intraperitoneal injections of OVA (20 µg per mouse; Sigma, St. Louis, MO, USA) adsorbed with 500 µg alum at 14 day intervals (days 0, 14, 28 and 42). Beginning on the 21st day, sensitized mice received OVA challenge by 1%OVA aerosols during 30 min, three times per week (days 21–46). Challenge was carried out using an INQUA NEB plus (Omron Company limited, Dalian, Liaoning, China). The control group was given normal saline i.p. (0.2 ml per mouse) and challenged with normal saline at the same time points. Mice in the LGG-treatment group received 0.2 ml LGG drink orally (5×10^8 CFU/ml) from day 0 to day 46 (Fig. 1).

2.3. Airway hyperresponsiveness

AHR was assessed by measuring the total lung resistance (Rrs, cm H₂O.s/mL) using the FlexiVent system (SCIREQ, Montreal, Canada) on day 47, as described previously [25]. Anaesthetized (pentobarbital sodium, 90 mg/kg) and tracheostomized (stainless steel cannula, 18 G) mice were nebulized 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 obtain dose–response curves for each group.

2.4. Bronchoalveolar lavage

Bronchoalveolar lavage was performed on day 47 after mice were sacrificed. Briefly, the airways of the mice were lavaged three times with 0.8 ml of PBS via a tracheal cannula. The total number of inflammatory cell in the bronchoalveolar lavage fluid (BALF) was determined under light microscopy, and then the BALF were centrifuged at 2500g for 5 min at 4 °C. The cells at the bottom were stained with Wright Giemsa stain. One hundred cells per slide were counted to classify individual leucocyte populations using the standard

morphological criteria.

2.5. Enzyme-linked immunosorbent assay

BALF and serum samples were collected to assay the presence of cytokines, mouse mast cell protease 1 (mMCP-1) and immunoglobulins (Igs). The levels of BALF IL-4, IL-13, IL-10, mMCP-1, and serum OVA-specific IgE and IgG1 were measured by enzyme-linked immunosorbent assay (ELISA) Kits (ReyBiotech, Norcross, GA, USA; eBioscience, San Diego, CA, USA; Chondrex, Redmond, WA, USA) following the the instructions of the manufacturer.

2.6. OVA-specific stimulation of spleen lymphocytes

Spleen cell suspensions were obtained as previously described [26] by passing the tissue through a 40 µm-cell strainer. Erythrocytes from the spleen were eliminated with ACK lysis buffer. Spleen cells were cultured in RPMI-1640 medium with 10% fetal calf serum (FCS), 1% L-Glutamine, and 2% Pen/Strep in round bottom 96-well plate (2×10^5 cells per animal/well) and re-stimulated with 100 mg/ml OVA for 4 days at 37 °C, 5% CO₂. As an additional readout for differences in the immune status of the animals, levels of IL-4 and IL-13 in culture supernatants were measured by ELISA (ReyBiotech).

2.7. Flow cytometry

Peribronchial lymph nodes (PLN), mesenteric lymph nodes (MLN), or trachea were processed in RPMI 1640 medium containing 2% fetal bovine serum (FBS), 400 U of type I collagenase. Cells were strained through a 70-mm cell strainer. Erythrocytes were lysed with ACK lysis buffer. Small intestine lamina propria were obtained after digestion in RPMI containing 5% FCS, 5 mM EDTA, and 2 mM dithiothreitol, as described previously [27]. Single-cell suspensions isolated from the above tissues were stained for FACS analyses. Cells were first stained for surface markers including CD4-PerCP, CD25-APC, CD11c-PerCP-Cy5.5, I-A/I-E-APC, CD103-PE, CD86-PE, CD40-PE, CCR9-FITC (BioLegend, San Diego, CA, USA). If required, cells were then fixed and permeabilized by Fixation/Permeabilization reagent (BioLegend) and stained for intracellular expression markers, Foxp3-PE. Data were acquired with BD FACSCanto (BD Biosciences, San Jose, CA, USA) and analyzed by FlowJo 10.0.7 soft (Tree Star Inc., Ashland, OR).

2.8. Histopathology

The lungs were fixed with 10% formalin for at least 24 h, after which the lungs were embedded in paraffin. Four-micrometer sections were stained with hematoxylin and eosin (H&E) for inflammation, periodic acid–schiff reagent (PAS) for goblet cell hyperplasia and Masson's trichrome for collagen fibers according to standard methods. Slides were reviewed in blinded fashion for histologic assessment using light microscopy.

2.9. Microbiome analysis

Feces and lung tissue samples from individual mice were collected

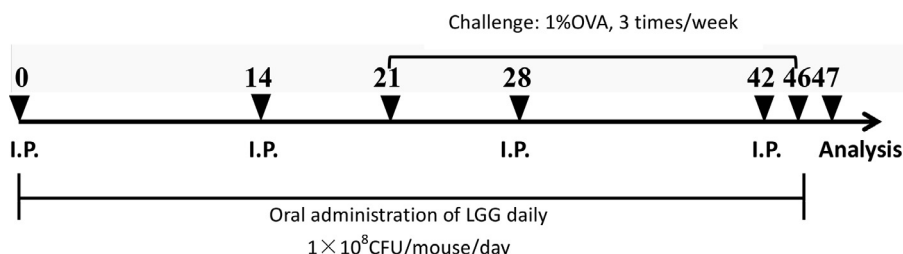


Fig. 1. Experimental procedure. Mice were sensitized with four intraperitoneal injections of ovalbumin (OVA, 20 µg per mouse) at 14-day intervals (days 0, 14, 28 and 42) and challenged with 1%OVA aerosols three times per week (days 21–46). Mice in the treatment group received bacterial suspension with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

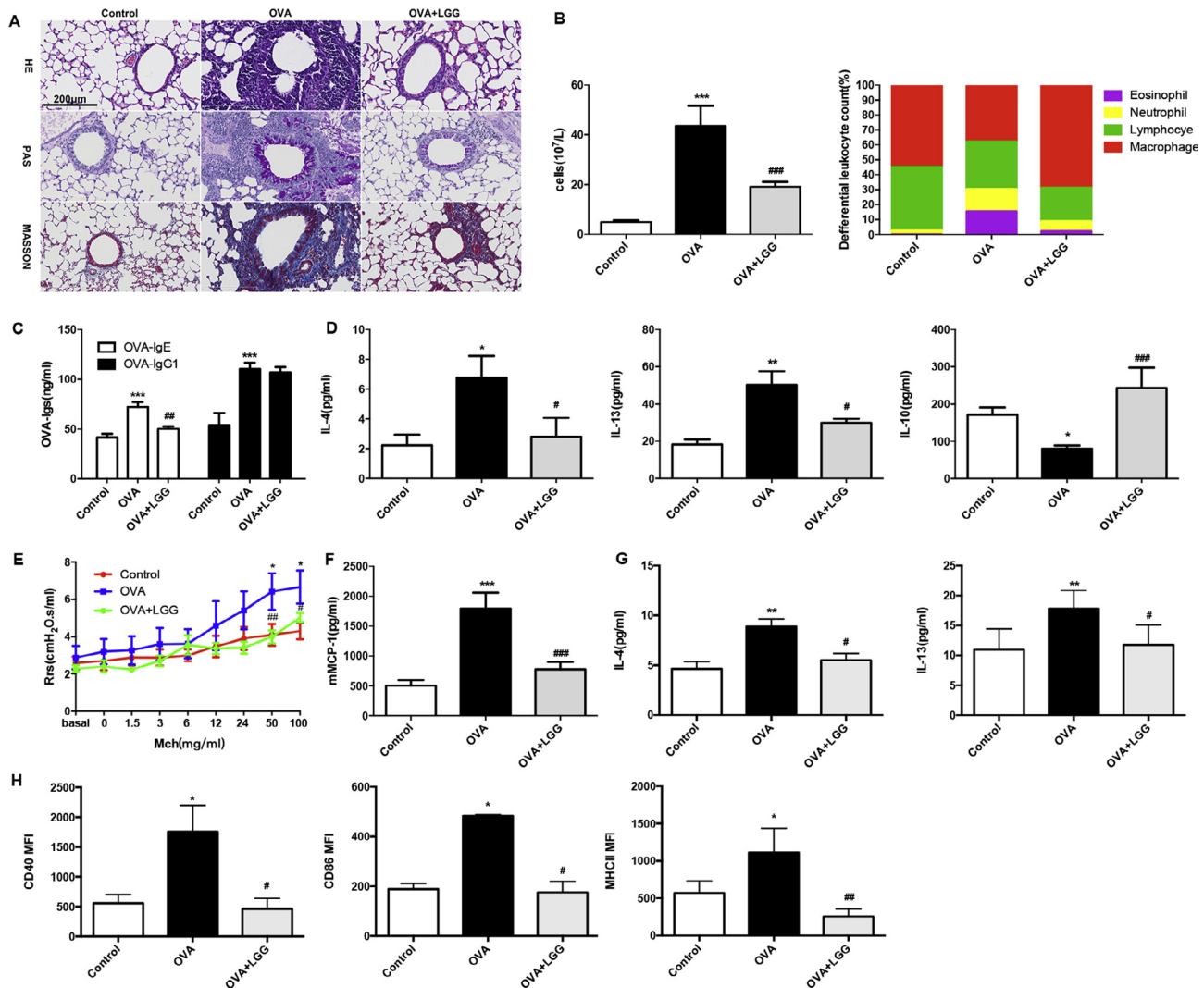


Fig. 2. Oral administration of LGG reduced OVA-induced allergic airway inflammation. A. Lung sections were stained with hematoxylin/eosin (H&E) or periodic acid-Schiff reagent (PAS) or Masson's trichrome (n = 6). B. Cellular infiltration of BALF. C. Serum levels of ovalbumin (OVA)-specific immunoglobulins (n = 5–7). D. Concentrations of IL-4, IL-13, and IL-10 in bronchoalveolar lavage fluid (BALF), (n = 5–7). E. Airway hyperresponsiveness (AHR) was assessed by measuring resistance in response to increasing doses of methacholine (MCh) after the last challenge (n = 5–6). F. BALF levels of mMCP-1 (n = 5–8). G. Levels of IL-4 and IL-13 in spleen supernatants (n = 6–8). H. Mean fluorescence intensity (MFI) expression of the co-stimulatory molecules CD40, CD86, and histocompatibility complex class II (MHCII) on CD11c⁺ dendritic cells (DCs) in peribronchial lymph nodes (PLN, n = 4–7). All 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.

under sterile conditions and stored at -80°C for microbiota analysis. The V3-V4 region of the bacterial 16S ribosomal RNA (rRNA) was amplified using nested PCR. Amplicons were sequenced using the MiseqPE2x300bp platform. Clean data was extracted from raw data using USEARCH 8.0. Operational Taxonomic Unit (OTU) were classified based on 97% similarity after chimeric sequences removed using UPARSE (version 7.1 <http://drive5.com/uparse/>). The phylogenetic affiliation of each 16S rRNA gene sequence was analyzed by RDP Classifier (<http://rdp.cme.msu.edu/>) against the Silva (SSU123) 16S rRNA database using confidence threshold of 70%. Sample diversity metrics were assessed on the basis of the nonparametric Shannon-Wiener (SW) diversity index and Simpson diversity index. Ecologic distances were calculated in QIIME. The QIIME pipeline was also used to generate principal coordinate analysis (PCoA) plots to visualize the distance dissimilarity. Permutational MANOVA (PERMANOVA) was used to test for statistical significance between the groups using 10,000 permutations (QIIME package). The linear discriminant analysis (LDA) effect size (LEfSe) was used to detect taxa with differential abundance among groups. Bar plots, PCoA plots and receiver operating

characteristics (ROC) curves and the area under the ROC curve (AUC) values were all generated in R (<http://www.R-project.org/>).

2.10. Statistical analysis

Data were expressed as means ± SEMs. All data were analyzed with SPSS software (Version 17.0; IBM, Armonk, NY, USA) or GraphPad Prism Software (Version 6.0, [Inc. La Jolla, CA, USA](http://www.graphpad.com)). One-way analysis of variance (ANOVA), PERMANOVA, and non-parametric Mann-Whitney *U* test were conducted to determine the statistical significance, where appropriate. A *P*-value of < 0.05 was considered statistically significant.

3. Results

3.1. LGG-treatment protects against OVA-induced allergic airway inflammation

To address whether LGG-treatment inhibited pulmonary

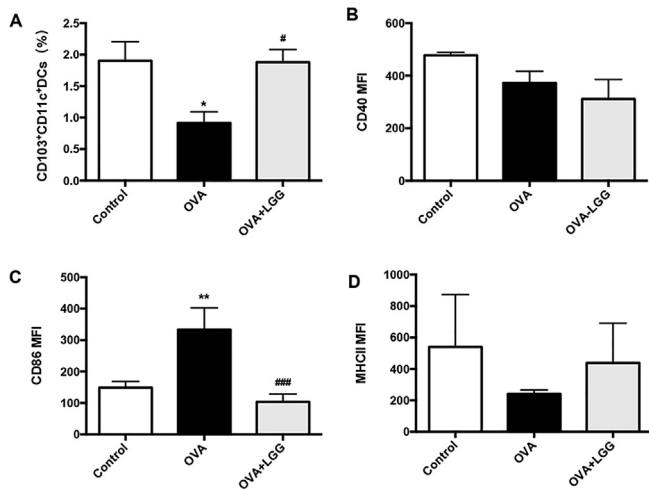


Fig. 3. LGG expands mesenteric CD103⁺CD11c⁺DCs to protect against OVA-induced allergic airway inflammation. A. Frequency of mesenteric lymph nodes (MLN) CD103⁺CD11c⁺DCs cells among lymphocytes. B-D. Mean fluorescence intensity (MFI) expression of the co-stimulatory molecules CD40(B), CD86(C), and histocompatibility complex class II (D. MHCII) on MLN CD11c⁺dendritic cells (DCs). n = 4–6. All 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.

inflammatory responses generated in asthma, we exposed BALB/c mice to OVA, and treated with a daily intragastric administration of LGG for 46 days. Mice treated with LGG display reduced peribronchial and perivascular cellular infiltration of the lungs, mucus hypersecretion and collagen deposition compared to those untreated (Fig. 2A). Similarly, inflammatory cell infiltration of airways shows a significant decrease in eosinophilic and neutrophilic infiltrates (Fig. 2B, both $p < 0.05$) upon LGG treatment, as were OVA-specific IgE serum titers compared to those of the asthmatic (Fig. 2C). In line with the reduced inflammatory cell infiltration, protein level of IL-4 and IL-13 were decreased whereas IL-10 increased by LGG-intervention (Fig. 2D). Lung resistance was significantly improved by LGG administration (Fig. 2E, at 50 and

100 mg/ml MCh, both $p < 0.05$), as were the quantities of BALF mMCP-1 protein (Fig. 2F). Both cytokines (IL-4 and IL-13) of spleen supernatants were also obviously decreased in the LGG-treatment group (Fig. 2G). DCs isolated from PLNs of LGG-administration mice displays a less activated phenotype, as demonstrated by reduced surface expression of CD40, CD86 and major histocompatibility complex class II (MHCII) molecules (Fig. 2H).

3.2. LGG expands mesenteric CD103⁺CD11c⁺DCs to protect against OVA-induced allergic inflammation

To further explore LGG-induced anti-inflammatory mechanism when administered systemically. MLN were collected for the expression of DCs. CD103⁺DCs have been widely described for their highly specific tolerogenic function in the mesenteries [5,28,29]. In the present study, LGG administration led to marked increases in the expression of CD103⁺DCs (Fig. 3A). However, phenotypic characterization of these DCs showed no difference in expression of CD40 and MHCII but significant decrease of CD86 by LGG-intervention (Fig. 3B–D). Take together, these data demonstrate that MLN CD103⁺DCs are critical for LGG-induced protective effects on allergic airway inflammation.

3.3. LGG induces Tregs in the mucosa to protect against OVA-induced allergic inflammation

MLN CD103⁺DCs are noted for their abilities to induce and maintain tolerance via induction of regulatory T cells [28,30]. Consequently, we examined the impact of LGG-treatment on CD4⁺CD25⁺Foxp3⁺Treg populations. We observed a remarkable increase in the Treg cells in the airway and intestinal mucosa expressing the gut-homing chemokine receptor CCR9 upon LGG-treatment in comparison to that of the asthmatic (Fig. 4).

3.4. Protective effects induced by LGG relates to gut microbiota

As recent evidence has indicated that microbiota can influence immune cell homeostasis [31] and susceptibility to allergic inflammation [32–35], we next analyzed LGG-induced protective effect on the microbiota. Taxonomic diversity was calculated using the Shannon

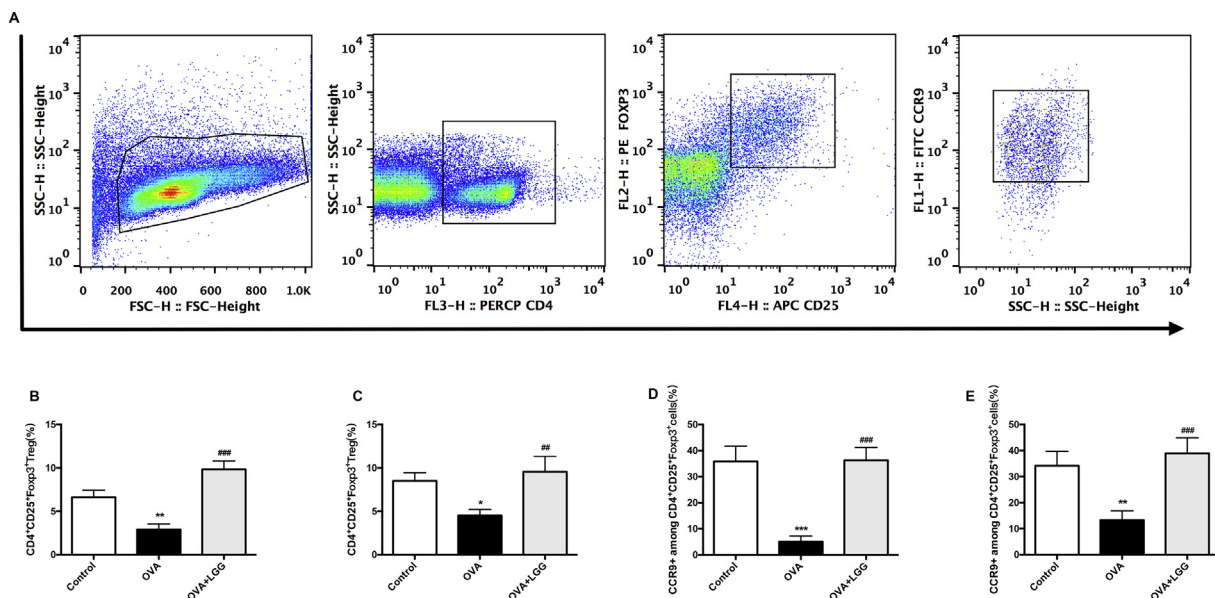


Fig. 4. LGG induces Tregs in the mucosa to protect against OVA-induced allergic inflammation. A-B. Frequency of CD4⁺CD25⁺Foxp3⁺Treg cells among CD4⁺T cells in trachea (A, n = 5–7), and small intestine lamina propria (B). C–D. Frequency of CCR9-expressing cells among the CD4⁺CD25⁺Foxp3⁺Treg population in trachea (C), and small intestine lamina propria (D, n = 5–6). All data are presented as means ± SEM. n = 4–7. *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.

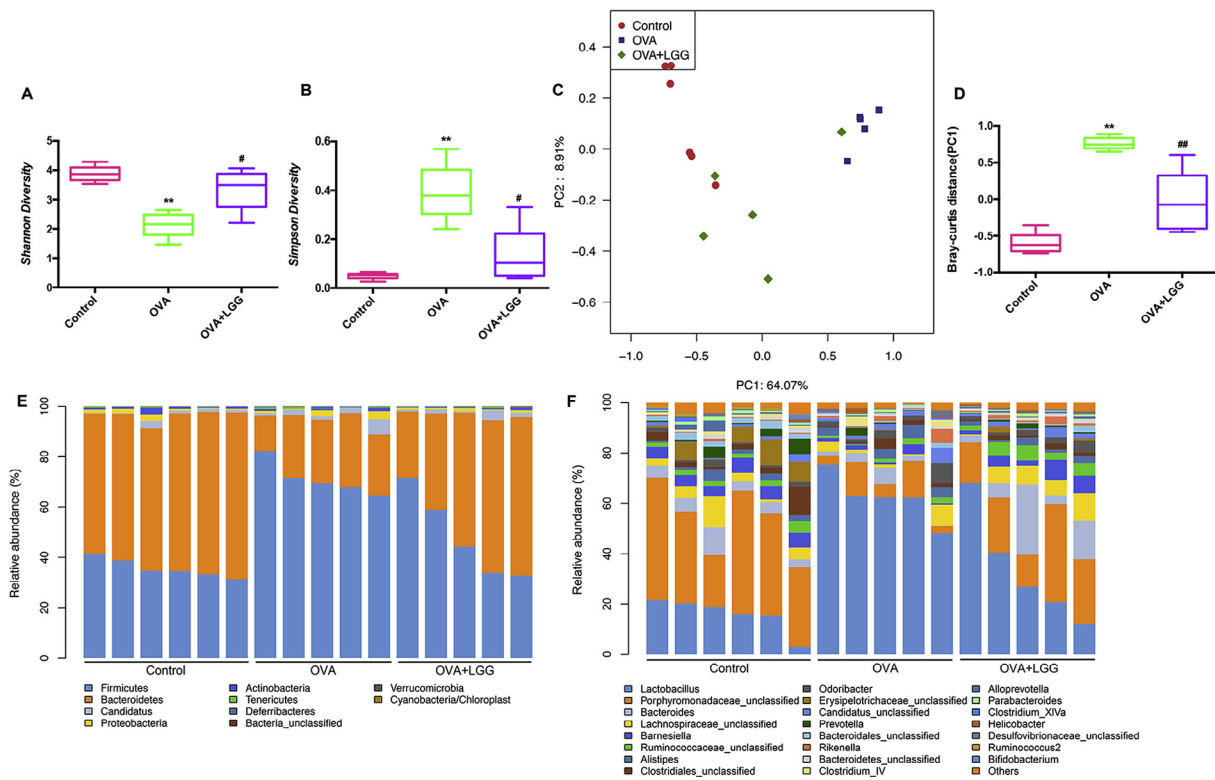


Fig. 5. LGG-induced protective effects relate to gut microbiota. A-B. Alpha-diversity was calculated with Shannon index (A) and Simpson index (B). C. Principal coordinate analysis (PCoA) plot based on the Bray-Curtis distance of feces samples (nonparametric Mann-Whitney test). D. Mean Bray-Curtis distance between each group based on the first dimension (PC1) of PCoA (nonparametric Mann-Whitney test). E-F. Bacterial composition of fecal samples at phylum (E) and genus (F) level. Error bars represent the standard error of the mean. $n = 5$ to 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.

(Fig. 5A) and Simpson (Fig. 5B) diversity index by non-parametric Mann-Whitney U test. By comparison, bacterial diversity was significantly different between the control and the asthmatic group or the asthmatic and LGG-treatment group. Both Shannon and Simpson index revealed that diversity was lowest in the asthmatic group and LGG-treatment contributes to increase the diversity. To evaluate the similarities of all samples, ecologic distances, calculated on the basis of the Bray-Curtis distances, were visualized by PCoA plot (Fig. 5C). We observed an obvious separation between control group and asthmatic group or between asthmatic group and LGG-treatment group, while paired control and LGG-treatment group were much closer to each other than to the any two group. The Bray-Curtis distance based on the first dimension (PC1) of PCoA quantified these differences (Fig. 5D). Marked difference was observed in the composition of gut microbes at phylum (Fig. 5E) and genus (Fig. 5F) level among the three groups.

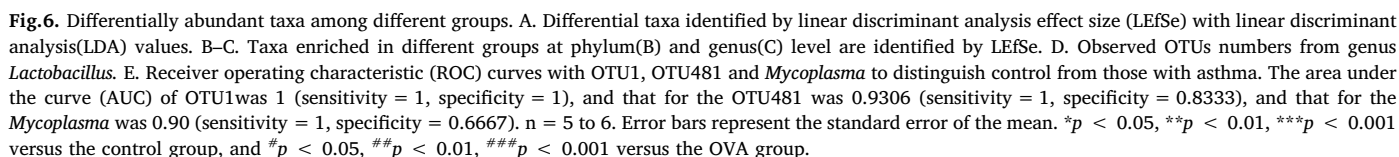
In order to compare the relative contribution of difference taxa, we used LEfSe to detect the key taxa responsible for the discrepancy among the three groups. A total of 33 different taxa at different levels with significant abundance differences across three groups were identified, of which 2 differentially abundant taxa at phylum level and 19 at genus were noted (Fig. 6A). We further analyzed the differential abundance of two discriminative phylum across three groups using the nonparametric Mann-Whitney test and *Bacteroidetes* were significantly decreased but *Firmicutes* increased in asthmatic mice, while LGG-treatment reversed this difference except for the *Firmicutes* (Fig. 6B). Among the nineteen discriminative genera, significant decrease in *Ruminococcus2*, *Barne-siella*, *Alloprevotella*, *Parasutterella*, *Clostridium_XIVb*, *Lachnospir-acea_incertae_sedis*, *Hydrogenoanaerobacterium*, *Oscillibacter* and *Bifido-bacterium* and increase in *Lactobacillus* and *Mycoplasma* were detected in asthmatic mice, while only *Alloprevotella* and *Oscillibacter* changed remarkably after LGG intervention (Fig. 6C). Interestingly, genus *Lacto-bacillus* and *Mycoplasma* displayed prominently increased trend in the

asthmatic, as were in the Fig. 5F. Five OTUs (OTU1, OTU3, OTU9, OTU133, and OTU481) from genus *Lactobacillus* have been identified in our samples (Fig. 6D). Further analysis manifested that the numbers of OTU1 and OTU481 increased dramatically in asthmatic mice but remarkably decreased by LGG-treatment except for the latter, which may be the potential screening tools for distinguishing the asthmatic from control. On the contrary, OTU9 presents a noticeably increased trend after LGG administration in comparison with that of the asthmatic group, which may be the bacterial strain we administered orally. Classification performance of underlying prediction strains was evaluated by ROC curve. Prediction models based on the random forests algorithm suggested that the area under curve (AUC) of OTU1 (*Lacto-bacillus*), OTU481 (*Lactobacillus*) and genus *Mycoplasma* was 1, and 0.931, and 0.90, respectively (Fig. 6E). However, in our study, OVA-induced allergic airway inflammation does not seem to result in alteration of lung microbiota (Fig. S1).

4. Discussion

In this study, We have shown that LGG-treatment restores lung function, reduces specific antibody and suppress eosinophil and neutrophil infiltration into the airways, mucus hyperplasia, and collagen deposition in the lungs upon systemic or mucosal delivery. Lung Th2 cytokine production including IL-4 and IL-13 was also significantly decreased by LGG-administration, as were in spleen supernatants, while anti-inflammatory cytokine IL-10 increased markedly in protein expression. Therefore, we have demonstrated that LGG can significantly suppress OVA-induced allergic airway inflammation.

Host-microbial mutualism is an integral part of maintaining health and immune homeostasis. Accumulating scientific evidence from both human and animal studies has suggested that an association may exist between microbiota and allergic disease. An abnormal composition of



[43]. In our study, gut microbiota from asthmatic mice has significantly lower community diversity compared with samples from the control. Moreover, our analysis suggested that gut microbial communities of asthmatic mice were also distinguishable from that of the control. More importantly, LGG treatment was effective to correct the disorder of gut microbiota similar to that of the control mice. However, what makes us confused is that *Lactobacillus* increased in the asthmatic mice while decreased by LGG-treatment. One possible explanation is that genus *Lactobacillus* contains some different species, not all of which are beneficial to organism. In the analysis of genus-OTU level, all the five OTUs came from genus *Lactobacillus* present an incompletely consistent trend among the three groups, which may explain our doubts. Perhaps the harmful *Lactobacillus* (maybe OTU1 and OTU481) increased in asthmatic mice which was neutralized by a nearly number of the beneficial ones (maybe OTU9), finally resulting in a reduced level of *Lactobacillus*. Prediction of asthma evaluated using the ROC curve manifested that OTU1(AUC = 1) or OTU481(AUC = 0.931) from genus *Lactobacillus* and *Mycoplasma*(AUC = 0.90) may possess certain classification potential, however, which still need to be further verified due to our relatively small sample size. Recently, the altered composition of intestine microbiota characterized with less phylum *Bacteroidetes* and more phylum *Firmicutes* has also been found to company with allergy diseases such as asthma [44,45], which were similar to that of our present study. More importantly, LGG-intervention reverses this trend by more

Bacteroidetes and less *Firmicutes* than that of the asthmatic mice except for it is not statistically significant of the latter. These results have good agreements with the previous studies to support that the intestine microbiota could influence the development allergopathology, and most importantly, LGG can protect OVA-induced allergic airway inflammation through mediating gut microbial communities.

In the recent years, airway microbiota has also been reported to be associated with allergic asthma [46–48]. Another recent study showed that that lung microbial colonization early in life can boost the frequency of lung Treg cells and promote tolerance to foreign aero-antigens through a mechanism involving PD-L1-expressing lung CD11b⁺DCs [49]. However, there is no difference between the three groups of lung microbiota in our study. The possible reason is that the bacteria in partial lung lobes failure to fully reflect the changes in the flora of the whole lung, which is the limitation of our study, and it is necessary to collect the BALF for further study.

5. Conclusion

In summary, these data demonstrate that LGG induced protective effect on allergic airway inflammation through regulating gut microbiota which could promote the expression of Tregs and MLN CD103⁺DCs to suppress Th2 inflammation. Taken together, our findings provide a new evidence of probiotics application in the allergic airway inflammation and support the concept that intervention strategies targeting gut microbiota are a valuable approach for not only intestinal diseases but also respiratory inflammatory diseases.

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Declarations of interest

None.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <https://doi.org/10.1016/j.cellimm.2018.08.002>.

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