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ORIGINAL ARTICLE

# Oral administration of *Lactobacillus delbrueckii* subsp. *lactis* LDL557 attenuates airway inflammation and changes the gut microbiota in a Der p-sensitized mouse model of allergic asthma

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## Abstract

**Background:** Lactic acid bacteria may be used as probiotics to prevent or treat various diseases, and *Lactobacillus delbrueckii* has an inhibitory effect on the development of atopic diseases.

**Objective:** This study explored the effects of *L. delbrueckii* subsp. *lactis* strain LDL557 administration on a mouse asthma model resulting from *Dermatophoides pteronyssinus* (Der p) sensitization and investigated the associated gut microbiota.

**Methods:** Der p-sensitized and challenged BALB/c mice were orally administered with three different doses of live (low, $10^7$ colony-forming units (CFU); medium, $10^8$ CFU; high, $10^9$ CFU) and heat-killed ($10^9$ cells) LDL557 in 200 μL of PBS daily, starting 2 weeks before Der p sensitization and lasting 4 weeks. After the allergen challenge, airway responsiveness to methacholine and the influx of inflammatory cells to the lungs were assessed. The gut microbiome was obtained by sequencing the V3-V4 region of the 16S rRNA gene from mice stool samples.

**Results:** LDL557 in the live ($10^9$ CFU) and heat-killed ($10^9$ cells) conditions reduced the airway hyper-responsiveness after stimulation with methacholine, inflammatory cell infiltration, and mucus production. These effects were similar to those in groups treated with dexamethasone. No significant change in the gut microbiota was observed after LDL557 treatment, except for the tendency of heat-killed LDL557 to change the gut microbial profile to a greater extent than live LDL557.

**Conclusion:** In summary, we found that live and heat-killed LDL557 had the beneficial effect of preventing Der p-induced allergic inflammation in a mouse model of asthma.

**Key words:** *Lactobacillus delbrueckii*, allergic asthma, house dust mite allergen, microbiota

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## Introduction

Allergic asthma is the most common chronic disease in affluent countries, and its prevalence is increasing in developing countries.[1,2] Presently, treatment consists of symptom control and use of anti-inflammatory agents, and the only effective immune regulatory and prevention approach is allergen-specific immunotherapy by subcutaneous or sublingual routes.[3] The hygiene hypothesis,[4] proposed in the last century, emphasizes that the lack of nosocomial or exogenous bacterial stimulation in early life leads to an altered host immune response and the development of allergic disease, as supported by recent clinical observations[5,6] and laboratory findings.[7,8]

Meta-analyses have demonstrated a preventive effect of probiotics on eczema but not on other allergic conditions.[9,10] Probiotics adhere to the intestinal mucosa, increase the local production of metabolites and short-chain fatty acids (SCFAs) that act as transducing signals for immune cell development, and have anti-inflammatory effects.[11,12] Probiotics such as *Lactobacillus* prevent the early development of allergic diseases in children and experimental asthma in mice.[13,14] Trompette et al. reported that the microbiota and dietary fibers modulate allergic inflammation in the lungs by directing immune cell development in the bone marrow (BM) towards a less inflammatory phenotype, and treatment with the SCFAs propionate leads to BM-derived dendritic cells with impaired type 2 T helper cell-stimulatory abilities.[11]

We previously reported that the oral intake of *L. gasseri* for 2 months improved mucosal allergic symptoms in asthmatic children with allergic rhinitis in a double-blind, randomized controlled trial.[15] Many *Lactobacillus* spp. are beneficial to human health. Oral administration of *L. delbrueckii* subsp. *lactis* inhibits the development of atopic disease.[16] Heat-killed *L. delbrueckii* subsp. *lactis* has an inhibitory effect on the development of atopic dermatitis in an ovalbumin-induced type I allergy mouse model.[17] In this study, we evaluated the effects of live and heat-killed *L. delbrueckii* subsp. *lactis* LDL5557 (LDL557) on a *Dermatophoides pteronyssinus* (Der p) sensitization asthma mouse model. The effects of LDL557 treatment on the gut microbiota were also investigated.

## Methods

### *Animals and reagents*

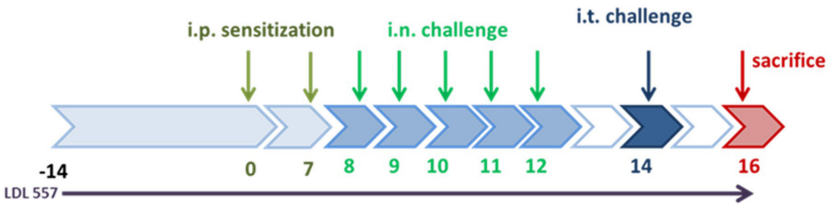
Female BALB/c mice aged 6–8 weeks were obtained from the National Applied Research Laboratories, Taipei, Taiwan. Mice were housed under specific pathogen-free conditions in the Laboratory Animal Center of the National Cheng Kung University, Tainan, Taiwan. Der p extract (1 g lyophilized whole-body extract in diethyl ether; Allergon, Engelholm, Sweden) was dissolved in pathogen-free isotonic saline, filtered through a 0.22 mm filter, and stored at -70°C before use. The lipopolysaccharide concentration of the Der p preparations was < 0.96 ng/mL (Limulus amebocyte lysate test; E-Toxate; Sigma-Aldrich, St Louis, MO, USA).

### *Probiotic bacterial preparations*

LDL557, which exhibits probiotic characteristics, was isolated from corn and provided by Synbio Tech (Kaohsiung, Taiwan). LDL557 bacterial powder was prepared by mixing freeze-dried bacterial cells with the appropriate amount of maltodextrin to a concentration of $1 \times 10^{10}$ colony-forming units (CFU)/g and stored at −20°C until use. Heat-killed LDL557 cells were prepared by heating the liquid bacterial culture at 100°C for 30 min.

### *Treatment protocols*

The animal experimental procedures were approved by the Animal Ethics Committee of National Cheng Kung University (IACUC No. 107225). The experiments involved seven groups: naïve (N), asthma (A), low dose of LDL557-treated asthma (L), medium dose of LDL557-treated asthma (M), high dose of LDL557-treated asthma (H), heat-killed LDL557-treated asthma (S), and dexamethasone-treated asthma (D). Each group consisted of 12 mice. Mice without induced allergic asthma were included in the N group as a control. Allergic asthma was induced in mice of the A, L, M, H, S, and D groups by intraperitoneal (i.p.) sensitization with a mixture of 50 μg Der p and 1 mg aluminum hydroxide (ThermoFisher, Waltham, MA, USA) on days 0 and 7. The mice were further anesthetized and intranasally (i.n.) challenged with 10 μg Der p from days 8 to 12 and intratracheally (i.t.) challenged with 100 μg Der p on day 14. Treatment groups received oral administration of $10^9$ (H group), $10^8$ (M group), or $10^7$ (L group) CFU of LDL557 or $10^9$ cells of heat-killed LDL557 (S group) in 200 μL PBS daily via a gavage needle, starting 14 days before the first immunization with Der p (day 0) and continuing until the day of sacrifice (day 16). Mice of the D group were subjected to i.p. administration of 10 ug dexamethasone (Sigma-Aldrich, St. Louis, MO, USA) on day 14. Airway hyperresponsiveness (AHR) in mice was measured and animals were sacrificed 48 hours after the last Der p exposure. The mice were sacrificed upon completion of the experiment. The fecal samples from the intestinal tract were collected from sacrificed mice (**Figure 1**).




| Groups | LDL557 | Der p sensitization & challenge |
|---|---|---|
| Naïve (N) | - | - |
| Asthma (A) | - | + |
| Low (L) | $10^7$ | + |
| Medium (M) | $10^8$ | + |
| High (H) | $10^9$ | + |
| Heat killed (S) | $10^9$ | + |
| Dexamethasone (D) | - | + |

**Figure 1. Schematic outline of the asthma animal model.**
The experiments involved seven groups: naïve (N), asthma (A), low-dose LDL557-treated asthma (L), medium-dose LDL557-treated asthma (M), high-dose LDL557-treated asthma (H), heat-killed LDL557-treated asthma (S), and dexamethasone-treated asthma (D). Each group consisted of 12 mice. The naïve (N) group served as a control, comprising mice without induced allergic asthma. Allergic asthma was induced in groups A, L, M, H, S, and D through intraperitoneal (i.p.) sensitization with Der p, followed by intranasal (i.n.) and intratracheal (i.t.) challenge with Der p. Treatment groups received daily oral administration of $10^9$ CFU (H), $10^8$ CFU (M), $10^7$ CFU (L) of LDL557, or $10^9$ cells of heat-killed LDL557 (S) daily, starting 14 days before the first Der p sensitization (day 0). Mice of group D were subjected to i.p. administration of dexamethasone on day 14.

***Measurement of AHR***

Mice were anesthetized with an intraperitoneal injection of pentobarbital sodium (50-90 mg/kg body weight). Mice airway function was obtained with an invasive measure of AHR to increasing concentrations of aerosolized methacholine (Sigma-Aldrich, MO, USA) using the flexiVent FX system (Scireq, Montreal Qc, Canada).

***Lung histochemistry***

The lungs of mice were inflated with 0.5 mL of formalin (Sigma-Aldrich) via the trachea, excised, and fixed in 4% buffered formalin overnight at room temperature. Tissues were embedded in paraffin, cut into 5-mm sections, and stained with hematoxylin and eosin (H&E). Inflammatory infiltrates and lung architecture were assessed using light microscopy. The infiltrated immune cells were counted in 144 (×400 magnification) microscopic fields per group, and the mean ± standard error of the mean (SEM) values was presented.

***Total and Der p-specific IgE measurements***

Serum samples were collected from the axillary vein 48 hours after the intratracheal allergen challenge. Total IgE serum antibody titers were determined using enzyme-linked immunosorbent assay (ELISA; Mouse IgE ELISA Quantitation Set, E90-115), with the required reagents and monoclonal antibodies purchased from Bethyl Laboratories, Montgomery, TX, USA. To detect Der p-specific IgE, the plates were initially coated with 10 μg of Der p and left at 4°C for 18 hours. After blocking, diluted sera (1:20 dilution) were applied and incubated overnight at 4°C. Subsequently, the plates were treated with horseradish peroxidase (HRP)-conjugated secondary antibodies (Bethyl Laboratories, Inc.), and then developed with tetramethylbenzidine solution. The optical density (OD) was measured at 450 nm.

***Isolation of fecal DNA and 16S rRNA gene sequencing and analysis***

Fecal DNA was extracted using an AllBio EasyPure Stool Genomic DNA Kit (AllBio, ABTGEE301-01, Taichung, Taiwan) following the manufacturer's instructions. The quality of the extracted DNA was assessed using agarose gel electrophoresis and quantified on a Qubit fluorometer using a high sensitivity dsDNA assay kit (Thermo Fisher Scientific, Waltham, MA, USA). Then, the V3-V4 region of the 16S rRNA gene was amplified using specific primers (806R, 5′- GGACTACNVGGGTWTCTAATCC -3′; 338F, 5′- CCTACG GRRBGCASCAGKVRVGAAT -3′). Amplicon pools were obtained for sequencing analysis using an Illumina MiSeq™ sequencing platform (Illumina, San Diego, CA, USA), and the data were analyzed with QIIME 2 2020.08.[18] The raw reads were demultiplexed with the q2-demux plugin and subjected to denoising and quality filtering with q2-dada2,[19] resulting in an amplicon sequence variants (ASVs) table.

ASVs were then assigned to taxonomic categories using the q2-feature-classifier plugin,[20] which employs the classify-sklearn naïve Bayes taxonomy classifier against the SILVA database (release 138). A representative sequence was selected from each ASVs followed by combining identical sequencing reads, and abundance was calculated at all taxonomic levels. Alpha diversity from Richness and Shannon indices, as well as beta-diversity indices from weighted and unweighted unifrac distances, were also calculated with the phyloseq package. The PERmutational Multivariate ANalysis of VAriance (PERMANOVA)/Adonis test was conducted using vegan: Community Ecology Package (R package version 2.6.2; http://cran.r-project.org/web/packages/vegan/index.html). The Linear discriminant analysis Effect Size (LEfSe) (http://huttenhower.sph.harvard.edu/galaxy/)[21] was performed to identify bacterial taxa differentially represented between groups.

### *Statistical analysis*

AHR, IgE titers, and infiltrated immune cells (counts from lung sections) are presented as mean ± SEM and analyzed using one-way ANOVA, followed by a post-hoc comparison between groups. A $P < 0.05$ was considered statistically significant. Microbiota data are expressed as the mean ± standard deviation (SD). Statistical analyses were performed using GraphPad Prism 7.04 (GraphPad Software, San Diego, CA, USA). For multiple group comparisons, data were analyzed using the Kruskal–Wallis test with Dunn's post-hoc test. Non-metric multidimensional scaling (NMDS) plot data were analyzed using PERMANOVA. Differences were considered statistically significant if $P < 0.05$.

## Results

### *Effects of LDL557 on Der p-induced allergic inflammation*

To investigate the protective effects of LDL557 in Der p-induced allergic asthma, low- ($10^7$ CFU, group L), medium- ($10^8$ CFU, group M), and high- ($10^9$ CFU, group H) doses of live LDL557 and heat-killed LDL557 ($10^9$ cells, group S) were orally administered for 2 weeks before and after allergen sensitization. Serum levels of total and Der p-specific IgE were significantly increased in Der p-induced asthmatic mice of group A compared with those of control group N. Although no significant change was observed in serum levels of total IgE (**Figure 2a**), asthmatic mice that administered different doses of live LDL557, heat-killed LDL557, or dexamethasone showed notably decreased levels of Der p-specific IgE compared with those of group A (**Figure 2b**). Furthermore, all treatment groups, including L, M, H, S, and D, exhibited a significant reduction in airway resistance (Rrs) and airway elastane (Ers) measurements after stimulation with methacholine in comparison with group A (**Figure 2c, d**). Histological analysis demonstrated that increased inflammatory cell infiltration, mucus production, and bronchial epithelial damage occurred in group A. In contrast, inflammatory cell infiltration and mucus production were significantly reduced in groups M, H, S, and D but only slightly improved in group L (**Figure 3**).

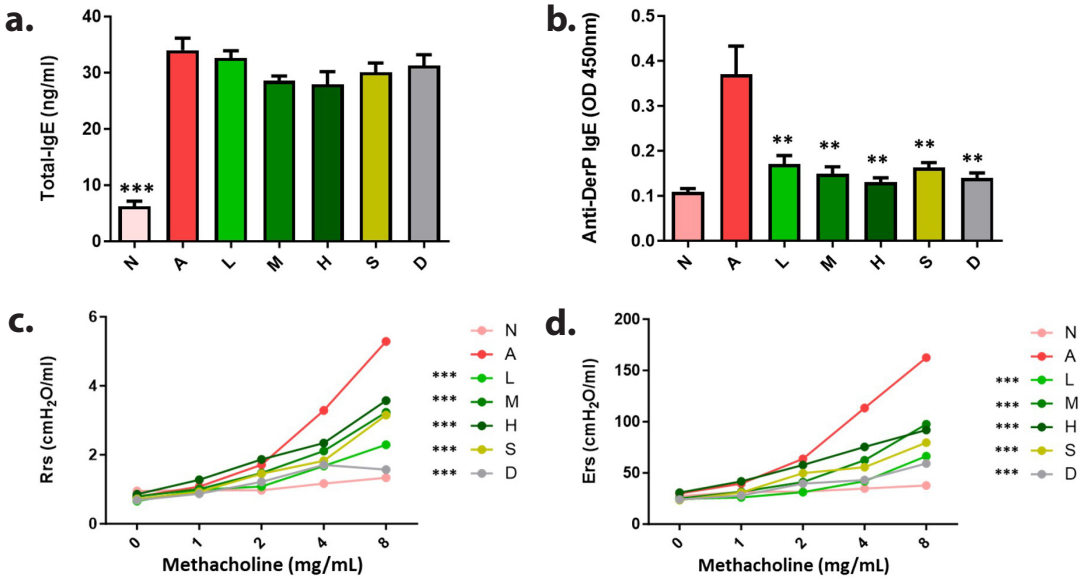



**Figure 2. Serum IgE levels and AHR in Der p-sensitized mice treated with different doses of LDL557 and dexamethasone.** **a** Total and **b** Der p-specific IgE serum concentrations were measured using ELISA. **c** Rrs and **d** Ers for increasing dosages of aerosolized methacholine were measured using the flexiVent FX system. Asterisk (*) indicates a significant difference compared to group A (n = 12. $^{**}p < 0.01$, $^{***}p < 0.001$).

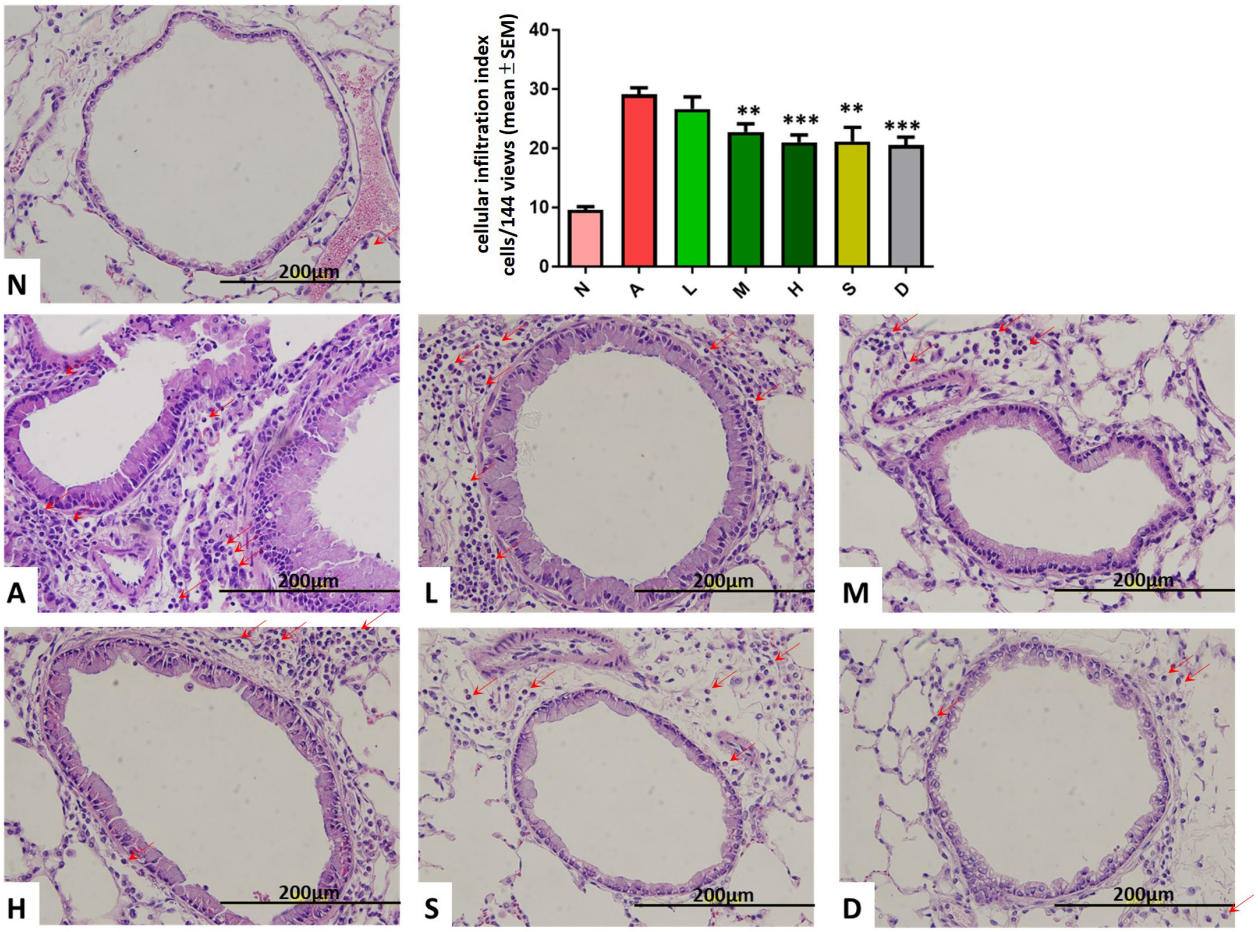



**Figure 3. Histopathological examination of representative lungs from mice treated with different doses of LDL557 and dexamethasone.**

Mice were sacrificed 2 days after the last Der p challenge. Lung sections were prepared and stained with hematoxylin and eosin (scale bar = 200 μm). Infiltrated immune cells (red arrows) were counted in ×400 magnification microscopic fields per group, and the average numbers were calculated. Asterisk (*) indicates a significant difference compared to group A (n = 12. $^{**}p < 0.01$, $^{***}p < 0.001$).

***Effects of LDL557 on the gut microbiota***

To assess the impact of LDL557 on the gut microbiota, the V3-V4 region of 16S ribosomal RNA genes in the DNA extracted from mice fecal samples were sequenced and analyzed. Levels of α-diversity were estimated using the Richness and Shannon indices. Richness ranged from 39.7 to 58.7, and there were no significant differences between group A and the other groups. The Shannon index ranged from 1.34 to 1.60, and there were no significant differences among the five groups (**Figure 4a**). To evaluate the differences in microbial community structures in each group, β-diversity was determined using NMDS ordinations of unweighted and weighted UniFrac distance matrices followed by PERMANOVA. There were no significant differences among groups using NMDS analysis of the unweighted UniFrac metric. However, the microbial profile of S group mice was distinctly different (PERMANOVA, $P = 0.040$) from that of group A after NMDS analysis of the weighted UniFrac metric (**Figure 4b**). In addition, after analysis of the unweighted UniFrac distance, bacterial communities of group A were significantly different from those of group N, L, S, and D (N vs. A, $P = 0.0003$; L vs. A, $P = 0.0002$; S vs. A, $P = 0.0264$; D vs. A, $P = 0.0008$). Moreover, a significant decrease in weighted UniFrac distance was observed in group S compared to that of group A (S vs. A, $P < 0.0001$) (**Figure 4c**).

Furthermore, LEfSe analysis were used to compare the microbiota between groups N and A. The cladogram showed the significant differences in taxonomic composition between groups N and A, with a hierarchy reflecting the taxonomic rank from phylum to genus. The taxonomy cladogram indicated that the relative abundances of *Monoglobaceae*, *Monoglobales*, *Monoglobus*, and *Turicibacter* were significantly higher in group N than in group A ($P < 0.05$) (**Figure 5a, b**). In contrast, the relative abundances of *Ruminococcus* and *Ruminococcaceae* were significantly higher in group A compared to group N ($P < 0.05$) (**Figure 5a, b**).

To further investigate the alterations in microbial composition, the relative abundances of major taxa at the phylum and family levels were analyzed (**Figure 6a, b**). At the phylum level, the relative abundances of *Actinobacteria* and *Deferribacterota* and the *Firmicutes/Bacteroidetes* ratio were slightly higher in group A and remained unchanged in groups L and S compared to group N (**Figure 6c**).

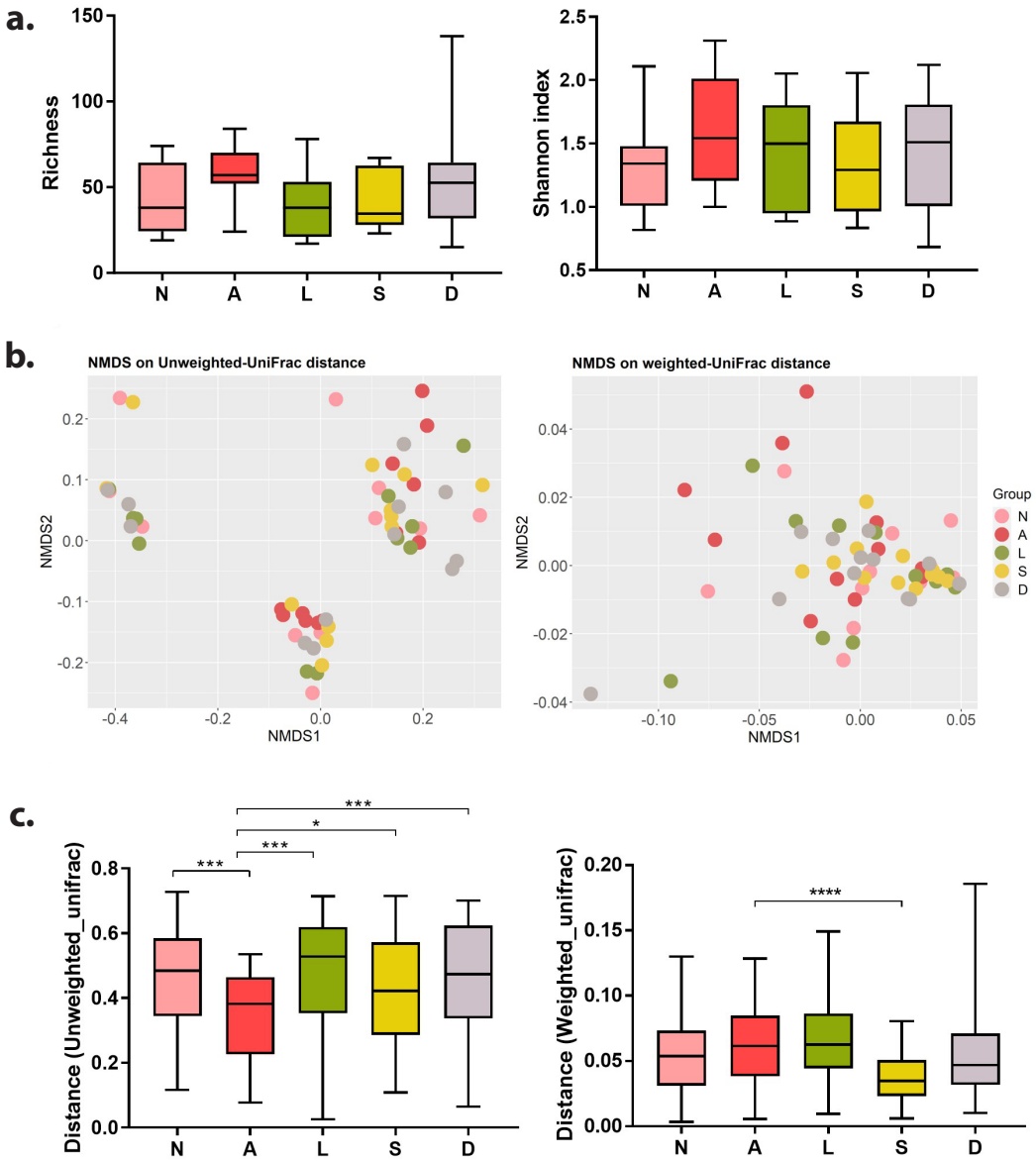



**Figure 4. Alpha and beta-diversity within the gut microbiota.**
**a** Box plots of richness and Shannon indices of alpha diversity. **b** NMDS plot of beta-diversity based on unweighted and weighted UniFrac distances. **c** Distances of beta-diversity based on unweighted and weighted UniFrac distances. Statistical differences were analyzed using the Kruskal–Wallis test with Dunn's post-hoc test. Asterisk (*) indicates a significant difference compared to group A (*$p < 0.05$, ***$p < 0.001$, ****$p < 0.0001$). NMDS plot data and statistical significance between the values of each group were analyzed using PERMANOVA.

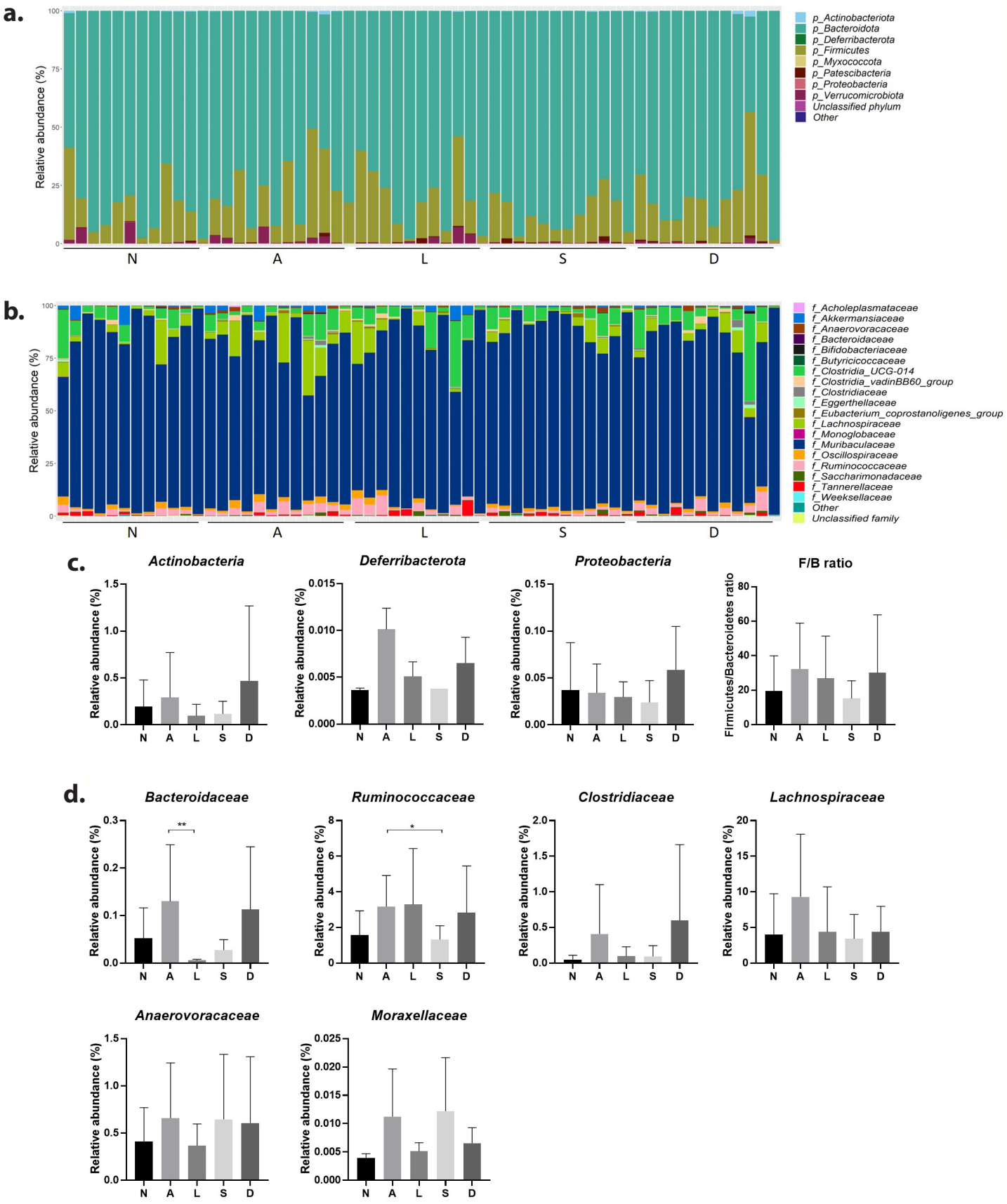



**Figure 5. LEfSe comparison of the gut microbiota.**
**a** Cladogram of different taxonomic compositions between the naive and asthma groups. **b** Bar graph of LDA scores showing taxa with significant differences between the two groups.

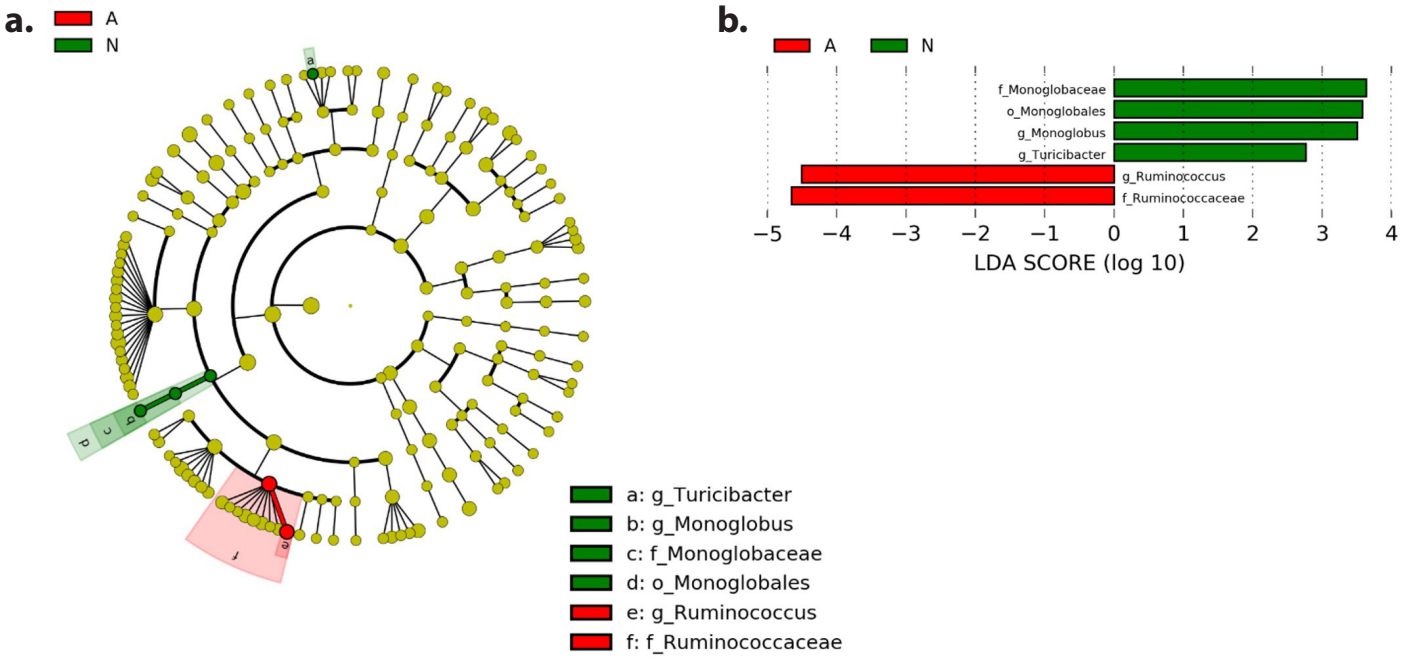



**Figure 6. Relative bacterial abundance at the phylum and family levels in the gut microbiota.**
Comparison of bacterial composition based on the **a** top 10 phyla and **b** top 20 families of bacteria on all samples. Relative abundance of specific taxa at the **c** phylum, **d** family levels was calculated. n = 12. Data are presented as mean ± SD. Statistical differences were analyzed using the Kruskal–Wallis test with Dunn's post-hoc test. Asterisk (*) indicates a significant difference compared to group A (*$p < 0.05$, **$p < 0.01$)

At the family level, the relative abundances of *Bacteroidaceae* and *Ruminococcaceae* were slightly increased in group A compared to group N. In contrast to group A, a decrease in *Bacteroidaceae* abundance was observed in groups L and S, especially in group L ($P = 0.0039$). In addition, the relative abundance of *Ruminococcaceae* was significantly decreased ($P = 0.0486$) in group S compared to group A. The relative abundances of *Clostridiaceae*, *Lachnospiraceae*, *Anaerovoracaceae*, and *Moraxellaceae* were slightly higher in group A than in group N. However, the relative abundances of *Clostridiaceae* and *Lachnospiraceae* were slightly decreased in groups L and S compared to group A (**Figure 6d**).

## Discussion

*L. delbrueckii*, an important industrial lactic acid bacteria, is a facultative anaerobic, non-motile, non-spore-forming, and rod-shaped gram-positive bacteria with a cell size range of 0.5-0.8 × 2.0-9.0 mm. Like other lactic acid bacteria, *L. delbrueckii* has acid resistance, cannot synthesize porphyrin, and has a strict fermentation metabolism with lactic acid as the main metabolic end product.[22] As a probiotic, it may help produce a better vaccination response and greater resistance to various infectious diseases.[23,24] *L. delbrueckii* can help treat gastrointestinal diseases such as inflammatory bowel disease and Crohn's disease.[25] One study evaluated whether adding *L. delbrueckii* to the diet can enhance the immune response of older individuals, which suggested that *L. delbrueckii* may help preserve an appropriate immune response mainly by slowing down the aging of T cell subsets and increasing the number of immature T cells that may respond to new antigens.[26] Moreover, animal studies suggested that *L. delbrueckii* may improve a variety of mental health conditions such as attention deficit hyperactivity disorder and depression.[27] In the present study, we employed i.p., i.n., and i.t. routes for Der p exposure in our mouse model, as detailed in our prior publication.[28] The i.p. route was utilized to induce systemic sensitization, leading to the production of IgE. Through i.n. exposure to Der p, we aimed to mimic localized, chronic environmental allergen exposure, specifically promoting the production of allergen-specific IgE in the mucosal environment. Finally, the i.t. challenge strategically involved introducing a substantial amount of allergen directly into the airways. This approach was designed to induce airway constriction and, consequently, foster the development of allergic asthma in the mice. In this model, we showed that orally administration of *L. delbrueckii* LDL557 attenuated allergen-induced airway inflammation and modulated the gut microbiota sampling from intestinal feces. Our findings may help explain the beneficial effects of probiotic supplements in the prevention of allergic asthma.

Live LDL557 at different doses ($10^7$-$10^9$ CFU) and dead LDL557 ($10^9$ heat-killed cells) reduced the Rrs and Ers measurements after stimulation with methacholine and the effects were similar to those of dexamethasone treatment. Der p-specific, but not total, IgE titers decreased after LDL557 and dexamethasone treatments of sensitized mice. Dead or inactive probiotics, mainly lactic acid bacteria, still prevent or treat various diseases.[29] In the present study, the effects on Der p-induced allergic asthma were observed after both heat-killed and live LDL557 treatments.

Inactive (including heat-killed) probiotics have the advantages of safety, physiological effects, and pharmaceutical characteristics compared to live probiotics.[30] Heat-killed or inactivated probiotics, such as heat-killed *Lactobacillus* species, have potential health benefits, including immune-modulating and anti-inflammatory effects. These effects result from the release of bacterial cell components, such as cell wall fragments, that activate immune cells and stimulate the production of cytokines. For example, heat-killed lactobacilli modulate immune responses, induce IL-12 secretion, and enhance innate immunity.[31] A comparison of heat-killed lactobacilli showed that *L. paracasei* had the highest ability to induce IL-12 secretion compared to other lactobacilli such as *L. reuteri*, *L. casei*, and *L. plantarum*.[31] Heat-killed probiotics may be more effective than live probiotics in certain situations, such as in the treatment of certain types of diarrheas or in preventing the development of allergies in infants. This is thought to be because heat-killed probiotics are more stable and can survive the harsh conditions of the digestive tract, allowing them to reach the target site in the gut intact. Testing for changes in IL-4, IL-13, or IFN-γ through ELISA in the study did not yield statistically significant results (data not shown). These findings suggest that the impact of *L. delbrueckii* on asthma may not be mediated through T cell immunity. In a prior investigation, we suggested that allergic reactions progress through the innate immune pathway, thereby impacting adaptive immunity.[32] Moreover, heat-killed *L. rhamnosus* strain OLL2838 protects against mucosal barrier permeability defects in mice with induced colitis, indicating that heat-killed bacteria help preserve gut barrier integrity.[33] Our study revealed that heat-killed LDL557 has the potential to alter the composition of the gut microbiota because the statistical difference was observed in both unweighted and weighted UniFrac distances (**Figure 4c**).

Compared to group A, both live and heat-killed LDL557 decreased the abundance of *Bacteroidaceae* (**Figure 6d**). In addition, the relative abundance of *Ruminococcaceae* was significantly decreased in the heat-killed LDL557 administered group than in the asthma group (**Figure 6d**). Lower abundance of *Ruminococcaceae* in the gut microbiota is found in polycystic ovarian syndrome[34] and nonalcoholic fatty liver disease.[35] Low abundance of *Ruminococcaceae* at baseline in the gut correlates with the risk of antibiotic-associated diarrhea.[36] However, it is important to note that the effectiveness of heat-killed probiotics may depend on the specific strain and the condition being treated. The current study, while providing valuable insights, has inherent limitations. A more comprehensive understanding of the probiotic properties and potential benefits of heat-killed LDL557 can only be attained through rigorous exploration in human clinical settings. Therefore, future research endeavors should aim to address these limitations and contribute to a more robust evaluation of the probiotic potential of heat-killed LDL557.

## Conclusions

In this study, LDL557 showed benefits as a probiotic supplement in the prevention of Der p allergen-induced airway inflammation in experimental asthma. Der p sensitization and challenge not only induced airway inflammation but also significantly changed the gut microbiota. Both live and heat-killed LDL557 significantly attenuated allergen-induced airway inflammation, but no significant change in the gut microbiota in intestinal feces was induced by LDL557 treatment, although the tendency to alter the gut microbiota was greater for heat-killed than for live LDL557.

## Conflict of interests

The authors have declared that no competing interest exists.

## Funding

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## Authors' contributions

- P.C.C and J.Y.W conceived and designed the study.
- P.C.C, H.Y.H, and M.H.H, performed the immunologic and mouse lung function experiments.
- Y.C.L, C.C.L, and L.S.H.W performed the 16S rRNA gene sequencing and analysis.
- W.S.K and L.S.H.W provided critical analysis and discussion.
- P.C.C, L.S.H.W, and J.Y.W wrote the manuscript.
- All authors approved the final manuscript for submission.

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