DOI: 10.26599/FSHW.2025.9250848

Contents lists available at SciOpen

Food Science and Human Wellness

journal homepage: https://www.sciopen.com/journal/2097-0765

China Food Publishing Co.

Bifidobacterium longum DPUB-15, screened by an *ex vivo* mesenteric lymph nodes cell model, alleviates allergic responses in casein-allergic Sprague-Dawley rats

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ABSTRACT: Cow's milk allergy is a global health issue, and probiotic-based treatments may offer a promising therapeutic approach. Mesenteric lymph nodes (MLNs) play a crucial role in the development of food allergies. The present study aims to establish an *ex vivo* model based on rat-derived MLN cells to screen probiotic strains with potential to alleviate casein allergy and to investigate the effects in the casein-allergic rat model. The results showed that *ex vivo* screening *via* normal and casein-sensitized SD rat MLN cells identified three strains, with *Bifidobacterium longum* DPUB-15 selected as target strain demonstrating optimal probiotic properties. Subsequently, the present study demonstrated that both live and heat-killed *Bifidobacterium longum* DPUB-15 could alleviate casein allergy in rats. Notably, heat-killed *Bifidobacterium longum* DPUB-15 demonstrated superior effects, further downregulating IgG2a and histamine levels, improving ileal immune balance and colonic barrier function (ZO-1), and modulating intestinal microbiota by reducing the abundance of casein allergy-associated Lachnospirales_CAG-274 and increasing the abundance of *Paraprevotella*. In conclusion, this study provides an *ex vivo* model for screening anti-allergic probiotic strains and highlights *Bifidobacterium longum* DPUB-15 as a promising candidate for alleviating casein allergy, with heat-killed *Bifidobacterium longum* DPUB-15 showing potential for postbiotic development in allergy treatment.

Keywords: Probiotics; Casein allergy; Mesenteric lymph nodes; Intestinal immunity; Intestinal microbiota

1. Introduction

Food allergy refers to the adverse effects that arise from a specific immune response following repeated exposure to particular foods or certain ingredients within foods [[1]]. The occurrence of food allergy is mainly affected by genetic predisposition and environmental exposures [[2]]. Cow's milk allergy (CMA) is one of the most prevalent type of food allergy, primarily caused by proteins found in milk [[3]]. IgE-mediated immediate hypersensitivity represents the most prevalent form of CMA, resulting from the overactivation of Th2 immune responses in response to allergens. Published studies indicate that approximately 60% patients with CMA

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Received 16 June 2025
Received in revised from 24 July 2025
Accepted 12 August 2025

experience IgE-mediated allergic reactions, which manifest with symptoms of varying severity across multiple systems, including skin redness and swelling, urticaria, sneezing, asthma, abdominal pain, and diarrhea [[4], [5]]. Currently, CMA has become a global health issue that warrants more extensive and in-depth research.

Studies have revealed significant differences in the structure of intestinal microbiota between allergic individuals and normal individuals indicating that a relatively healthy intestinal microbiota promotes the natural development of immune tolerance in the host [[6]-[8]]. Early studies on CMA provide detailed examples indicating that the gut microbiota of infants with CMA differ significantly from those of healthy infants [[9], [10]]. Furthermore, colonizing the intestines of germ-free (GF) mice with fecal bacteria from healthy, but not CMA, infants effectively prevented CMA allergic reactions [[11]]. At present, microbiota intervention methods, mainly probiotics and fecal bacterial transplantation, have become emerging measures to alleviate allergies [[12]]. Considering people's acceptance of cost-effectiveness and side effects, the prospects for ingesting probiotics are particularly noteworthy. Existing results indicate that the intake of single probiotics or mixed probiotic preparations can prevent or alleviate food allergy. The potential mechanism is mainly based on the regulation of the intestinal mucosal immune system by probiotics and their metabolites. For example, taking a formula supplemented with *Lacticaseibacillus rhamnosus* GG can promote the acquisition of immune tolerance in CMA infants by changing the composition of intestinal microbiota [[10]]. *Lactiplantibacillus plantarum* ZDY2013 increases the expression of tight junctions (TJs) in the Intestinal epithelial cell layer of the mouse colon, reduces serum IgE levels, and alleviates allergic symptoms induced by β-lactoglobulin (β-LG) [[13]]. *Clostridia* colonization in GF mice enhances intestinal barrier function and prevent CMA by inducing RORγt^+ ILCs and T cells in the intestinal lamina propria to produce IL-22 [[14]]. Oral administration of *Lacticaseibacillus casei* BL23 induces the production of $Foxp3^+$ RORγt^+ Treg subsets in mice and alleviate allergic symptoms [[15]]. *Lactiplantibacillus plantarum* CJLP133 and CJLP243 alleviate OVA-induced allergy by downregulating Th2 cytokines and improving mast cell infiltration [[16]].

The small intestine is the primary site where food allergies occur. Food allergens are taken up into the intestinal lamina propria by microfold cells covering Peyer's patches (PPs) located in the small intestine, they can also reach the mesenteric lymph nodes (MLNs) from PPs through their efferent lymphatic vessels leading to food allergic reactions [[17]]. In addition, allergens can also be absorbed by small intestinal villi and presented by dendritic cells (DCs) *via* lymphatic vessels to the MLNs, and this pathway has been proven to play a decisive role in oral immune tolerance [[18], [19]]. In fact, as the largest lymph node in the gut-associated lymphoid tissue (GALT), MLNs are also known as sentinel sites for intestinal immunosurveillance and maintenance of immune homeostasis due to their important contribution to the intestinal immune system [[20]]. At present, the *in vitro* model of screening probiotics capable of alleviating allergy from immunological perspective mainly include the splenocyte model, RBL-2H3, and KU812 cell models. However, due to the cell origin and the immune patterns reflected by those models, there are theoretical limitations to use.

As the major component (80%) of cow's milk protein, casein exhibits high sensitization rates (> 90%) among allergic individuals, superior heat stability compared to whey proteins, and its specific IgE levels predict clinical outcomes like baked milk tolerance and disease persistence [[21]]. Therefore, casein was selected as the allergenic protein for investigation. This study aims to use the MLNs-derived cells model as an *ex vivo* screening tool, based on the mechanisms underlying the development of food allergy in the intestine. An *ex vivo* co-culture system was established using MLNs-derived cells obtained from normal and casein-sensitized rats respectively. Probiotics that have the potential to alleviate casein allergy were screened, with relevant cytokines serving as evaluation indicators. Furthermore, a preliminary study was conducted to assess the effective strain form in alleviating allergy symptoms, and the allergy-relieving effects of the strain were validated and further explored in a rat model of casein allergy.

2. Materials and Methods

2.1 Chemical reagents and cell

Casein, L-cysteine and sterile ovine blood were purchased from Solarbio Science and Technology Co., Ltd. (Beijing, China). The rat IFN-γ, IL-4, IL-10, IL-12, IL-17A, total IgE, IgG1, IgG2a, ZO-1, Claudin-1, Occludin, secretory immunoglobulin A (sIgA) and histamine ELISA kit were purchased from Enova Biotechnology Co., Ltd (Wuhan, Hubei, China). The HEPES, Penicillin/Streptomycin, Fetal bovine serum and RPMI-1640 medium were purchased from Dalian Meilun Biotechnology Co., Ltd (Dalian, Liaoning, China). Freund's adjuvant, pepsin and trypsin were purchased from Sigma (Missouri, USA). The MRS, LB and Columbia medium were purchased from Qingdao Hi-Tech Industrial Park Hope Bio-Technology Co., Ltd (Qingdao, Shandong, China). The Wright-Giemsa Stain, 1,1-diphenyl-2-picryl-hydrazyl radical (DPPH), 2,2'-Azinobis-(3-ethylbenzthiazoline-6-sulphonate) (ABTS), bile salt, and the antibiotic sensitive tablets were purchased from Shanghai Yuanye Bio-Technology Co., Ltd. (Shanghai, China). IL-4 and IFN-γ antibodies used in immunohistochemistry (IHC) analysis were purchased from ProteinTech Group (Wuhan, Hubei, China). The HT-29 cell was purchased from National Collection Authenticated Cell Cultures (Shanghai, China).

2.2 Source and Culture of bacteria

The bacterial strains included in the present study were isolated from infant feces. The collection of infant fecal samples was approved by the Ethics Committee of Peking Union Medical College Hospital, Chinese Academy of Medical Sciences (Approval Number: I-23PJ1912), with written informed consent obtained from their parents or legal guardians. All samples were processed anonymously in strict accordance with the Declaration of Helsinki. The inclusion criteria for the infants mandated that they had no prior history of allergies, antibiotic use, or exposure to probiotic products, and that both parents also had no history of allergies. The bacteria selected for testing were deposited at the Dalian Key Laboratory of Functional Probiotic and Protein (Dalian, Liaoning, China). The purified strain is inoculated into MRS broth and cultured anaerobically for 3 generations at 37℃ for a duration of 18 h to ensure complete activation. The resulting

bacterial suspension can then be utilized for subsequent experiments. The activated bacterial solution was washed with phosphate-buffered saline (PBS) and resuspended to create a viable bacterial cell suspension. This suspension was subsequently heated and sterilized in a water bath at 75℃ for 30 min to prepare heat-killed bacterial cell suspension.

The other strains utilized in the study, including *Bifidobacterium animalis* subsp. *lactis* Bb12, *Listeria monocytogenes* ATCC19115, *Staphylococcus aureus* ATCC29213, *Escherichia coli* ATCC25922, and *Salmonella enterica* ATCC25928, are deposited at the Dalian Key Laboratory of Functional Probiotic and Protein as well.

2.3 Animal and establishment of the casein-sensitized rat model

This study was approved by the Dalian Polytechnic University Experimental Animal Ethics Committee (DLPU2024SP013). Five-week-old specific pathogen-free female Sprague-Dawley (SD) rats were utilized in the experiment and were procured from Liaoning Changsheng Biotechnology Co., Ltd (Benxi, Liaoning, China). At the time of purchase, the rats weighed between 140 and 150 g. The animals were maintained under controlled conditions: room temperature was set at (25 ± 2)℃, relative humidity at 45% ± 5%, and a light-dark cycle of 12 h each. The rats had ad libitum access to water and food.

The establishment of the casein-sensitized rat model was adapted with minor modifications from Song [[22]], as illustrated in Figure S1. During the first sensitization, 0.2 mg of casein, supplemented with 200 μL of complete Freund's adjuvant (cFA), was injected intraperitoneally. For the second sensitization, 0.1 mg of casein, along with 200 μL of incomplete Freund's adjuvant (iFA), was injected intraperitoneally. One week later, MLN cells were isolated for subsequent experiments.

2.4 Ex vivo screening of strains with potential to alleviate casein allergy through MLNs-derived cells model

This study references the method employed by Kim et al. for the screening of strains that significantly induce IL-10 in recent years, with modifications made to adapt the present protocol [[23]-[25]].

2.4.1 Isolation of MLNs-derived cells and preparation of cell suspension

SD rats (6-week-old rats after one week of adaptive feeding or casein-sensitized rats constructed as described above) were euthanized. The MLNs were then excised and placed in pre-cooled RPMI-1640 culture medium. The MLNs were ground, filtered through a cell sieve, washed, and subsequently resuspended in RPMI-1640 complete medium. The cell concentration was determined using a hemocytometer and adjusted to 2×10^6 cells/mL. HEPES and β-mercaptoethanol were added to achieve final concentrations of 10 mmol/L and 0.055 mmol/L, respectively. The mixture was thoroughly mixed to prepare the cell suspension, which was then added to the cell culture plate.

2.4.2 Optimization of casein stimulation conditions in ex vivo screening model of MLNs-derived cells

Add casein solutions of different concentrations (sterile PBS as solvent) to the cell culture plate and mix with the cell suspension until the final protein concentrations are 0, 50, 100, 200, 400, 600, and 800 μg/mL respectively (for normal rats-derived cell model). And for the casein-sensitized rat-derived cell model, the

final concentrations of casein were adjusted to 0, 50, 100, 150, 200, 250, and 300 μg/mL respectively. The culture plate was placed in a carbon dioxide incubator (SANYO, Japan) at 37℃ with a 5% CO_2 concentration and incubated for 72 h. Following the incubation, the culture was centrifuged at 845 ×*g* for 20 min (FA-45-24-11, Eppendorf 5424R) to collect the cell culture supernatant. The corresponding cytokines were then measured according to the instructions provided in the ELISA kit. In the normal rats-derived cell model, we measured the concentrations of IL-10 and IL-12 in the cell supernatant to identify the appropriate casein concentration for simulating the inflammatory environment based on the ratio of IL-10 to IL-12. In the casein-sensitized rat-derived cell model, we assessed the levels of IFN-γ and IL-4 in the cell supernatant, utilizing the ratio of IFN-γ to IL-4 to determine the optimal concentration for simulating an allergic environment.

2.4.3 Evaluation of the effects of different strain samples on MLNs-derived cells

As mentioned above, prepare MLNs-derived cells suspensions from normal and casein-sensitized rats respectively and transfer them into cell culture plates. Introduce a bacterial suspension until the cell-to-bacterial liquid concentration ratio reaches 1:10 (number of cells to bacterial CFU). Subsequently, add a casein solution and adjust the protein concentration to match the casein concentration established in the "2.4.2" experiment. The three-way co-culture system comprising cells, bacteria, and proteins was then placed in a carbon dioxide incubator set at 37℃ with 5% CO2 concentration and incubated for 72 h. Following incubation, the levels of IL-10, IL-12, IFN-γ, and IL-4 in the cell culture supernatant were measured.

2.5 In vitro gastric and intestinal fluid tolerance experiment

After activating the bacterial solution, it was washed twice with sterile saline, resuspended in sterile saline, and adjusted to a concentration of 1×10^9 CFU/mL. The solution was centrifuged at 2057 ×*g* for 5 min (FA-45-6-30, Eppendorf 5804R) and the bacterial pellet is resuspended in pre-filtered artificial gastric fluid (0.22 μm filter). The suspension is incubated anaerobically at 37℃ for 2 h. Bacterial suspensions at 0 h and 2 h are then plated for counting. Gastric fluid tolerance is calculated as:

Gastric fluid tolerance (%) = $(\log N_1) / (\log N_0) \times 100$

After 2 h in gastric fluid, 0.5 mL of the suspension is added to 4.5 mL of pre-filtered artificial intestinal fluid and incubated anaerobically for 8 h. Plate counting is done, and intestinal fluid tolerance is calculated as:

Intestinal fluid tolerance (%) = $(\log N_2) / (\log N_1) \times 100$

In the formula: N_2: the bacterial count after 8 h of incubation in intestinal fluid; N_1: the bacterial count after 2 h of incubation in gastric fluid; N_0: the bacterial count after 0 h of incubation in gastric fluid.

2.6 Auto-aggregation ability

The auto-aggregation ability of the strains was evaluated and calculated with reference to Kos et al [[26]]. The activated bacteria were washed and resuspended with sterile PBS, adjusting the viable bacterial count to approximately 10^9 CFU/mL. Subsequently, the bacterial suspensions were vortexed for 30 s and incubated

under static conditions at 37℃. Supernatants from each bacterial suspension were collected at 0, 2, 4, 8, 16, and 24 h, and their absorbance values at 600 nm were measured.

2.7 Adhesion to HT-29 cells

The method for assessing the adhesion ability of the bacterial strain to HT-29 cells was adapted from Jacobsen et al [[27]]. HT-29 cells were activated in RPMI-1640 complete medium, and the culture supernatant was discarded. The cells were washed 3 times with PBS, followed by digestion with trypsin. The cells were resuspended in RPMI-1640 complete medium, and the concentration was adjusted to 5×10^5 cells/mL. The cell suspension was then added to a 12-well cell culture plate pre-inserted with cell coverslips, and incubated for 6 h until the cells adhered to the surface. The supernatant was discarded. The bacteria in the logarithmic growth phase were resuspended in RPMI-1640 complete medium at 1×10^7 CFU/mL. Equal volumes of bacterial and cell suspension were co-incubated in a carbon dioxide incubator for 2 h. Non-adherent bacteria were removed with PBS washing, and cells were fixed in methanol for 10 min, followed by Wright-Giemsa staining. After washing with PBS and allowing the slides to dry, the cells were examined under a 100× oil immersion objective.

2.8 Antibacterial activity

The antibacterial activity of probiotics was assessed using the method described by Feng et al [[28]]. The cell-free supernatant (CFS) was obtained by filtering the probiotic supernatant through a 0.22 μm filter for subsequent use. The activated pathogenic bacterial suspension was diluted to achieve a viable count of 10^7 CFU/mL. A layer of water agar was pre-poured into the plates, with 3 Oxford cups placed in each. The prepared pathogenic bacterial dilution was inoculated at a 2% inoculation volume into LB solid medium cooled to approximately 50℃, mixed thoroughly, and then poured into the plates until solidified. Subsequently, 200 μL of the CFS from the test strain was added to each Oxford cup. After pre-diffusion in a chromatography refrigerator at 4℃ for 6 h, the plates were incubated at 37℃ for 24 h, and the diameter of the inhibition zone was measured.

2.9 In vitro antioxidant potential

Three sample forms were prepared for each strain[[29]]:

(1) CFS: Filter the activated bacterial culture through a 0.22 μm filter to obtain the supernatant, which is the CFS.

(2) Intact cells: Centrifuge the bacterial culture, wash the pellet three times with sterile PBS, and then resuspend it in sterile PBS to an OD600 of 1.2 to obtain the intact cells.

(3) Cell-free extract: Resuspend the bacterial pellet in sterile PBS to an OD600 of 1.2. Use an ultrasonic cell disruptor to sonicate the suspension in an ice-water bath (600 W, 15 min, 3 s on, 5 s off). Then centrifuge at 10,000 ×*g* for 10 min at 4℃ and collect the supernatant to obtain the cell-free extract.

The method for ABTS radical scavenging rate is as follows, to prepare the ABTS working solution, mix 7 mmol/L ABTS with 2.45 mmol/L potassium persulfate aqueous solution in a 1:1 (*v*/*v*) ratio. Allow the

mixture to stand at room temperature in the dark for 16 h. Subsequently, adjust the concentration with absolute ethanol to achieve an absorbance of 0.700 ± 0.005 at 734 nm. For the assay, take 0.5 mL of the sample to be tested and mix it with 3 mL of the prepared ABTS working solution. After allowing the mixture to stand at room temperature for 30 min, measure the absorbance of the sample at 734 nm. Use PBS as the control group. The ABTS radical scavenging rate can be calculated using the formula:

$$\text{ABTS radical scavenging rate (\%)} = (A_C - A) / A_C \times 100$$

where A represents the absorbance value of the sample and A_C represents the absorbance value of the control group.

The method for DPPH radical scavenging rate is as follows, mix the sample with a 0.2 mmol/L DPPH-anhydrous ethanol solution in a 1:1 (*v*/*v*) ratio, ensuring thorough and uniform mixing. Subsequently, place the mixture in a dark environment at room temperature for 30 min. After incubation, the mixture was centrifuged at 1503 ×*g* for 10 min (FA-45-24-11, Eppendorf 5424R) and was measured the absorbance at 517 nm. Use PBS as the control for the sample, and calculate the DPPH radical scavenging rate (%) using the following formula:

$$\text{DPPH radical scavenging rate (\%)} = (A_0 - A) / A_0 \times 100$$

where: A represents the absorbance value of the sample, and A_0 represents the absorbance value of the control group.

2.10 Establishment of casein allergy model

The casein allergy rat model was established with modifications based on the work of Feng et al[[30]], and the process is illustrated in Figure 3. A total of 42 SD rats were randomly assigned to six groups: blank control group (Control group), casein allergy group (Allergy group), live bacteria intervention group (A+DPUB-15 group), heat-killed bacteria intervention group (A+HK-15 group), live bacteria control group (DPUB-15 group), and heat-killed bacteria control group (HK-15 group). Initial sensitization was conducted on day 0, followed by booster immunizations on days 14, 21, and 28, culminating in an oral challenge on day 35. For the initial sensitization of the Allergy group, A+DPUB-15 group and A+HK-15 group, a casein solution supplemented with an equal volume of cFA was administered *via* intraperitoneal injection. During the booster immunizations, an iFA supplemented casein solution (with protein quantity halved) was used for intraperitoneal injection. In contrast, the remaining three groups received an equivalent volume of PBS in place of the casein solution. From day 0 to day 35, the four groups (A+DPUB-15 group, A+HK-15 group, DPUB-15 group, and HK-15 group) were treated with a bacterial solution three times a week, while the other two groups received an equal volume of sterile saline *via* intragastric administration. On day 35, following a 30-min oral challenge, allergic symptoms were assessed and body temperature was recorded. The rats were euthanized by cervical dislocation after being anesthetized with 4% isoflurane. Subsequently, serum, cecal contents, colon contents, jejunum, ileum, colon, spleen, and mesenteric lymph nodes were collected. All samples, except for histological analysis, were rapidly frozen in liquid nitrogen for over 20 min immediately after collection and stored at -80°C.

2.11 Allergy symptom scoring criteria

Allergic symptoms in rats were recorded and scored according to the following criteria: 0: Asymptomatic; 1: nasal/head scratching or rubbing; 2: hair bristling with reduced activity and tachypnea; 3: hunched posture with periorbital/perioral edema; 4: post-stimulation immobility with shivering and muscle contractions; 5: Death.

2.12 Determination of antibodies and histamine

The levels of IgE, IgG1, and IgG2a in rat serum, along with the level of histamine in rat plasma, were quantified following the protocols outlined in the ELISA kit.

2.13 Histopathological analysis

The jejunums were fixed in a 4% paraformaldehyde solution, routinely embedded in paraffin, and sliced to a thickness of 5 μm [[31]]. Following this, hematoxylin-eosin (H&E) staining was performed. The images of the sections were captured using an inverted fluorescence microscope (Leica Microsystems Wetzlar GmbH，Wetzlar，Germany) under standard optical conditions at a magnification of 200×.

2.14 Measurement of cytokines secreted by the spleen and MLNs

The rats were dissected in a clean bench and the spleen and MLNs were removed with forceps and placed in pre-cooled RPMI-1640 medium. Refer to "2.4.1" for the preparation of the cell suspension. A casein solution was added to achieve a final protein concentration of 100 μg/mL, with PBS used as a substitute in the Control, DPUB-15, and HK-15 groups. The cell culture plate was then placed in a carbon dioxide incubator and incubated at 37℃ with 5% CO_2 for 72 h. After incubation, the cell supernatant was collected. Following the instructions of the ELISA kit, the mixture was centrifuged at 845 ×*g* for 20 min (FA-45-24-11, Eppendorf 5424R), and the supernatant was collected for subsequent detection of cytokine levels, including IL-4, IL-10, IL-12, IL-17A, and IFN-γ.

2.15 Immunohistochemical analysis

The jejunum and ileum of rats were fixed, embedded in paraffin, and sections were sliced to a thickness of 4 μm, with the labeling procedure referenced in He et al [[32]]. The sections were photographed under an inverted fluorescence microscope with normal optical conditions at a magnification of 200×. Five fields of view were selected for each section, and quantification was performed using ImageJ 1.51K (NIH, USA).

2.16 Analysis of intestinal microbiota

The cecal contents of rats were rapidly frozen in liquid nitrogen and sent to Shanghai Personal Biotechnology Co., Ltd. for microbial community diversity and composition analysis. The analysis procedure is as follows: First, total bacterial DNA was extracted from the cecal contents, and the bacterial 16S rRNA V3-V4 region was amplified using the specific primers 338F (5’-ACTCCTACGGGAGGCAGCA-3’) and 806R (5’-GGACTACHVGGGTWTCTAAT-3’). The DNA fragments were then subjected to paired-end sequencing on the Illumina MiSeq platform. Sequences were processed using the DADA2 plugin in QIIME2

(2022.11) for primer removal, quality filtering, denoising, merging, and chimera removal to obtain Amplicon Sequence Variants (ASVs). Taxonomic classification of ASVs was performed using the Greengenes database.

The Chao1 index, Observed_species index, Shannon index, and Simpson index were calculated using QIIME2 to characterize the richness and diversity among groups. Nonmetric multidimensional scaling analysis, based on the Weighted UniFrac distance metrics, was conducted using R packages (v3.2.0) and QIIME2 to illustrate β-diversity. The composition at various taxonomic levels is represented using bar charts. Linear discriminant analysis effect size (LefSe) is employed to identify differential species among groups, which are visualized through bar charts of LDA value distribution for differential species and cladograms of classification. The LDA threshold is set to greater than 3.0.

The abundance values of each species, obtained from the sequencing results, were used as the 'Composition' label for a joint analysis with a heatmap of allergy indicators, visualized as a network heatmap. Additionally, Spearman correlation analysis was performed between the relative abundance values at the family, genus, and species levels and the allergy indicators, with the results visualized in the form of association heatmaps. All images were generated using the Personalbio Genescloud platform.

2.17 Statistical analysis

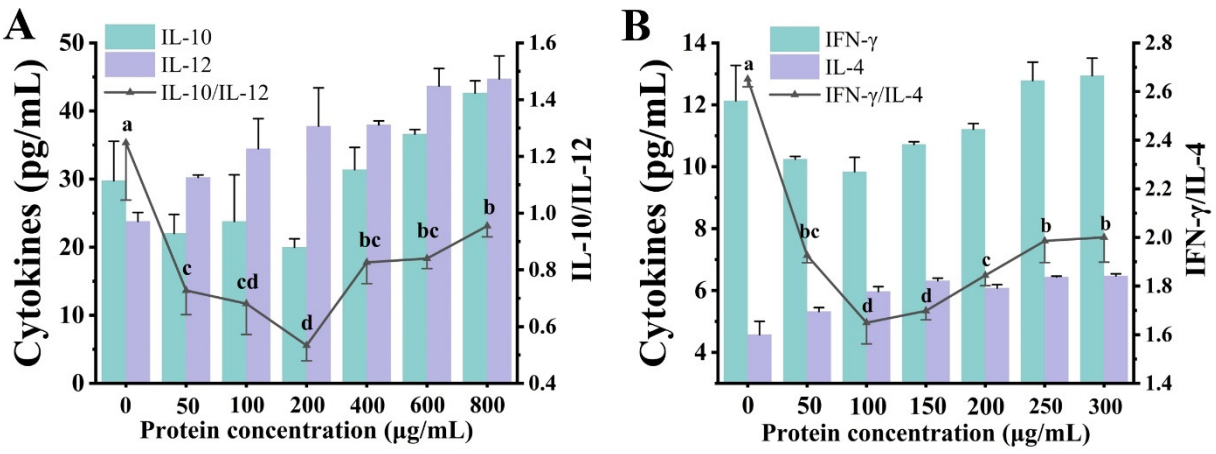
Data analysis was conducted using IBM SPSS Statistics 26 and GraphPad Prism 10. For comparisons among multiple groups, statistical analyses were performed using one-way analysis of variance (ANOVA). Distinct letters (a-d) denote statistically significant differences with P -values (P) < 0.05. For pairwise comparisons between groups, statistical analyses were conducted using t-tests. The symbols "*" or "#" indicate $P < 0.05$, "**" or "##" indicate $P < 0.01$, "***" indicates $P < 0.001$, and "****" indicates $P < 0.0001$. Identical letters or "ns" denote no significant difference. Results for "3.1" and "3.2" are presented as mean ± standard deviation, while the remaining of the Results are presented as mean ± standard error. Graph and figures were created using Origin 2019 and GraphPad Prism 10.

3. Results

3.1 Ex vivo screening of strains with the potential to alleviate casein allergy using a MLNs-derived cells model

In order to screen strains with the potential to alleviate casein allergy, MLNs-derived cells isolated from normal and casein-sensitized SD rats were respectively used to construct *ex vivo* screening models. We stimulated cells from normal rat MLNs *ex vivo* with casein to activate naïve T cells. The anti-inflammatory factor IL-10 and the pro-inflammatory factor IL-12 were utilized as indicators to identify the optimal cell concentration for simulating an inflammatory environment. As shown in Figure 1A, when the final concentration of casein in the system is set at 200 μg/mL, the ratio of IL-10 and IL-12 reaches its lowest value. These findings suggest that at this specific protein concentration, MLNs-derived cells are in an "optimal" inflammatory state. When the final concentration of casein was 100 μg/mL as shown in Figure 1B, the expression level of IFN-γ/IL-4 was lowest in the *ex vivo* culture system of MLN cells derived from casein-sensitized rats, indicating that the optimal allergic state was achieved at this concentration. The

bacterial strains were subsequently added to the *ex vivo* co-culture system. It was observed that, upon stimulation with 200 μg/mL casein, 30 strains of bacteria were able to elevate the ratio of IL-10 and IL-12 in the system to above 2.5 (where 2.5 represents twice the value of the PBS group). We thought of these 30 strains as possessing anti-inflammatory potential. After co-culturing the 30 strains of bacteria initially screened with sensitized cells and casein, the study identified three strains (DPUB-5, DPUB-15, DPUB-22) that significantly increased the expression of IFN-γ/IL-4. The expression levels were notably higher than those observed in the PBS group, which lacked added protein for *ex vivo* challenge ($P < 0.05$, Figure 1D). Given the potential variability in immune responses among different SD rats, this study aimed to assess the reproducibility of the allergy-relieving effects of three strains. To achieve this, MLNs were isolated from various sensitized batches, and repeated experiments were conducted. Due to significant differences in cytokine secretion levels among the rats, the ratio of IFN-γ to IL-4 is considered as a single value. In each individual rat experiment, the four experimental groups (casein group and three bacterial groups) were compared against the control group based on their IFN-γ/IL-4 ratios, which were plotted on the Y-axis. The results, illustrated in Figure 1E, indicate that the three bacterial strains significantly elevated the ratio of IFN-γ and IL-4 in sensitized rats, surpassing the levels observed in the protein stimulation group. Specifically, after casein stimulation, the IFN-γ/IL-4 ratio secreted by MLN cells was approximately 0.74 times that of the control group. In contrast, the three bacterial strains significantly enhanced the IFN-γ/IL-4 level in the co-culture system ($P < 0.05$), achieving values of 0.94 (*B. breve* DPUB-22), 1.00 (*B. longum* DPUB-15), and 1.09 (*B. pseudocatenulatum* DPUB-5) times that of the control group. Consequently, these three strains were initially selected as strains for alleviating casein allergy.



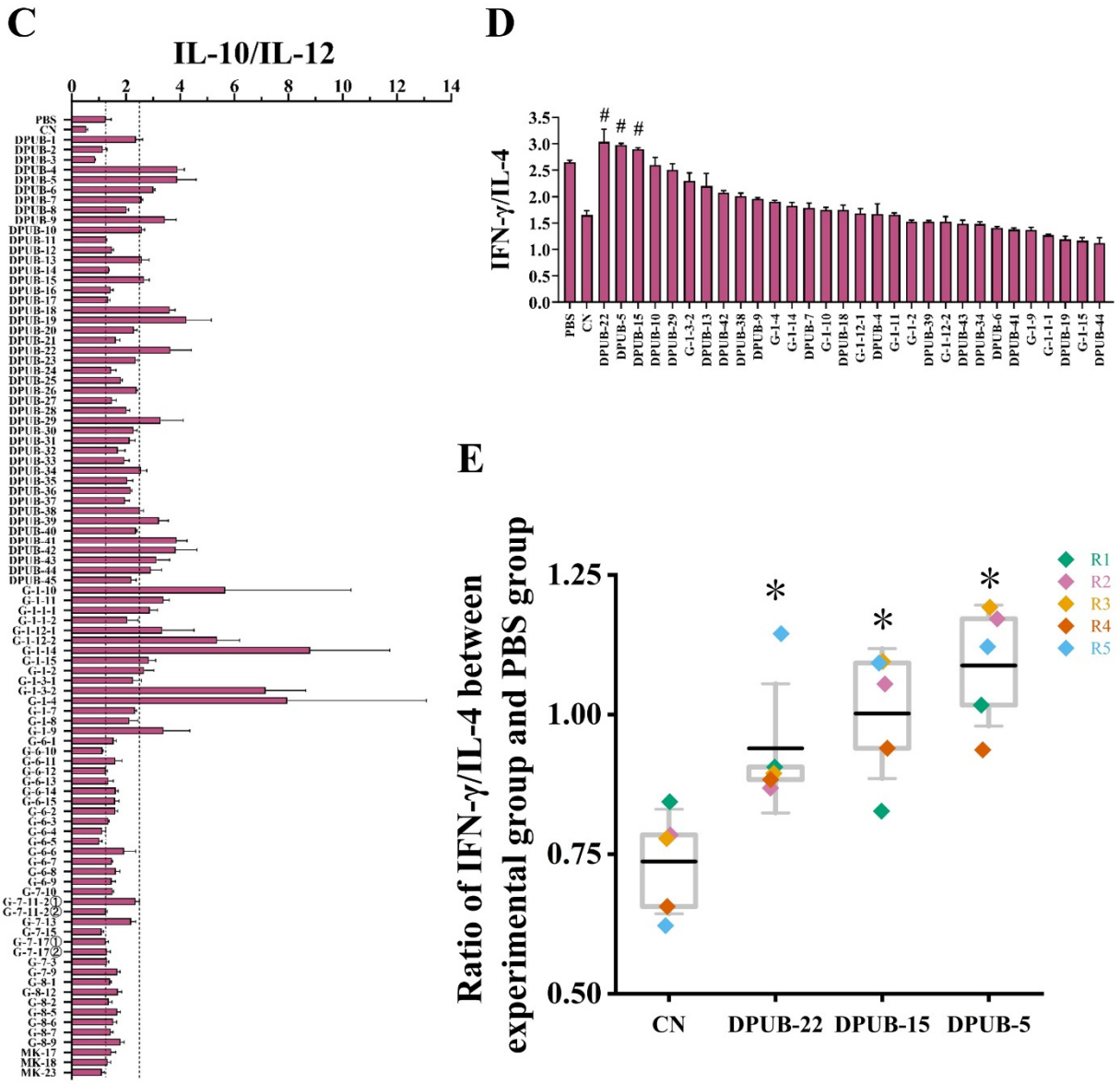


Figure 1 MLNs-derived cells model was constructed to *ex vivo* screen strains that alleviate casein allergy. (A) Inflammatory effects of casein at different concentrations on MLNs-derived cells from normal rats; (B) stimulating effect of casein at different concentrations on MLNs-derived cells from sensitized rats; (C) regulation of IL-10/IL-12 in MLNs-derived cells by different strains, the first vertical dashed line from the left indicates the IL-10/IL-12 ratio in the PBS control group, while the second vertical dashed line from the left represents a threshold value of 2.5 for screening bacterial strains with anti-inflammatory potential; (D) effect of potential anti-inflammatory strains on IFN-γ/IL-4 secretion on MLNs-derived cells from sensitized rats; (E) effects of 3 strains on IFN-γ/IL-4 in different MLNs co-culture system compared with PBS group. Different letters represent statistical significance between groups ($P < 0.05$). "#" indicates significant differences ($P < 0.05$) compared with the PBS group. "*" indicates significant differences ($P < 0.05$) compared with the CN group.

3.2 Selecting targeted strains through evaluating in vitro probiotic properties

For ingested probiotics, successfully reaching and colonizing in the intestine are essential for the probiotic effects of the strains. In this section, we evaluated three candidate strains initially selected for their potential to alleviate casein allergy through *in vitro* experiments assessing their probiotic properties. *Bifidobacterium animalis* subsp. *lactis* Bb12 (*B. lactis* Bb12) was used as a reference probiotic.

As shown in Figure 2, after sequential exposure to gastric and intestinal fluid, the reference strain *B. lactis* Bb12 demonstrated ideal tolerance with an overall survival rate of 69.34%. In contrast, *B. pseudocatenulatum* DPUB-5 exhibited poor tolerance to gastrointestinal fluids, with an overall survival rate of

only 9.50%, which was significantly lower than that of other strains ($P < 0.05$). Among three strains, *B. longum* DPUB-15 displayed higher tolerance to gastric fluid, with a survival rate of 55.09%, although this remained lower than that of *B. lactis* Bb12 ($P < 0.05$). Its tolerance to intestinal fluid was comparable to that of *B. lactis* Bb12, achieving an overall survival rate of 84.02%. The survival rate of *B. breve* DPUB-22 in gastric and intestinal fluid was slightly lower than that of *B. longum* DPUB-15, resulting in an overall survival rate of 29.34%.

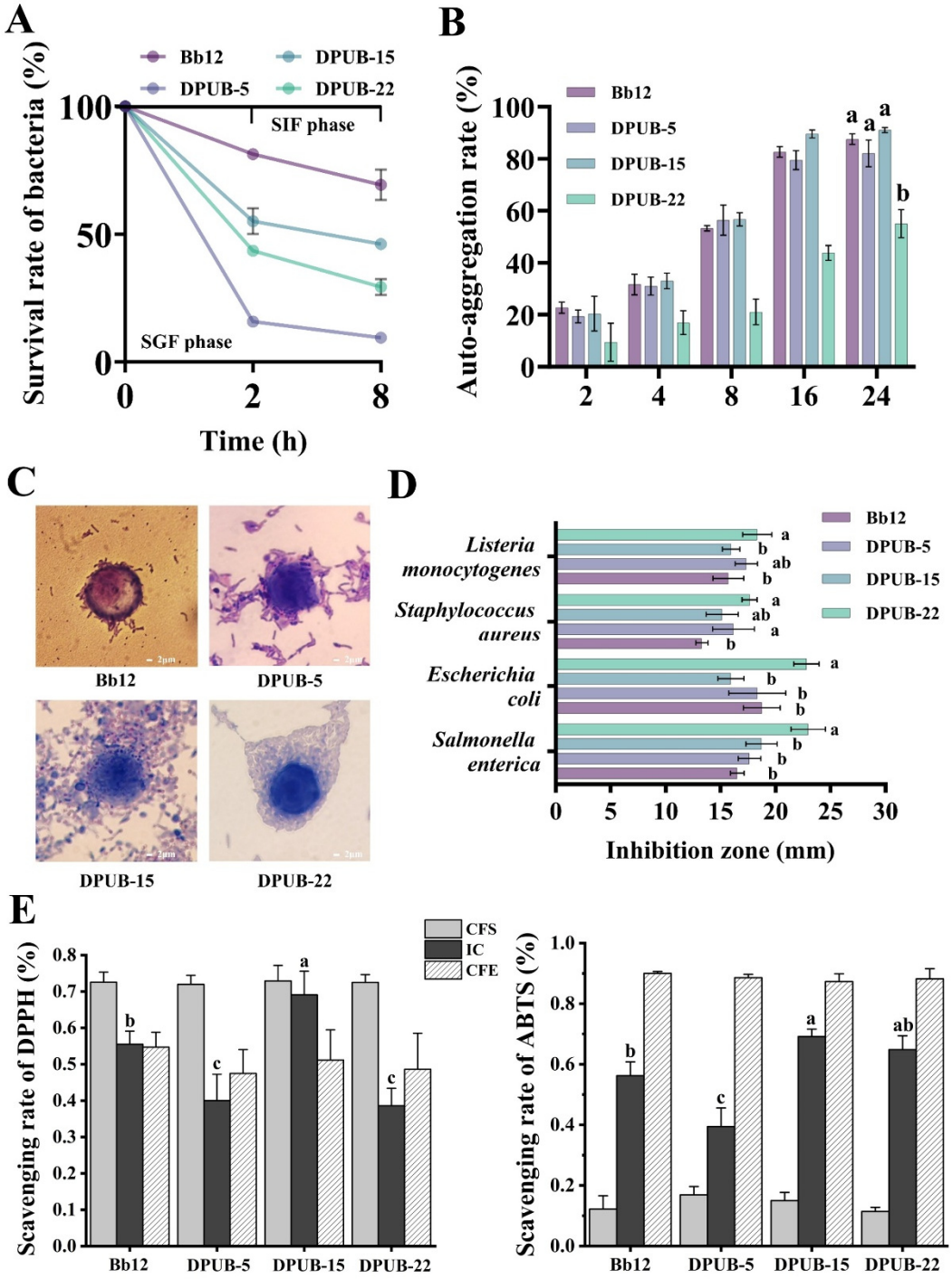


Figure 2 Evaluation of *in vitro* probiotic properties of candidate strains. (A) Tolerance of strains to simulated gastric and intestinal fluid; (B) auto-aggregation ability of strains; (C) adhesion of strains to HT-29 cells; (D) inhibitory ability of strains on pathogenic bacteria; (E) strain's ability to scavenge free radicals. "CFS" represent "cell-free supernatant", "IC" represent "intact cells", "CFE" represent "cell-free extract". Different letters (a-c) represent statistical significance between groups ($P < 0.05$).

After digestion by gastrointestinal fluids, surviving probiotics reached the intestines. The ability to adhere to the intestinal lining is a crucial factor in determining whether probiotics can establish long-term colonization. Consequently, we next evaluated the adhesion potential of three strains through HT-29 cell adhesion and bacterial auto-aggregation experiments. Similar to *B. lactis* Bb12, *B. pseudocatenulatum* DPUB-5 and *B. longum* DPUB-15 exhibited well auto-aggregation capabilities, with their 24 h auto-aggregation rates reaching 82.06% and 91.03%, respectively (Figure 2B). Furthermore, all three strains intuitively demonstrate adhesion to HT-29 cells (Figure 2C), with adhesion levels exceeding 100 cells per cell. This indicates that these strains possess robust adhesion potential to intestinal epithelial cells.

In vitro testing of the common functional properties of probiotics showed that the fermentation supernatant of *B. breve* DPUB-22 demonstrated antimicrobial activity against Gram-positive foodborne pathogenic bacteria, as well as Gram-negative foodborne pathogenic bacteria (Figure 2D). Regarding antioxidant function, the strains were evaluated based on their ability to scavenge free radicals. Among the three sample forms of the four *Bifidobacterium* strains, only the intact cells (IC) exhibited significant differences among the strains. Notably, *B. longum* DPUB-15 displayed superior scavenging abilities for DPPH and ABTS free radicals compared to the other two strains, achieving scavenging rates of 69.08% and 69.15%, respectively, which surpassed the free radical scavenging ability of *B. lactis* Bb12 ($P < 0.05$, Figure 2E). These findings indicate that *B. longum* DPUB-15 possesses good antioxidant potential *in vitro*.

In summary, we conclude that *B. longum* DPUB-15 demonstrated superior *in vitro* probiotic properties compared to the other two candidates. Consequently, this study identifies *B. longum* DPUB-15 as a promising probiotic strain capable of alleviating casein allergy.

3.3 B. longum DPUB-15 alleviates allergic symptoms in casein-allergic rats

Considering that *Bifidobacterium* is an anaerobic bacterium that rapidly loses viability in the cell culture environment, we speculate that the substance in *B. longum* DPUB-15 regulating cytokines may be its bacterial structure. Consequently, we evaluated the *in vivo* alleviation effects of both live and heat-killed forms of *B. longum* DPUB-15 on casein allergic rats through animal experiments as shown in Figure 3A. The results indicated that a significant number of rats in the casein allergy group (Allergy) exhibited symptoms, including facial swelling and frizzy hair. Allergy symptom scores were significantly higher in Allergy group compared to the Control group ($P < 0.0001$). The scores of rats gavaged with live bacteria (A+DPUB-15) and those gavaged with heat-killed bacteria (A+HK-15) were significantly lower than those of the Allergy group ($P < 0.001$). Furthermore, the weight gain over the course of 5 weeks and the change in body temperature following the oral challenge in the Allergy group were significantly lower than those observed in the Control group. Both live and heat-killed *B. longum* DPUB-15 partially alleviate the phenomenon (Figure 3C, D). Morphological analysis of the jejunum revealed that rats in the Allergy group exhibited damage to the submucosal muscle layer, disordered structure of the small intestinal villi, and cell shedding. Following intervention with either live or heat-killed *B. longum* DPUB-15, the morphological changes associated with

the jejunum were alleviated (Figure 3E). Briefly, the results in this section demonstrate that live or heat-killed *B. longum* DPUB-15 can reduce symptoms caused by casein allergy.

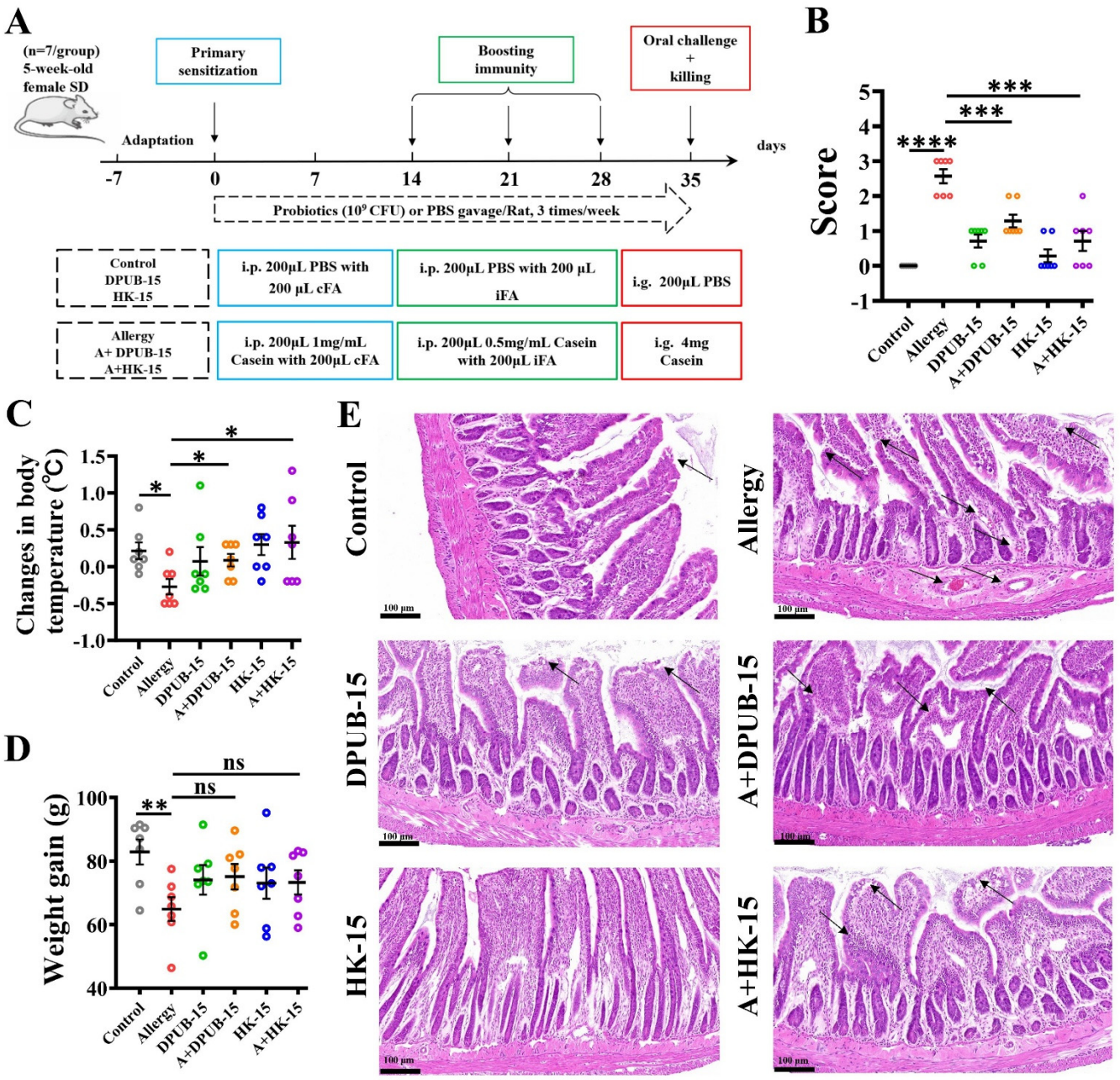


Figure 3 *B. longum* DPUB-15 alleviates allergic symptoms in casein-allergic rats (n=7). (A) Establishment of casein allergy model; (B) allergy symptom scores; (C) changes in body temperature before and after oral challenge; (D) monitoring of weight gain; (E) morphological changes of jejunum tissue in rats, the arrow pointing to the lower right indicates inflammatory cell infiltration and hemorrhage, accompanied by structural disruption in the mucosal muscle layer within the Allergy group, the arrow pointing to the upper left highlights villous structural damage. "ns" indicates no significant differences; "*" indicates significant differences ($P < 0.05$) compared with the Allergy group; "**" indicates $P < 0.01$; "***" indicates $P < 0.001$; "****" indicates $P < 0.0001$.

3.4 B. longum DPUB-15 regulates humoral and cellular immune responses in casein-allergic rats

Food protein-induced allergic reactions typically involve IgE-mediated humoral immune responses accompanied by Th1/Th2 imbalance. Therefore, we measured the levels of antibodies in rat serum using ELISA. The results indicated that the serum levels of IgE, IgG1, and IgG2a in the rats from the Allergy group

were significantly higher than those in the Control group. However, the antibodies levels of rats in the A+HK-15 group were significantly lower than those in the Allergy group. In the A+DPUB-15 group, the serum levels of IgE and IgG1 antibodies were reduced, whereas the level of IgG2a did not show a significant difference when compared to the Allergy group (Figure 4A). Furthermore, there was no significant difference in the levels of the three antibodies among the DPUB-15 group, the HK-15 group, and the Control group. These results suggest that intervention with either live or heat-killed *B. longum* DPUB-15 can modulate the humoral immune response induced by casein sensitization.

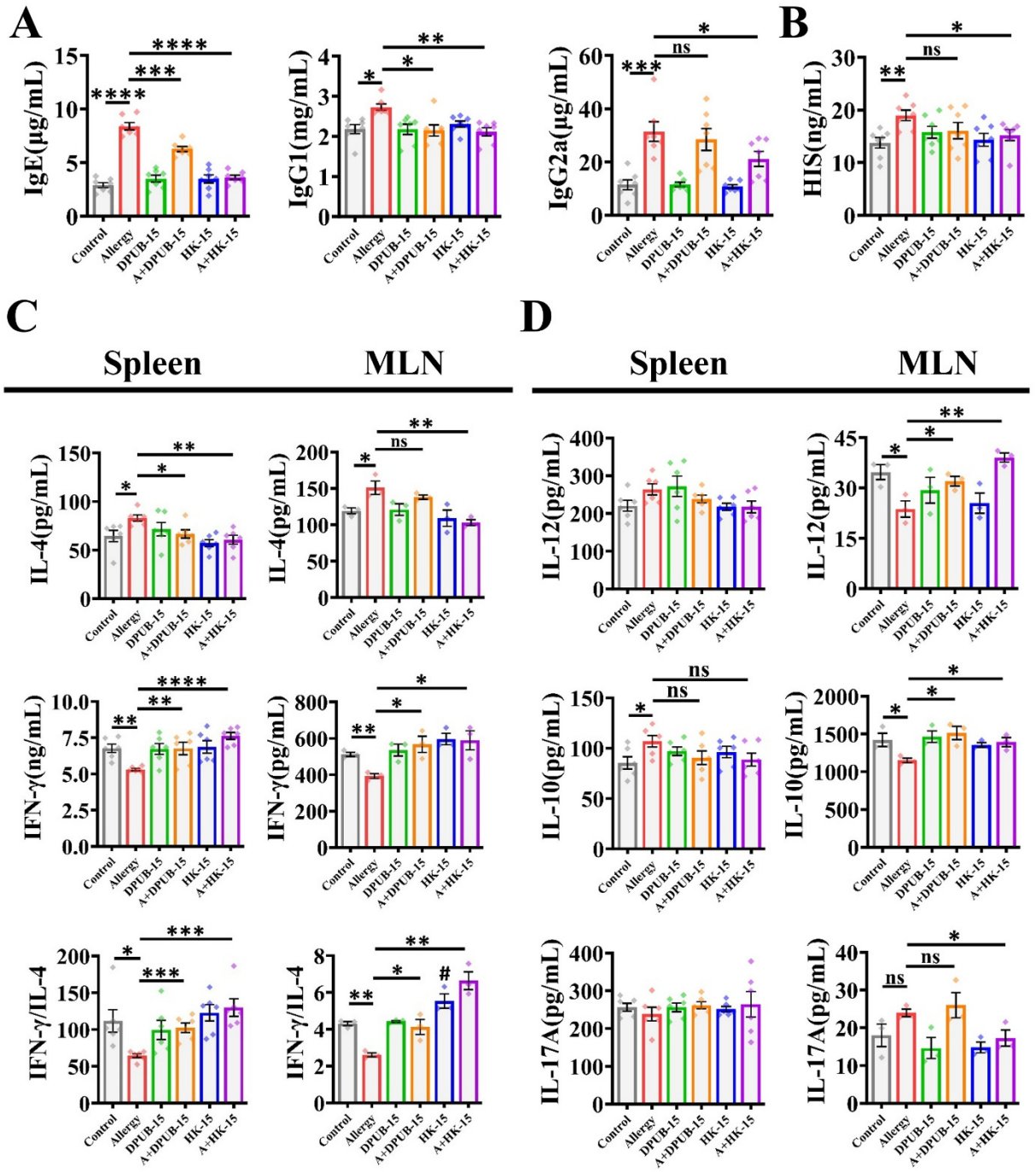


Figure 4 *B. longum* DPUB-15 regulates humoral and cellular immune responses in casein-allergic rats. (A) Serum antibody levels in rats (n=7); (B) plasma histamine level in rats (n=7); (C) levels of Th1 and Th2 cytokines secreted by spleen (n=6) and MLNs (n=3) in rats; (D) levels of allergy-induced inflammatory cytokines secreted by spleen (n=6) and MLNs (n=3) in rats. "ns" indicates no significant differences; "*" indicates significant differences ($P < 0.05$) compared with the Allergy group; "**" indicates $P < 0.01$; "***" indicates $P < 0.001$; "****" indicates $P < 0.0001$; "#" indicates significant differences ($P < 0.05$) compared with the Control group.

Histamine serves as the primary inflammatory mediator released during mast cell degranulation and is directly responsible for the clinical symptoms associated with allergies. The results indicated that the intragastric administration of heat-killed *B. longum* DPUB-15 could effectively regulate histamine levels in rat plasma (Figure 4B), but the live *B. longum* DPUB-15 administered showed no significant effect.

Both the spleen and the intestine serve as crucial peripheral immune organs in the body. In the previous study, we utilized the MLNs cell co-culture model to screen *B. longum* DPUB-15, a bacterial strain with the potential to alleviate casein allergy. In order to assess the impact of *B. longum* DPUB-15 on cellular immunity, the levels of Th1, Th2, Treg and Th17 cytokines in both the supernatants of MLNs and spleen were measured by ELISA; and the relevant transcription factors (T-bet, GATA3, Foxp3, and RORγt) in MLNs were determined by RT-qPCR. As shown in Figure 4C, the IFN-γ to IL-4 ratio in the Allergy group was significantly lower than that in the Control group, both in spleen and MLN cell culture supernatants. However, the IFN-γ to IL-4 ratios in the A+DPUB-15 and A+HK-15 group were significantly higher compared to the Allergy group. There was no significant difference in the levels of IFN-γ and IL-4 between the DPUB-15 group and the Control group. Notably, the study found that the ratio of IFN-γ to IL-4 secreted by MLNs in the HK-15 group was significantly higher than that in the Control group.

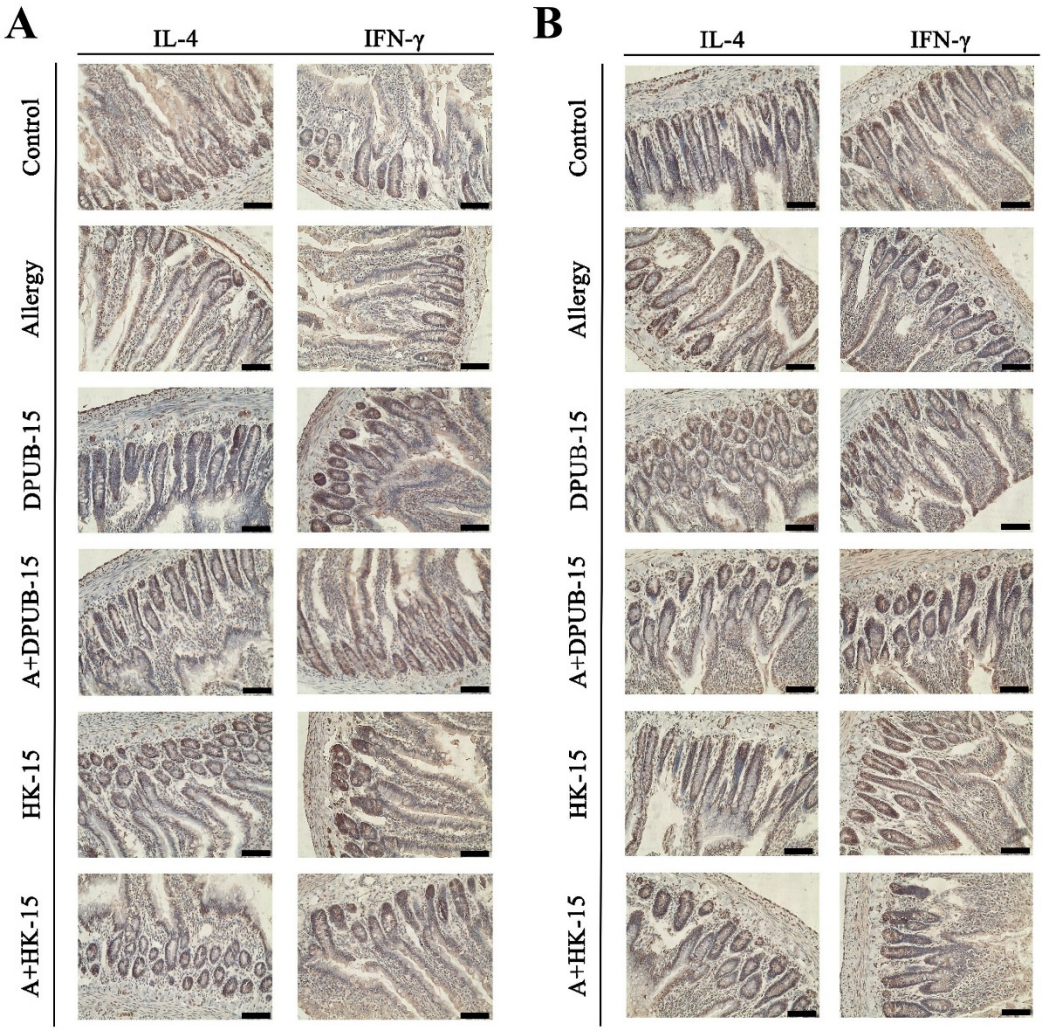
In the MLN cell culture supernatants, the IL-12 levels in the Allergy group were significantly lower than those in the Control group. The A+DPUB-15 and A+HK-15 groups exhibited significantly higher IL-12 levels compared to the Allergy group. No significant differences were observed among groups in the spleen cell culture supernatant. Regarding IL-10 content in the spleen, the Allergy group demonstrated significantly higher levels than the Control group; however, the A+DPUB-15 and A+HK-15 groups did not show significant differences when compared to either the Allergy or Control groups. In the MLN cell culture supernatants, the IL-10 levels in the Allergy group were significantly lower than those in the Control group, while the A+DPUB-15 and A+HK-15 groups showed significantly elevated levels compared to the Allergy group, which were different with the findings in the spleen. Regarding the levels of IL-17A in the MLNs, the Allergy group exhibited an upward trend compared to the Control group, although this difference was not statistically significant. There were no significant changes observed in the A+DPUB-15 group relative to the Allergy group; however, the A+HK-15 group demonstrated a significantly lower level than the Allergy group. Similar to Figure 4C, neither the DPUB-15 group nor the HK-15 group showed significant alterations in the levels of IL-12, IL-10, and IL-17A within the spleen and MLN cell culture supernatants (Figure 4D).

As shown in Figure S2, in the Allergy group, the relative expression levels of the characteristic transcription factors for Th1 and Treg cells, namely *Tbx21* and *Foxp3*, were significantly lower compared to the Control group ($P < 0.05$). In contrast, the relative expression level of the Th2 cells associated transcription factor *Gata3* was markedly higher in the Allergy group (Figure S2B, $P < 0.05$). The expression of the Th17 transcription factor *Rorc* showed a trend towards elevation, although this was not statistically significant ($P > 0.05$). Notably, in the A+DPUB-15 and A+HK-15 groups, the abnormal expression patterns of *Tbx21*, *Foxp3*, and *Gata3* were reversed when compared to the allergic group (Figure S2A-C). The relative expression of

Rorc in the A+HK-15 group was significantly reduced. Interestingly, the expression of *Foxp3* in the MLNs of rats in the DPUB-15 and HK-15 groups was significantly higher than in the Control group, suggesting that both live and heat-killed *B. longum* DPUB-15 might enhance Tregs differentiation in the MLNs. Overall, the expression patterns of key transcription factors for the four cell types in the MLNs closely correlate with the levels of corresponding cytokines secreted by the MLNs (Figure 4C, D).The above results indicate that the administration of either live or heat-killed *B. longum* DPUB-15 to casein-allergic rats can restore the immune balance between Th2 and Th1 cytokines in the body, as well as regulate the associated inflammatory factors in the intestinal immune.

3.5 B. longum DPUB-15 regulates the secretion of IFN-γ and IL-4 in the small intestine

To evaluate the secretion of Th1 and Th2 cytokines in the intestine, the level of the IFN-γ and IL-4 in the jejunum and ileum was measured by IHC. In comparison to the Control group, the Allergy group exhibited a increase in IL-4 levels and a decrease in IFN-γ levels in both the jejunum and ileum. The expression levels of IL-4 and IFN-γ in the jejunum and ileum of the A+HK-15 group were comparable to those of the Control group. In the A+DPUB-15 group, abnormal cytokine secretion was observed to be modulated exclusively in the jejunum (Figure 5). The results indicated that heat-killed *B. longum* DPUB-15 significantly modulated the intestinal allergic response induced by casein sensitization in rats, whereas viable *B. longum* DPUB-15 was limited to regulating the allergic condition specifically in the jejunum.



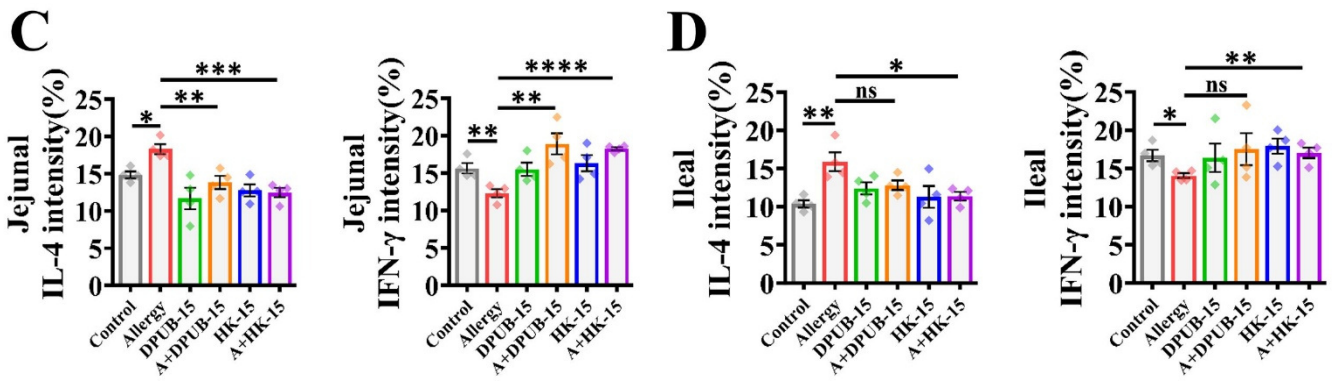


Figure 5 Immunohistochemical analysis of IL-4 and IFN-γ in the rats gut (n=4). (A) and (B) illustrate the immunohistochemical staining of IL-4 and IFN-γ in the jejunum and ileum, respectively; (C) and (D) provide a quantitative analysis of IL-4 and IFN-γ positivity in the jejunum and ileum. "ns" indicates no significant differences; "*" indicates significant differences ($P < 0.05$) compared with the Allergy group; "**" indicates $P < 0.01$; "***" indicates $P < 0.001$; "****" indicates $P < 0.0001$.

3.6 Effects of B. longum DPUB-15 on the intestinal barrier of casein-allergic rats

To evaluate the regulatory effect of *B. longum* DPUB-15 on the intestinal barrier of casein-allergic rats, the levels of 3 TJs (ZO-1, Claudin-1, and Occludin) as well as sIgA in the intestine was measured by ELISA. Compared to the Control group, the levels of ZO-1 in the jejunum and colon of rats in the Allergy group were significantly reduced. The ZO-1 contents in the jejunum of rats in the A+DPUB-15 group, as well as in the jejunum and colon of rats in the A+HK-15 group, was significantly higher than that observed in the Allergy group. Additionally, the ZO-1 content in the jejunum of rats in the HK-15 group exceeded that of the Allergy group, while the Control group exhibited the highest levels (Figure S3A, B). For both Claudin-1 and Occludin, no significant changes were noted in the jejunum of rats in the Allergy group (Figure S3C, E); however, a decrease in Claudin-1 was observed in the colon of rats in the Allergy group (Figure S3D). No significant changes in these two proteins were detected in the intestines of the other groups (Figure S3C-F). These results indicate that heat-killed *B. longum* DPUB-15 has the potential to enhance the expression of ZO-1 in the jejunum of rats, thereby contributing to the alleviation of allergies. In addition, the secretion of sIgA was reduced in the colon of casein-allergic rats, thereby affecting mucosal immunity. The administration of both live and heat-killed *B. longum* DPUB-15 to allergic mice resulted in a significant increase in sIgA levels (Figure S3G).

3.7 B. longum DPUB-15 modulates intestinal microbiota in casein-allergic rat

Various probiotic strains and preparations have been demonstrated to treat allergic diseases by modulating the composition of intestinal microbiota [[33]]. To investigate whether *B. longum* DPUB-15 can alleviate casein allergy through the regulation of intestinal microbiota, this study performed a comprehensive analysis of the intestinal microbiota across different groups.

3.7.1 B. longum DPUB-15 changes the richness and composition of intestinal microbiota in casein-allergic rats

Microbial α-diversity refers to the richness and diversity of various microbial species within a microecological system. The Chao1 index for rats in the Allergy group was significantly lower than that of the Control group, whereas the Chao1 index for the A+HK-15 group was higher than that of the Allergy group. Similarly, the number of observable species in each group exhibited a comparable trend. However, there were no significant differences in the Shannon and Simpson index among the groups (Figure 6A). These results indicate that heat-killed *B. longum* DPUB-15 may upregulate the intestinal microbiota richness in casein-allergic rats. The β-diversity results indicated significant differences in the bacterial community structure between the Allergy group and the Control group. However, no significant differences were observed among the other groups or between these groups and either the Allergy group or the Control group (Figure 6B).

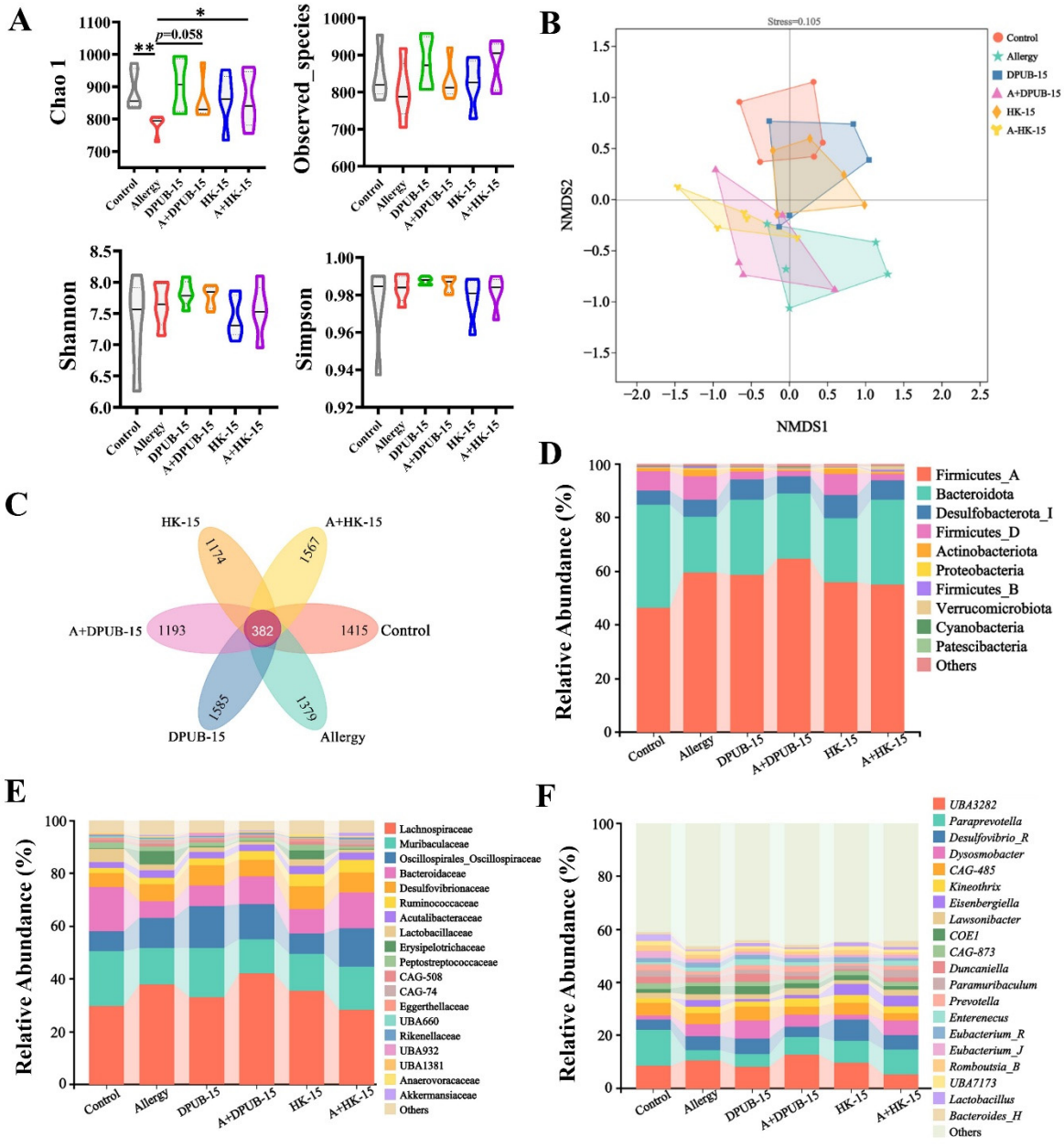


Figure 6 Impact of *B. longum* DPUB-15 on intestinal microbiota diversity and composition in casein allergy rats (n=5). (A) α-diversity, (B) β-diversity and (C) community analysis of intestinal microbiota in rats. Composition of intestinal microbiota at the (D) phylum, (E) family, (F) genus level. "*" indicates significant differences ($P < 0.05$) compared with the Allergy group; "**" indicates $P < 0.01$.

The relative abundance of intestinal microbiota at the phylum level is presented in Figure 6D. In the Control group, Firmicutes_A, Firmicutes_D (which represent Clostridia and Bacilli, respectively, in taxonomic annotation), and Bacteroidota were the dominant bacterial phyla. In the Allergy group, there was a increase in the abundance of Firmicutes, accompanied by a decrease in the abundance of Bacteroidetes. Compared to the Control group, the abundance of Bacteroidetes in both the DPUB-15 and HK-15 groups decreased, while the abundance of Firmicutes_D in the DPUB-15 group was significantly reduced. In comparison to the Allergy group, the abundance of Bacteroidetes in the A+HK-15 group was higher, and a notable decrease in the abundance of Firmicutes_D was observed in both the A+DPUB-15 and A+HK-15 groups.

At the family level (Figure 6E), the dominant bacterial families in the Control group included Lachnospiraceae, Muribaculaceae, Bacteroidaceae, Oscillospiraceae, Desulfovibrionaceae, and Lactobacillaceae. The study found that in the intestines of rats in the Allergy group, the abundance of Lachnospiraceae and Oscillospiraceae, which belong to the Firmicutes _A, significantly increased. Conversely, the abundance of Muribaculaceae and Bacteroidaceae, classified under Bacteroidota, significantly decreased. This trend aligns with the observed changes at the phylum level. Notably, the abundance of Oscillospiraceae in the DPUB-15 group was significantly higher than that in the Control group, while the abundance of Bacteroidaceae was lower. Additionally, the abundance of Bacteroidaceae in the HK-15 group was lower than that in the Control group, with minimal differences observed in the other bacterial families. In comparison to the Allergy group, the abundance of Bacteroidaceae in both the A+DPUB-15 group and the A+HK-15 group increased to varying degrees.

At the genus level (Figure 6F), the changes in the bacterial communities of each group are not much different from those of the corresponding family level. For instance, *UBA3282* from Lachnospiraceae, *Paraprevotella* from Bacteroidaceae, and *Dysosmobacter* from Oscillospiraceae exhibit patterns that are consistent with those of their respective higher taxa. The relative abundances at these levels are similar.

3.7.2 The alleviating effect of B. longum DPUB-15 on allergic rats is associated with alterations in the composition of intestinal microbiota

This part of the study focused on analyzing the differences in intestinal microbiota among the Allergy group, the Control group, the A+DPUB-15 group, and the A+HK-15 group. Consequently, the sample data from the DPUB-15 group and the HK-15 group were excluded from the analysis. LefSe analysis results (Figure 7A) showed that Bacteroidaceae, Haloplasmatales, Turicibacteraceae, *Turicibacter* and *Yaniella* were significantly enriched in the Control group. Erysipelotrichales, Erysipelotrichaceae, *Dubosiella*, Actinomycetia, Proteobacteria, Gammaproteobacteria, Burkholderiales and *CAG-603* (originating from Lachnospiraceae) were significantly enriched in the Allergy group. Firmicutes_A, Clostrdia, *Acetatifactor* and *Bilophila* were significantly enriched in the A+DPUB-15 group. Dysosmobacter and Acutalibacter were enriched in the A+HK-15 group.

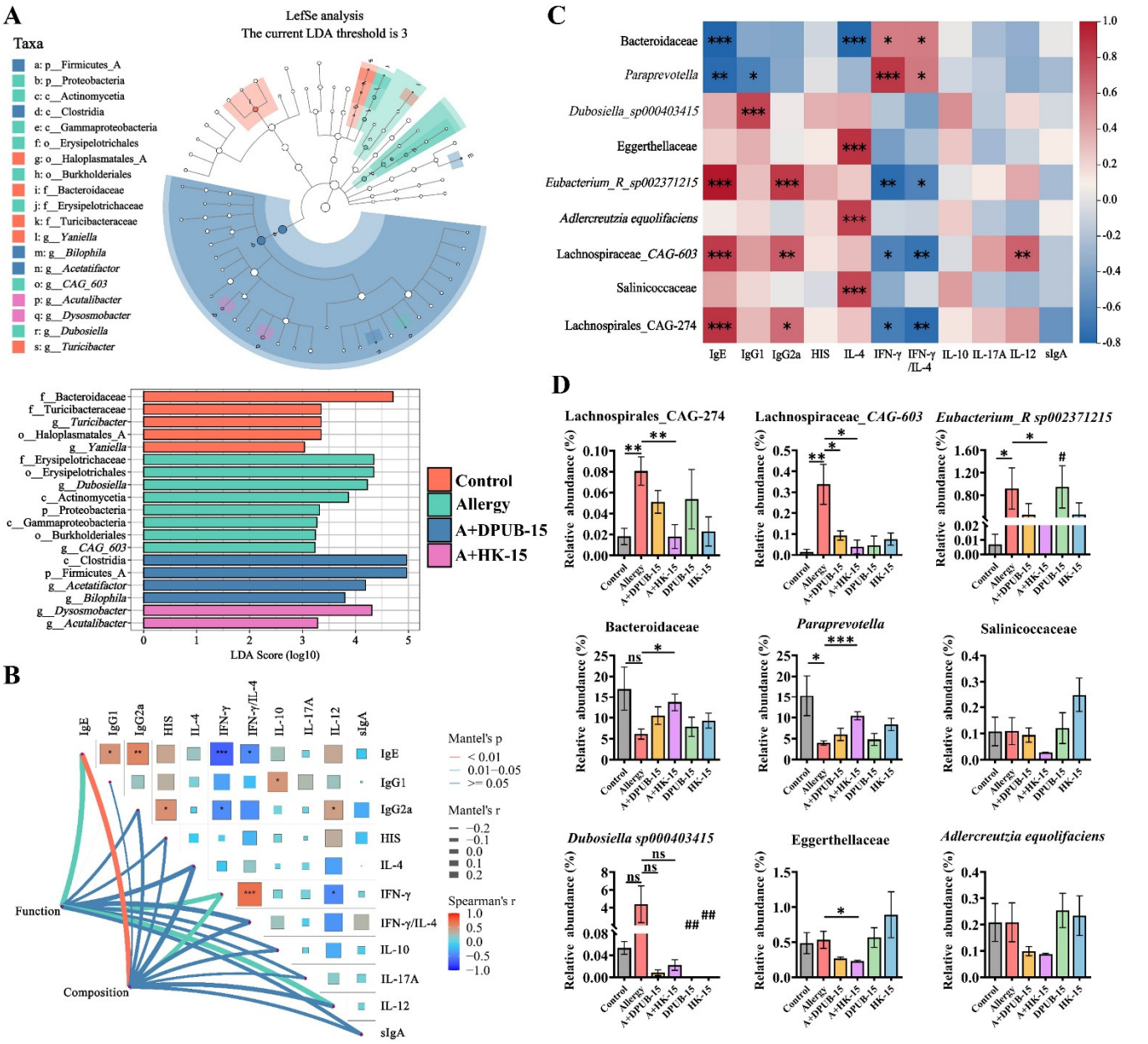


Figure 7 Association analysis between intestinal microbiota and allergy-related indicators across experimental groups in rats. (A) LefSe analysis (LDA > 3.0) in Control, Allergy, A+DPUB-15, and A+HK-15 Groups; (B) network heatmap analysis of the correlation between intestinal microbiota and allergic indicators; (C) correlation analysis between intestinal microbiota abundance and allergic indicators; (D) the relative abundance values of bacteria associated with allergic indicators in the rat intestinal microbiota. In (C), "*" indicate the significance level ($P < 0.05$) of correlations between individual microbial taxa and allergic indicators; **$P < 0.01$, and ***$P < 0.001$; italicized text represents microbial genus or species names, while non-italicized text denotes family names. In (D), "ns" indicates no significant differences; "*" indicates significant differences ($P < 0.05$) compared with the Allergy group; "**" indicates $P < 0.01$; "***" indicates $P < 0.001$; "#" indicates significant differences ($P < 0.05$) compared with the Control group; "##" indicates $P < 0.01$.

In order to analyze whether intestinal microbiota plays a role in the development of casein allergy, the study first visualized the correlation between allergy indicators and the composition or function prediction of intestinal microbiota using network heatmap. The composition of the microbiota was significantly positively correlated with serum IgE levels ($P < 0.01$) and also showed a significant positive correlation with the level of IFN-γ in the spleen ($P < 0.05$, Figure 7B). These findings suggest that alterations in the composition of the microbiota may be associated with the production of IgE and IFN-γ. Next, this study conducted Spearman

correlation analysis on the relative abundance values of microbiota at the family, genus, and species levels and allergy indicators, and visualized the data in the form of correlation heatmap. The results indicated that the abundance of Bacteroidaceae exhibited a negative correlation with serum IgE and IL-4 levels in the spleen, while demonstrating a positive correlation with the values of IFN-γ and the IFN-γ/IL-4 levels in the spleen. *Paraprevotella* from Bacteroidaceae showed a negative correlation with serum IgE and IgG1, alongside a positive correlation with IFN-γ and the IFN-γ/IL-4. Additionally, *Dubosiella_sp000403415* from Erysipelotrichaceae displayed a positive correlation with IgG1. The Eggerthellaceae, *Adlercreutzia equolifaciens*, and Salinicoccaceae were significantly positively correlated with IL-4 levels. Furthermore, *Eubacterium_R sp002371215*, Lachnospiraceae_*CAG-603*, and Lachnospirales_CAG-274 were significantly positively correlated with serum IgE and IgG2a, while exhibiting a negative correlation with IFN-γ and the IFN-γ/IL-4 levels.

Following the identification of microbes potentially associated with allergy indicators, this study analyzed the abundance of these microbes across each group to determine whether the intestinal microbiota was altered by the administration of *B. longum* DPUB-15 to alleviate casein allergy. In this study, both the DPUB-15 group and the HK-15 group were included in the analysis to assess whether the regulation of specific microbes through intragastric administration of live or heat-killed *B. longum* DPUB-15 exhibits common characteristics in allergic versus non-allergic individuals. The results (Figure 7D) show that the abundance of the CAG-274 family from Lachnospirales (Lachnospirales_CAG-274), the genus *CAG-603* from Lachnospiraceae (Lachnospiraceae_*CAG-603*), and the *Eubacterium_R sp002371215* is significantly higher in the Allergy group than the Control group, which is consistent with the conclusion of the heatmap. In the A+HK-15 group, the abundance of these three bacterial taxa was significantly lower than that observed in the Allergy group. It is worth noting that the abundance of *Eubacterium_R sp002371215* in the DPUB-15 group was significantly higher than that in the Control group. The abundance trends of Bacteroidaceae from Bacteroidota, as well as its subordinate *Paraprevotella*, are similar across each group. Notably, in the A+HK-15 group, the abundance of both taxa is significantly higher than in the Allergy group. Furthermore, the abundance of *Paraprevotella* in the Allergy group is significantly lower than that in the Control group. The study identified that the *Dubosiella_sp000403415* was enriched in the Allergy group, while its abundance was diminished in the A+DPUB-15 and A+HK-15 groups. However, it is important to note that these results were not statistically significant. Additionally, the relative abundance of this bacterium in both the DPUB-15 and HK-15 groups was recorded as “0”. For the relative abundance levels of Salinicoccaceae, Eggerthellaceae and *Adlercreutzia equolifaciens*, no meaningful changes were found among each group.

4. Discussion

Food allergies, including CMA, have emerged as a significant public health issue worldwide [[34]]. Taking probiotics may be a promising way to relieve allergies with low side effects. Probiotics regulate the interaction between intestinal immunity, intestinal microbiota, and the intestinal barrier through their own cells or their metabolites, using the intestinal mucosal immune system as the effector platform to alleviate food allergy [[35]].

Therefore, establishing an effective *ex vivo* model is crucial for screening allergy-relieving probiotics. Mesenteric lymph nodes (MLNs) function as "outposts" for food allergy and oral immune tolerance, cells derived from which may demonstrate intestinal immune patterns that more closely resemble actual allergic responses [[20], [36]]. In this study, we designed an *ex vivo* MLNs-derived cells model based on the development mechanism of food allergy, and a strain of *Bifidobacterium longum* was screened using this model. The allergy-relieving effects of this strain were subsequently validated in a rat model of casein allergy.

To screen strains with the potential to alleviate casein allergy, we sequentially isolated MLNs-derived cells from both normal rats and casein-sensitized rats. The primary approach involved measuring cytokine level changes induced by varying concentrations of casein, thereby establishing *ex vivo* environments that mimics inflammation and allergic responses, respectively. Naïve CD4+T cells in the lymph nodes of animals become activated after sensitization [[37]]. In order to simulate the sensitization and effect stages of milk allergy, casein was added for stimulation when culturing cells *ex vivo*. IFN-γ and IL-4 are the primary cytokines secreted by Th1 and Th2 cells, respectively. In the constructed *ex vivo* model designed to mimic allergic reactions, we assessed the immune response indirectly by measuring the levels of IFN-γ and IL-4, thereby reflecting the balance between Th1 and Th2 cells. Furthermore, the study revealed that when the final concentration of casein exceeded 100 μg/mL, there was a slight increase in the ratio of IFN-γ to IL-4. This phenomenon has been observed in other similar studies, suggesting that the elevated protein concentration may induce immune tolerance, leading to a termination of biased Th2 immune response [[28], [38]]. BALB/c mice are frequently employed as a model for food allergy research due to their propensity for Th2 immune responses and the production of elevated levels of IgE. However, the human immune system is more intricate, characterized by a greater number of interactions among immune cells and mediators, and may also involve alternative pathways, such as Th17 immune responses, which can lead to different clinical manifestations [[39]]. Furthermore, rats and humans share more similarities in their immune systems compared to mice, particularly regarding the complement system and the expression of major histocompatibility complex (MHC) molecules [[40]], thereby providing a more similar simulation of human immune responses. This study involved SD rats from various sensitized batches for *ex vivo* experiments and confirmed the reproducibility of 3 strains of *Bifidobacteria* in regulating the ratio of IFN-γ to IL-4. Probiotics are defined as live microorganisms that confer health benefits to the host when consumed in sufficient amounts. In this study, *Bifidobacterium longum* DPUB-15 was identified as a probiotic strain with the potential to alleviate casein allergy, due to its relatively better tolerance to gastrointestinal fluids and its adhesion capabilities, warranting further *in vivo* evaluation.

Postbiotics are defined by the International Scientific Association of Probiotics and Prebiotics (ISAPP) as “preparation of inanimate microorganisms and/or their components that confers a health benefit on the host’’, primarily including heat-killed microbial cells, extracellular vesicles, cell wall components and certain metabolites [[41]]. Postbiotics have garnered significant attention in recent years due to their beneficial properties. Unlike probiotics, however, postbiotics do not carry potential adverse effects commonly associated with probiotics, such as bacteremia or the transfer of antibiotic resistance genes. Several studies have explored

the potential of postbiotics derived from probiotics in alleviating food allergy. For instance, extracellular vesicles from *B. longum* KACC 91563 can specifically bind to and induce apoptosis in mast cells, thereby improving allergic diarrhea symptoms in OVA-induced mice [[42]]. Heat-killed *L. paracasei* JY56 has been shown to alleviate OVA-induced allergic reactions in mice through mechanisms such as inhibiting Th2 immune responses, repairing the intestinal barrier, and modulating gut microbiota composition [[43]]. Furthermore, inactivated *L. plantarum* DPUL-F232 can alleviate whey protein allergy by reshaping the gut microbiota and regulating intestinal metabolism [[30]]. In this study, the probiotic *B. longum* DPUB-15 and its derived postbiotic—heat-killed *Bifidobacterium longum* DPUB-15 both exhibited alleviating effects in a casein-allergic rat model.

In the sensitization phase of food allergy, food allergens induce the differentiation of naïve T cells into Th2 cells. Under the influence of Th2 cytokines, B cells are activated to produce allergen-specific IgE, which subsequently binds to mast cells. Upon re-exposure to the same allergen, it binds to specific IgE-FcεRI complexes on mast cells, triggering degranulation and the release of biologically active substances such as histamine [[1]]. Therefore, changes in the levels of IgE and histamine are crucial for evaluating food allergic reactions. Additionally, elevated levels of IgG1 are often observed in IgE-mediated allergic responses [[44], [45]]. Although IgG2a is generally associated with Th1 immune responses [[46]], some studies have also reported increased levels of IgG2a [[45], [47]]. Crucially, early research indicates that rat IgG2a can bind to antigens, forming antigen-antibody complexes that interact with the FcεRI receptor, thereby triggering histamine release [[48]]. Therefore, the lack of a significant decrease in histamine levels of the A+DPUB-15 group may be related to IgG2a levels. The above results indicate that the coordinated changes in the IgE-dominated antibody profile constitute a potential pathway regulating mast cell degranulation.

Cellular immunity plays a crucial role in food allergy progression by participating in key processes such as T cell differentiation and B cell activation. It works in concert with humoral immunity, primarily driven by IgE, to regulate the onset of allergic reactions [[49]]. The differentiation of naïve T cells is closely linked to the development of allergies, with an immune balance between Th1 cells, which predominantly produce IFN-γ, and Th2 cells, which mainly secrete IL-4. Additionally, Tregs that produce IL-10, as well as Th17 cells that produce IL-17A, also play crucial roles in the development of allergic responses [[35]]. This study demonstrates that both live and heat-killed *B. longum* DPUB-15 can reverse the Th1/Th2 immune imbalance in the spleen and MLN of casein-allergic rats. Furthermore, heat-killed *B. longum* DPUB-15 upregulated the IFN-γ/IL-4 ratio in the MLN of normal rats, suggesting its potential in preventing casein allergy. IL-12, primarily secreted by macrophages and DCs, is a cytokine that promotes the differentiation of naïve T cells into Th1 cells [[50]]. In non-allergic individuals, IL-12 is considered a pro-inflammatory factor. Previous studies in this research also utilized the IL-10/IL-12 ratio as a marker for screening anti-inflammatory strains. In allergic individuals, IL-12 is typically regarded as a Th1-type cytokine. In this study, both live and heat-killed *B. longum* DPUB-15 were able to correct the downregulation of IL-12, possibly promoting the differentiation of Th1 cells. Notably, changes in IL-12, IL-10, and IL-17A levels exhibited inconsistent patterns in the spleen and MLN, likely due

to their tissue specificity and distinct neuroimmune circuits [[51]]. The spleen lacks afferent lymphatic vessels, meaning it does not encounter antigens *via* lymphatic circulation but instead through blood circulation, receiving signals from other lymphatic tissues to participate in systemic immune responses [[52]]. The MLN is a critical site for adaptive immune responses to intestine-derived antigens and plays a central role in intestinal immunity [[53]]. Immune tolerance, mediated by Tregs and regulatory cytokines like IL-10, is one of the key mechanisms by which probiotics regulate intestinal immunity. Previous studies have shown that *Limosilactobacillus reuteri* enhances IL-10 secretion and $Foxp3^+$ Tregs expression in the MLN of OVA-sensitized mice, thereby inducing immune tolerance and alleviating allergies [[54]]. In this study, ELISA (Figure 4D) and RT-qPCR (Figure S2C) results suggest that both live and heat-killed *B. longum* DPUB-15 induce immune tolerance by promoting $Foxp3^+$ Tregs expression and IL-10 production, alleviating casein allergy. Regarding the pro-inflammatory cytokine IL-17A, the results from ELISA (Figure 4D) and RT-qPCR (Figure S2D) show that heat-killed *B. longum* DPUB-15 downregulates Th17 cells expression and IL-17A secretion. Additionally, immunohistochemical analysis of the jejunum and ileum (Figure 5) further supports the efficacy of both live and heat-killed *B. longum* DPUB-15 in alleviating casein allergy through modulation of gut immunity, with a stronger effect observed for the heat-killed form.

Food allergy-induced inflammatory responses can increase intestinal permeability, allowing allergens to penetrate the compromised intestinal mucosal barrier and trigger immune reactions [[55]]. The intestinal epithelial cells (IECs) layer is a crucial component of the mucosal barrier, with tight junctions (e.g., ZO-1, Claudin-1, and Occludin) effectively preventing antigens from crossing the IECs into the underlying lamina propria. ZO-1, a cytosolic scaffold protein, interacts with transmembrane proteins such as Claudin-1 and Occludin to maintain the structural integrity of tight junctions [[56]]. Certain probiotics can enhance intestinal barrier integrity by altering tight junction composition, such as increasing ZO-1 expression, thereby alleviating food allergy symptoms [[13],[57]]. Heat-killed *Lactiplantibacillus plantarum* L-137 can also upregulate the expression of ZO-1, thereby improving intestinal barrier function [[58]]. Secretory IgA, an antibody secreted by plasma cells into the intestinal mucosa, plays a vital role in maintaining mucosal integrity and in allergic defense [[59]]. Research on the role of heat-killed probiotics in modulating the intestinal barrier to improve allergies is limited. This study proactively demonstrates that heat-killed probiotics upregulate ZO-1 levels in the jejunum of both normal and allergic rats, enhance sIgA secretion, and alleviate casein allergy-induced intestinal barrier damage.

The intestinal microbiota is one of the most important components of the human microbiome. The hygiene hypothesis [[61]], the old friends hypothesis [[60]], and several cohort studies [[9], [10], [62], [63]]all indicate that the intestinal microbiota plays a critical role in the onset, progression, and tolerance of food allergies [[7]]. Previous studies have shown that the richness of the intestinal microbiota is often reduced in allergic individuals [[30], [43]], which is consistent with the findings of this study. Furthermore, gavage with heat-killed *B. longum* DPUB-15 can enhance the richness of the intestinal microbiota in rats. This study found that the abundance of Lachnospiraceae and Oscillospiraceae, which belong to the Clostridia class, was significantly

increased in the intestines of allergic rats, while the abundance of Muribaculaceae and Bacteroidaceae, which belong to the Bacteroidetes, was significantly reduced. These bacterial changes showed consistent trends at the phylum, family, and genus levels (Figure 6D-E). Clostridia is a common symbiotic bacterium in the mammalian gut, which plays an important role in maintaining intestinal homeostasis by producing immune-regulatory metabolites [[11]]. A cohort study of twin children showed that the abundance of Lachnospiraceae was significantly higher in the intestines of allergic infants compared to non-allergic infants [[62]]. Lachnospiraceae abundance was also found to be elevated in the intestines of mice sensitized to β-LG [[64]]. Another cohort study revealed that the abundance of Oscillospiraceae continued to increase during the progression of CMA in children [[10]]. Studies have shown that Muribaculaceae abundance is lower in the intestines of allergic mice [[65]]. Bacteroidaceae, also a common gut symbiont, has anti-inflammatory potential [[66]]. Clinical studies have demonstrated that for every quarter increase in the ratio of Enterobacteriaceae to Bacteroidaceae, the risk of allergy doubles [[67]]. Furthermore, this study identified some microbiota taxa potentially associated with casein allergy in rats (Figure 7C), and subsequently analyzed their relative abundances across the groups (Figure 7D). The results revealed that Lachnospirales_CAG-274, Lachnospiraceae_*CAG-603*, and *Eubacterium_R sp002371215*, which were positively correlated with rat serum IgE and IgG2a and negatively correlated with the IFN-γ/IL-4 secretion from MLNs, showed significantly increased abundance in the intestines of casein-allergic rats. Conversely, *Paraprevotella*, which was negatively correlated with serum IgE and positively correlated with the IFN-γ/IL-4 secretion, exhibited a significant decrease in abundance in the intestines of casein-allergic rats. Additionally, we found that the relative abundances of Lachnospirales_CAG-274, Lachnospiraceae_*CAG-603*, and *Eubacterium_R sp002371215* in the A+HK-15 group were significantly lower than those in the Allergy group, whereas *Paraprevotella* was significantly higher in the A+HK-15 group compared to the Allergy group. In the A+DPUB-15 group, however, the changes in these four bacterial taxa were not entirely significant. In line with the previous conclusions, we observed that IgE levels in the A+HK-15 group were closer to those of the Control group than those in the A+DPUB-15 group. The reduction in IgG2a by heat-killed DPUB-15 is likely mediated by its suppression of Lachnospirales_CAG-274, Lachnospiraceae_*CAG-603*, and *Eubacterium_R sp002371215*, which positively correlated with IgG2a. Crucially, lower IgG2a directly contributes to reduced histamine release [[48]]. Conversely, live DPUB-15 induced insufficient modulation of these key taxa, explaining its inability to significantly reduce IgG2a and consequently, histamine levels. This underscores the superior efficacy of heat-killed DPUB-15 in alleviating allergy via microbiota-driven suppression of IgG2a.Therefore, heat-killed DPUB-15 might alleviate casein allergy by modulating the intestinal microbiota composition. In both the DPUB-15 and HK-15 groups, with the exception of *Eubacterium_R sp002371215*, which showed a significant change in abundance compared to the Control group, the remaining three bacterial taxa did not exhibit significant differences in abundance relative to the Control group. Notably, this study preliminarily suggests that Lachnospirales_CAG-274, Lachnospiraceae_*CAG-603*, and *Paraprevotella* may serve as potential biomarkers for the casein allergy model.

5. Conclusion

In conclusion, this study established an *ex vivo* co-culture model to simulate intestinal inflammation and allergy environments by using MLN cells derived from normal and casein-sensitized rats. By combining this model with *in vitro* probiotic properties assessment, we identified a strain, *Bifidobacterium longum* DPUB-15, which demonstrates potential in alleviating casein allergy. The effectiveness of the selected model was subsequently confirmed through *in vivo* experiments. Furthermore, this study revealed that heat-killed *B. longum* DPUB-15 exhibited superior anti-allergic effects compared to live bacteria, operating through a multi-target synergistic mechanism (modulating intestinal immunity, enhancing intestinal barrier, and regulating intestinal microbiota). This suggests the potential of *B. longum* DPUB-15 as a functional postbiotic, providing a theoretical basis for the application of probiotic heat-killed preparations in allergy intervention. Future work may involve further analysis of the postbiotic components of *B. longum* DPUB-15 to identify the specific substances and mechanisms responsible for its alleviation of casein allergy.

Supporting Information

Detailed information about the information of strains in the study (Table S1), Sequences of primers (Table S2), Design of casein-sensitized rat model (Figure S1), Relative expression of allergy-related genes in mesenteric lymph nodes among different rats (Figure S2), Assessment of intestinal barrier among different rats (Figure S3).

Declaration of Interest Statement

The authors declare no competing financial interest.

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