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Original Article

Lactobacillus plantarum exopolysaccharides exert immunomodulatory function by regulating Th1 and Th2 responses in mice

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Abstract

Background: Our previous study demonstrated that exopolysaccharides (EPS) from *Lactobacillus plantarum* (*L. plantarum*) act as an effective adjuvant. They can enhance both the immune response elicited by the oprH recombinant subunit vaccine against *Pseudomonas aeruginosa* (*P. aeruginosa*) and the protective efficacy in experimental animals. However, it is not clear whether oral administration of *L. plantarum* EPS can promote immune function in experimental animals. **Aims:** The present study aimed to explore the immunoregulatory function of *L. plantarum* EPS in mice. **Methods:** EPS was extracted from *L. plantarum*. EPS was then orally administered to the mice at doses of 20, 40, and 60 mg/kg for 20 consecutive days. After 10 and 20 days, the immune organ index, delayed-type hypersensitivity (DTH), phagocytic ability of peritoneal macrophages, serum hemolysin, and splenic lymphocyte proliferation of mice were measured. The concentrations of IgG1, IgG2a, IFN-γ, IL-2, IL-4, and IL-10 in serum were measured using ELISA. The percentage of $CD4^+$ and $CD8^+$ T lymphocytes subpopulations and the expression of transcription factors T-bet and GATA-3 in the mouse spleen cells were measured by flow cytometry and qRT-PCR, respectively. **Results:** *L. plantarum* EPS could induce high levels of immune organ index, DTH reaction, phagocytic ability of peritoneal macrophages, serum hemolysin, splenic lymphocyte proliferation, serum IgG1, IgG2a, Th1-type cytokines IL-2, IFN-γ, Th2-type cytokines IL-4, IL-10, $CD4^+$ T lymphocyte, $CD8^+$ T lymphocyte, T cell transcription factors T-bet and GATA-3, and has a certain dose-dependency. **Conclusion:** *L. plantarum* EPS can concurrently upregulate both Th1 and Th2 immune responses in mice, with this effect being more pronounced, particularly at the high dose. Our study provides a theoretical basis for research on *L. plantarum* EPS in veterinary.

Key words: Exopolysaccharides, Immune indexes, Immunoregulatory function, *Lactobacillus plantarum*, Th1/Th2 immune response

Introduction

Lactobacillus plantarum is a gram-positive bacillus, that belongs to the lactic acid bacteria. It has many functions, such as inhibiting the growth of pathogenic bacteria, maintaining the balance of intestinal microbiota, promoting the absorption of nutrients, reducing the content of serum cholesterol, preventing cardiovascular disease, and so on (Naruszewicz *et al*., 2002; Zhang *et al*., 2021; Hu *et al*., 2022; Shang *et al*., 2024; Zhang *et al*., 2024). It is widely used in medicine, food, feed, and other fields. Therefore, it is one of the most essential probiotics. *L. plantarum* can produce a variety of metabolites during its growth, such as lactobacillin, indole-3-lactic acid, propionic acid, small molecular peptides, exopolysaccharides, and other bioactive substances (Maidana *et al*., 2021; Zhang *et al*., 2023). Secreted by *L. plantarum* during growth and metabolism, EPS is a crucial macromolecular compound with a unique structure and biological activity. It functions as both a stabilizer and a signaling molecule in biofilms (Abo-Saif and Sakr, 2020). Research has demonstrated that *L. plantarum* EPS enhances the texture and rheological properties of fermented dairy products, such as cheese and yogurt, and is widely used in the dairy industry as a tackifier, emulsifier, gelling agent, or stabilizer (Jiang and Yang, 2018; Wang *et al*., 2019). Furthermore, excellent functional properties of EPS, such as its stability, water retention, gelling, and emulsification capabilities, make it a promising candidate for applications in the cosmetics and pharmaceutical industries. Because of its good stability, water retention, gel, and emulsification, EPS also has potential application value in cosmetics and

pharmaceutical industries (Emamifar *et al*., 2011; Kowsalya *et al*., 2023). At the same time, it also has a variety of pharmacological effects, such as inhibiting bacterial biofilm, being anti-ulcer, reducing cholesterol, and so on (Pradeepa *et al*., 2016; Liu *et al*., 2017; Liu *et al*., 2024). In addition, microbial EPS also have essential uses in veterinary medicine as vaccine adjuvants, prebiotics, and pharmaceutical excipients. As adjuvants, they can enhance the immunogenicity of vaccines and promote the body to produce stronger and longer-lasting protection. As drug carriers, they can be used to realize slow-release and targeted delivery. Therefore, microbial EPS has been widely used to enhance vaccine efficacy, improve animal intestinal health and production performance, develop novel drug delivery systems, and enhance the overall immunity of animals, which have made significant contributions to the protection of animal health, the improvement of breeding efficiency, and the promotion of the sustainable development of the animal husbandry and veterinary industry. In our earlier work, we created an EP-rOprH vaccine of *P. aeruginosa* using EPS from *L. plantarum* as an adjuvant. Our animal studies demonstrated that the EPS of *L. plantarum* significantly enhanced the antibody response, cellular immunity, and protective efficacy conferred by the OprH vaccine (Tian *et al*., 2023). Furthermore, the immunomodulatory properties of *L. plantarum* EPS have been documented in several *in vitro* studies. For example, it has been shown to inhibit nitric oxide (NO), TNF-α, and IL-6 production in RAW264.7 macrophages (Huang *et al*., 2022; Huang *et al*., 2025). Another study showed that EPS from *L. plantarum* JCM1149 induced high secretion of IL-6, IL-10, and IL-12 in dendritic cells (DCs) (Kudo *et al*., 2023). While these findings highlight the immunomodulatory potential of EPS, its efficacy in enhancing animal immune function via oral administration has not been established. Based on this, we extracted EPS from *L. plantarum*. Using mice as experimental animals, the immunomodulatory function of *L. plantarum* EPS was evaluated by measuring a variety of immune indices. The primary objective of this study was to establish a foundation for future research on the use of *L. plantarum* EPS in immunoactive products.

Materials and Methods

Bacterial strain, reagents, and experimental animals

L. plantarum was preserved in the food microbiology laboratory of Henan University of Science and Technology. Concanavalin A (ConA), IFN-γ, IL-2, IL-4, IL-10, IgG1, and IgG2a mouse ELISA kits were purchased from Shanghai Sangon Biotech Co., Ltd. Anti-Mouse CD4 and CD8 antibodies were purchased from Cusabio Biotech Co., Ltd. BALB/c mice were obtained from the Experimental Animal Center of Henan University of Science and Technology.

Extraction of *L. plantarum* EPS

The EPS of *L. plantarum* was extracted following the method of Yue *et al*. (2025). In brief, *L. plantarum* was introduced into the de Man Rogosa Sharpe fermentation medium, and was incubated for 24 h at 37°C. Subsequently, 80% trichloroacetic acid (m/v) was added until the final concentration was 4%, followed by 2 h of agitation at 25°C. Then, the mixture was centrifuged at 4,000 ×g for 30 min. Following centrifugation, the upper phase was collected and combined with three volumes of absolute ethanol. This mixture was vortexed thoroughly and left overnight at 4°C. Next, the mixture was concentrated using a rotary evaporator operating at 50°C. Centrifugation of the concentrate was carried out at 10,000 ×g for 40 min. Post-centrifugation, the precipitate was harvested, dissolved in minimal ddH_2O, and subjected to dialysis for 48 h. After dialysis, the product was freeze-dried under vacuum. The yield of *L. plantarum* EPS was then determined colorimetrically and stored at -80°C for subsequent use.

Mice grouping and feeding

The mice were housed under controlled conditions of ambient light, temperature, and humidity. In this investigation, 120 healthy BALB/c mice (18-20 g) were employed. All mice had free access to drinking water and standard diet throughout the study. Animal health was observed daily throughout the study. A randomized grouping design was employed after the mice acclimated to their housing conditions. The cohorts included low-, middle-, and high-dose arms, plus an untreated control. The mice in the three treatment groups received daily oral gavage of *L. plantarum* EPS for 20 days at 20, 40, and 60 mg/kg body weight, respectively. Mice in the control group received 100 µL of sterile saline solution daily via oral gavage. A routine post-dosing observation was conducted to monitor the mice for immediate adverse effects. Mice exhibiting symptoms of depression, anorexia, or clinical evidence of disease were segregated and kept in a quiet feeding area until they recovered.

Detection of immune organs index

At the end of the 20-day gavage period, five mice from each group were randomly selected. These mice were then weighed and euthanized via cervical dislocation. After being removed under aseptic conditions, the spleen and thymus were dissected free of adherent fat and weighed. The immune organ index was assessed by dividing the fresh weight of the spleen or thymus (mg) by the body weight of the animal (g).

Detection of DTH reactions

At the terminal point of the 20-day gavage, a random subset of five mice per group was selected. The abdominal hair of these mice was shaved, and 50 µL of a 1% 2,4-dinitrofluorobenzene (DNFB) solution was applied to the shaved area. After five days, 10 µL of 1% DNFB solution was applied uniformly onto the right ear. Mice were sacrificed by cervical dislocation after 24 h. After the excision of the left and right ear pinnas, eight mm-diameter ear fragments were gathered using a hole

puncher and weighed. The degree of edema was represented by the difference in weight between the left and right ear pieces.

Phagocytic capacity of murine peritoneal macrophages

To assess phagocytic ability, a 0.5 ml suspension of 10% red blood cells (RBCs) was intraperitoneally administered to five randomly chosen mice per group, 2 h following the final gavage. Six hours post-injection, the mice were euthanized by cervical dislocation. Subsequently, 2 ml of normal saline was injected into the peritoneal cavity, and the abdomen was gently kneaded. Under aseptic conditions, the peritoneal fluid was collected, transferred to an aseptic glass slide, and incubated at 37°C for 30 min. Then, the slide was rinsed with normal saline and subjected to fixation using a 1:1 acetone: methanol solution after natural drying. Subsequent staining was carried out with 4% Giemsa phosphate buffer for 3 min, followed by a wash step with sterile water. Following natural drying, microscopic examination of the slide was performed using an oil immersion objective. The phagocytic index was derived from the total ingested chicken RBCs divided by the macrophage count. The phagocytic percentage was calculated by dividing the number of macrophages that had engulfed chicken RBCs by the total number of macrophages and multiplying the result by 100%.

Assessment of serum hemolysin in mice

The procedure began with the isolation of the guinea pig complement. Guinea pig complement was prepared by harvesting fresh blood from five animals and separating the serum. The serum (5 ml) was mixed with 1 ml of SRBCs and refrigerated at 4°C for 30 min. The resulting supernatant was collected and stored at -20°C for subsequent analysis. The half-hemolysis value (HC50) method was employed to quantify serum hemolysin levels. Following 20 days of oral administration, mice (n=5 per group) received an intraperitoneal injection of 0.2 ml of a 2% SRBC suspension. After a 5-day rearing period, the mice were euthanized by cervical dislocation. Blood was collected, and the serum was separated and diluted 200-fold with sterile normal saline. For the hemolysis assay, 1 ml of the diluted serum was combined with 0.5 ml of 10% SRBC and 1 ml of a 10-fold diluted guinea pig complements in a test tube. A serum-free control tube was prepared in parallel. The mixtures were incubated at 37°C for 30 min, and the reaction was immediately terminated in an ice bath. The supernatant was then collected and reacted with 3 ml of Drabkin's reagent. To generate the half-hemolytic reference, 0.25 ml of 10% SRBC was added to 4 ml of Drabkin's reagent; this mixture was designated as the half-hemolytic tube. After a 10-min incubation at room temperature, the absorbance of all samples was measured at 540 nm against appropriate blank controls. The HC50 value was calculated as: HC50 = [Sample OD / Half-hemolytic tube OD] × Dilution factor (Gong *et al*., 2020).

Splenic lymphocyte proliferation assay

Five mice from each group were randomly sacrificed by cervical dislocation on days 10 and 20 of the intragastric administration. The serum was separated from the mouse blood samples to detect concentrations of cytokines. Following the aseptic harvest of spleens, the generation of splenic lymphocyte suspensions was carried out, followed by concentration adjustment to 1×10^7 cells/ml. A 96-well culture plate was seeded with 50 µL of cell suspension. To stimulate the cells, 50 µL of 10 µg/ml ConA was added to the experimental wells, whereas 50 µL of RPMI 1640 medium was used as a negative control. The culture plates were then maintained at 37°C in a 5% CO_2 incubator for 72 h. Each well was then filled with 10 µL of 5 mg/ml MTT, and the mixture was cultured for an additional 4 h. Following centrifugation and removal of the supernatant, 150 µL of dimethyl sulfoxide (DMSO) was added to each well. The absorbance at 570 nm was measured after a 10-min incubation. The stimulation index (SI) was calculated as the ratio of the absorbance in the experimental well to that in the negative control well.

Determination of serum IgG subclasses and cytokine secretion

We measured the serum concentrations of IgG1, IgG2a, IFN-γ, IL-2, IL-4, and IL-10 in gavaged mice on days 10 and 20. Following the kit instructions (Sangon Biotech Co., Ltd.), all assays were conducted with three technical replicates per sample, and the mean value was used for analysis.

Detection of T lymphocyte subset and transcription factor expression levels in splenic lymphocytes

Using a similar protocol mentioned in Section 2.8, splenic lymphocyte suspensions from mice were prepared. After 10 and 20 days of gavage, the percentage of $CD4^+$ and $CD8^+$ T lymphocytes subpopulations in the mouse spleen cells was measured by flow cytometry. At the same time, qRT-PCR was performed to quantify the mRNA expression of transcription factors T-bet and GATA-3 in mouse spleen cells. The corresponding primer sequences are listed in Table 1.

Table 1: Primer sequences of qRT-PCR

Gene	Primer sequence
β-actin	Forward: 5´-GAG ACC TTC AAC ACC CCA GCC-3´
	Reverse: 5´-AAT GTC ACG CAC GAT TTC CC-3´
T-bet	Forward: 5´-CTG TTC CCA GCC GTT TCT AC-3´
	Reverse: 5´-CCG CTT CAT AAC TGT GTT CC-3´
GATA-3	Forward: 5´-TTA TCA AGC CCA AGC GAA G-3´
	Reverse: 5´-CAT TAG CGT TCC TCC TCC AG-3´

Statistical methods

Data were analyzed statistically with SAS (ver. 9.4; SAS Institute). ANOVA was employed to ascertain statistically significant variations in averages among the experimental groups. The significance levels were

Table 2: The effects of *L. plantarum* EPS on immune organs index and ear swelling in mice

Groups	Thymus index (mg/g)	Spleen index (mg/g)	Degree of ear swelling (mg)
Control group	1.96±0.22	3.94±0.37	4.28±0.52
Low dose group	2.40±0.37*	4.54±0.18*	5.93±0.66*
Medium dose group	2.95±0.42**	5.72±0.58**	7.74±0.71**
High dose group	3.29±0.69**	6.313±0.92**	8.91±0.75**

* Significant difference compared with the control group (P<0.05) and ** Extremely significant difference compared with the control group (P<0.01)

defined as P<0.05 for statistical significance and P<0.01 for high statistical significance.

Results

Results of the immune organ index measurement

The thymus and spleen were collected from the mice in each group after the final gavage, and the thymus and spleen indices were determined. *L. plantarum* EPS in each dose group can increase the thymus and spleen index. Mice in the low dose group exhibited a significant increase in thymus and spleen indices compared with the controls (P<0.05). In contrast, the indices in the middle- and high-dose groups were markedly elevated relative to the control group (P<0.01). Although the thymus and spleen index of the high dose group was slightly higher than that of the middle dose group, the two groups did not differ significantly (P>0.05) (Table 2). The results indicated that *L. plantarum* EPS can promote the development of the thymus and spleen.

Results of DTH reaction

Twenty days after the gavage, the DTH response of each group of mice was evaluated by measuring the degree of ear swelling after DFNB sensitization. The results are shown in Table 3. While all dose groups exhibited a significantly increased ear swelling response relative to the control (P<0.01), no statistically significant differences were detected among the low, middle, and high dose groups (P>0.05). Together, these findings indicate that *L. plantarum* EPS can induce a high level of DTH response in mice.

Table 3: The effects of *L. plantarum* EPS on ear swelling in mice

Groups	Degree of ear swelling (mg)
Control group	4.2±0.84
Low dose group	10.8±1.92**
Medium dose group	12.8±3.11**
High dose group	13.2±3.63**

** Extremely significant difference compared with the control group (P<0.01)

Determination of the phagocytic ability of peritoneal macrophages

The phagocytic function of peritoneal macrophages was evaluated across all mouse groups via the chicken erythrocyte phagocytosis assay (Table 4). As compared with the control group, the low-dose group exhibited a significant increase in both the phagocytic index and phagocytic percentage (P<0.05). This enhancement was more pronounced in the middle- and high-dose groups. Specifically, the phagocytic index was markedly elevated in these two groups (P<0.01). Regarding the phagocytic percentage, the middle and high doses demonstrated a significant (P<0.05) and highly significant (P<0.01) increase, respectively. The above results showed that *L. plantarum* EPS can enhance immune responses by improving the phagocytic ability of macrophages.

Table 4: The Effect of *L. plantarum* EPS on phagocytosis of peritoneal macrophages in mice

Group	Phagocytic index	Phagocytic percentage (%)
Control group	0.52±0.03	42.34±2.04
Low dose group	0.61±0.05*	45.80±2.62*
Medium dose group	0.68±0.06**	47.24±3.77*
High dose group	0.78±0.08**	50.32±4.11**

* Significant difference compared with the control group (P<0.05) and ** Extremely significant difference compared with the control group (P<0.01)

Serum hemolysin levels in mice

Serum haemolysin levels in each group of gavaged mice were determined by the half haemolysis value method, and the results are shown in Table 5. After intragastric administration of *L. plantarum* EPS, mice in all dose groups were able to produce high levels of serum hemolysin. The low dose group demonstrated a significant increase in serum hemolysin levels over the control group (P<0.05). While no significant difference was found between the middle and high dose groups, both exhibited markedly elevated serum hemolysin levels compared with the control group (P<0.01). These findings demonstrate that *L. plantarum* EPS has the capacity to potentiate the humoral immunity in mice.

Table 5: Results of the serum hemolysin levels

Groups	Half hemolysis value (HC_{50})
Control group	67.99±2.05
Low dose group	72.96±2.97*
Medium dose group	75.40±3.90**
High dose group	80.38±4.92**

* Significant difference compared with the control group (P<0.05) and ** Extremely significant difference compared with the control group (P<0.01)

Results of lymphocyte proliferation assay

The effect of *L. plantarum* EPS on murine cellular immune function was investigated by measuring splenic lymphocyte proliferation (Fig. 1). After 10 days of

administration, a significant increase in the stimulation index (SI) was observed only in the middle and high dose groups compared with the control (P<0.05). With advancing duration to 20 days, the SI value showed a significant rise in the low dose group (P<0.05), and further intensified to a highly significant level in the middle and high dose groups (P<0.01). Collectively, these findings demonstrate that *L. plantarum* EPS promotes splenic lymphocyte proliferation in a dose- and time-dependent fashion.

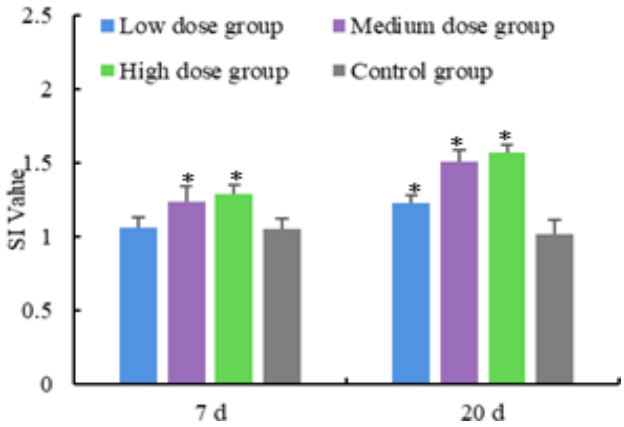

Fig. 1: Spleen lymphocyte proliferation assays from mice gavaged with *L. plantarum* EPS. * Significant difference compared with the control group (P<0.05) and ** Extremely significant difference compared with the control group (P<0.01)

Serum IgG subclasses

The dynamics of serum IgG1 and IgG2a concentrations following *L. plantarum* EPS administration are shown in Fig. 2. For IgG1, 10-day gavage resulted in a significant increase only in the high dose group compared with the controls (P<0.05), with no significant differences observed in the low and middle dose groups (P>0.05). By day 20, both the low and middle dose groups exhibited significantly elevated IgG1 levels (P<0.05), while the high dose group showed a further enhanced, highly significant increase (P<0.01) (Fig. 2A). A similar dose and time dependent effect was noted for IgG2a (Fig. 2B). After 10 days, the IgG2a level in the low dose group was comparable with the controls, but became significantly higher by day 20 (P<0.05). In contrast, the middle and high dose groups already displayed significantly elevated IgG2a at day 10 (P<0.05), which progressed to a highly significant difference after 20 days (P<0.01).

Results of the determination of cytokine secretion

To investigate the influence of *L. plantarum* EPS on cytokine production, serum levels of IL-2, IFN-γ, IL-4, and IL-10 were quantified following 10 and 20 days of administration (Fig. 3). On day 10, cytokine concentrations in the low dose group remained comparable with the controls (P>0.05). In contrast, the middle and high dose groups exhibited significant (P<0.05) and highly significant (P<0.01) elevations in all four cytokines, respectively. By day 20, a marked elevation in the Th1-type cytokines (IL-2 and IFN-γ) was observed. The levels in the low dose group were significantly increased (P<0.05), while those in the middle and high dose groups showed a highly significant rise (P<0.01). A similar trend was seen for the Th2-type cytokines (IL-4 and IL-10), with the low and middle doses showing significant increases (P<0.05) and the high dose demonstrating a highly significant upregulation (P<0.01). It is suggested that *L. plantarum* EPS can induce mice to secrete high levels of Th1 and Th2 cytokines.

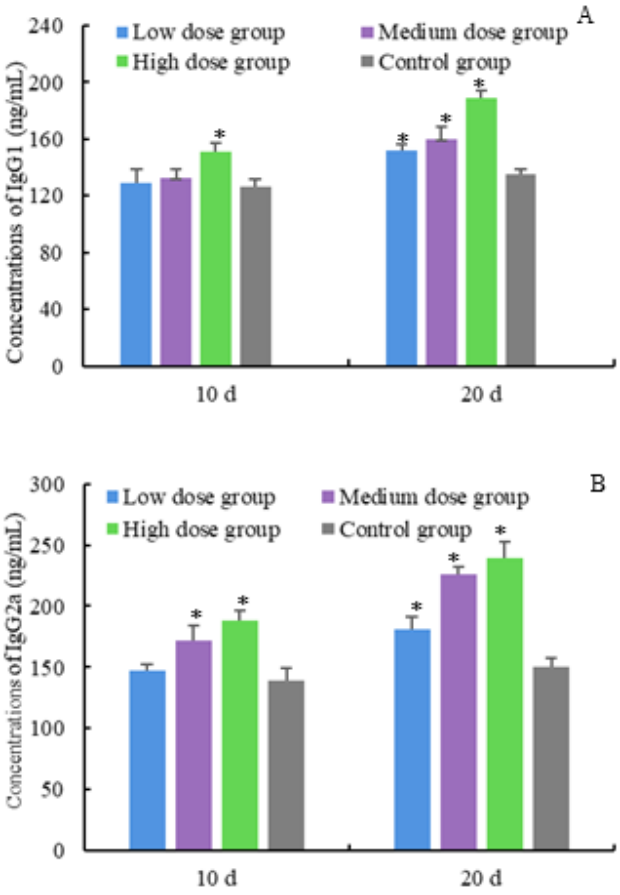

Fig. 2: Effect of *L. plantarum* EPS on the contents of IgG subclasses (**A**, **B**) in the serum of experimental mice. * Significant difference compared with the control group (P<0.05) and ** Extremely significant difference compared with the control group (P<0.01)

Detection of T lymphocyte subset

The content of T cell subsets in mouse splenocytes was measured by flow cytometry to analyze the effect of *L. plantarum* EPS on splenic T lymphocyte subsets in gavaged mice. For $CD4^+$ T cells (Fig. 4A), the low and high dose groups exhibited significant (P<0.05) and highly significant (P<0.01) increases over the controls, respectively. The middle dose group demonstrated a time-dependent enhancement, showing a significant rise at day 10 (P<0.05) that progressed to a highly significant level by day 20 (P<0.01). A similar dose and time

dependent effect was observed for $CD8^+$ T cells (Fig. 4B). On day 10, only the middle and high doses resulted in significant increases (P<0.05), while by day 20, the low and middle doses showed significant elevations (P<0.05), and the high dose group displayed a highly significant increase (P<0.01).

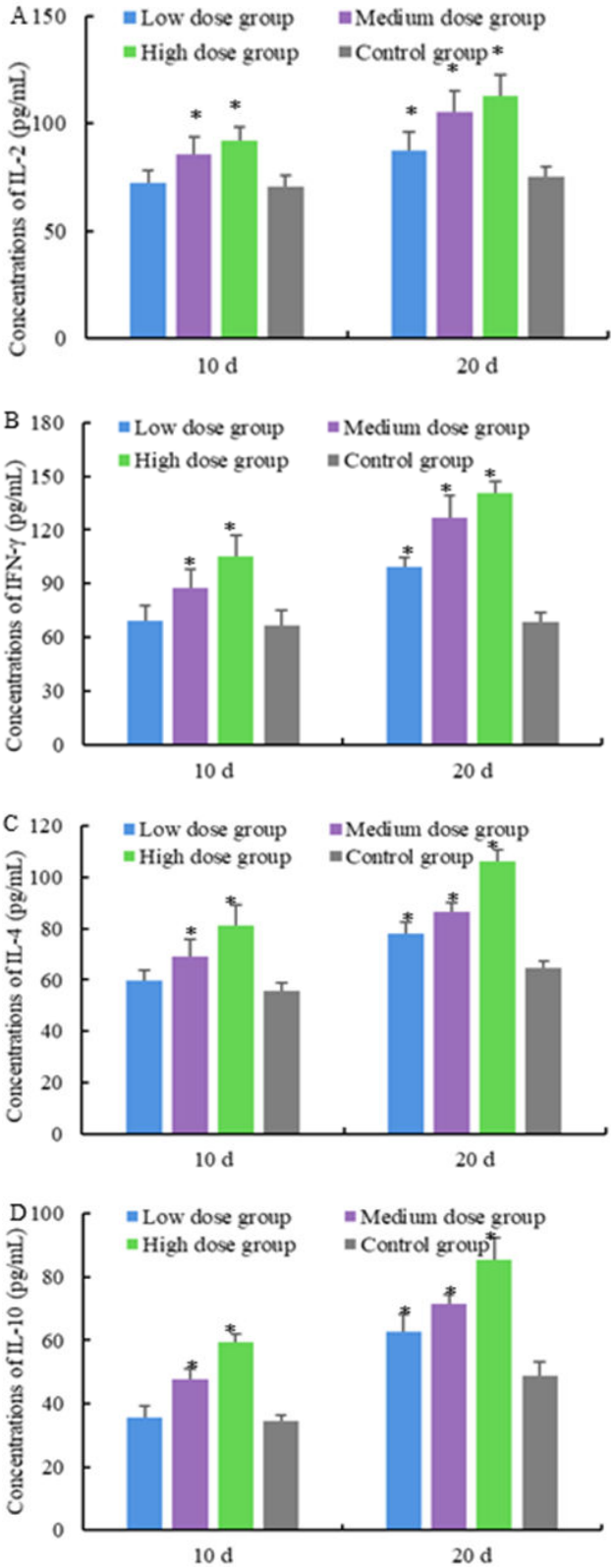

Fig. 3: Concentrations of (**A**) IL-2, (**B**) IFN-γ, (**C**) IL-4, and (**D**) IL-10 from the serum of mice gavaged with *L. plantarum* EPS. * Significant difference compared with the control group (P<0.05) and ** Extremely significant difference compared with the control group (P<0.01)

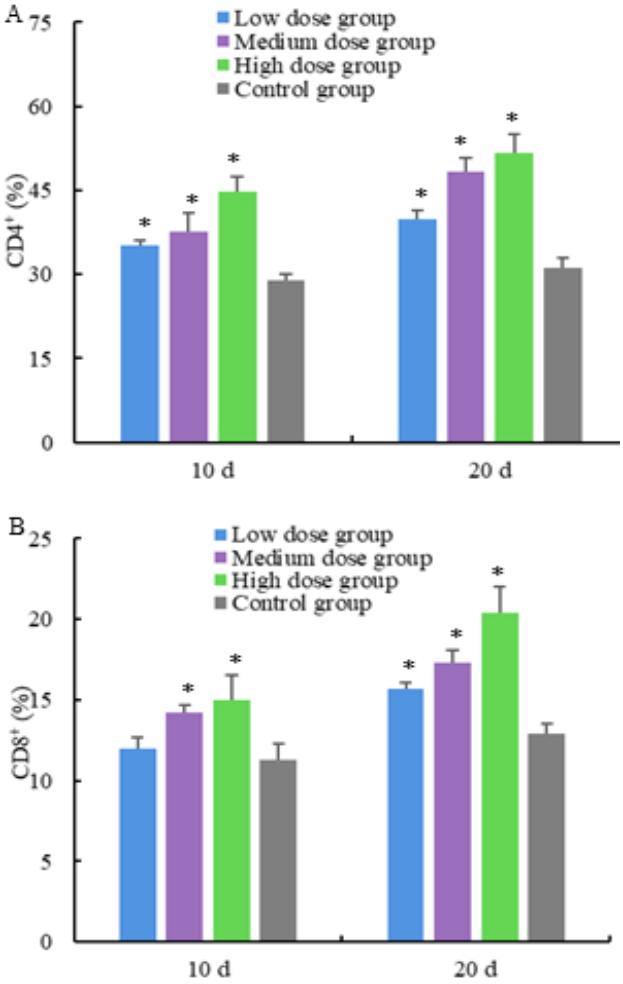

Fig. 4: Effect of *L. plantarum* EPS on the contents of spleen (**A**) $CD4^+$ and (**B**) $CD8^+$ T lymphocyte of experimental mice. * Significant difference compared with the control group (P<0.05) and ** Extremely significant difference compared with the control group (P<0.01)

Expression level of T-bet and GATA-3

To determine the effects of *L. plantarum* EPS on the transcription factors of T cells in gavaged mice, the mRNA transcript levels of T-bet and GATA-3 in splenic lymphocytes were examined after 10 and 20 days of gavage, respectively. The results are shown in Fig. 5. For T-bet, a 10-day administration led to a non-significant elevation in the low-dose group as compared with the controls (P>0.05), whereas the medium and high doses resulted in significant (P<0.05) and highly significant (P<0.01) upregulation, respectively. This effect was enhanced with prolonged administration to 20 days, at which point the low dose group also exhibited a significant increase (P<0.05), and the middle and high doses showed a highly significant upregulation (P<0.01). The expression of GATA-3 at the 10-day mark followed a similar pattern, with the low dose showing no

significant difference from the controls, while the middle and high doses induced a marked and highly significant increase (P<0.01). After 20 days of oral administration, the expression in the low dose group had also increased to a level significantly above the controls (P<0.05), while the middle and high dose groups remained markedly elevated (P<0.01). It is indicated that *L. plantarum* EPS can enhance the immune function of mice by promoting the expression of transcription factors T-bet and GATA-3.

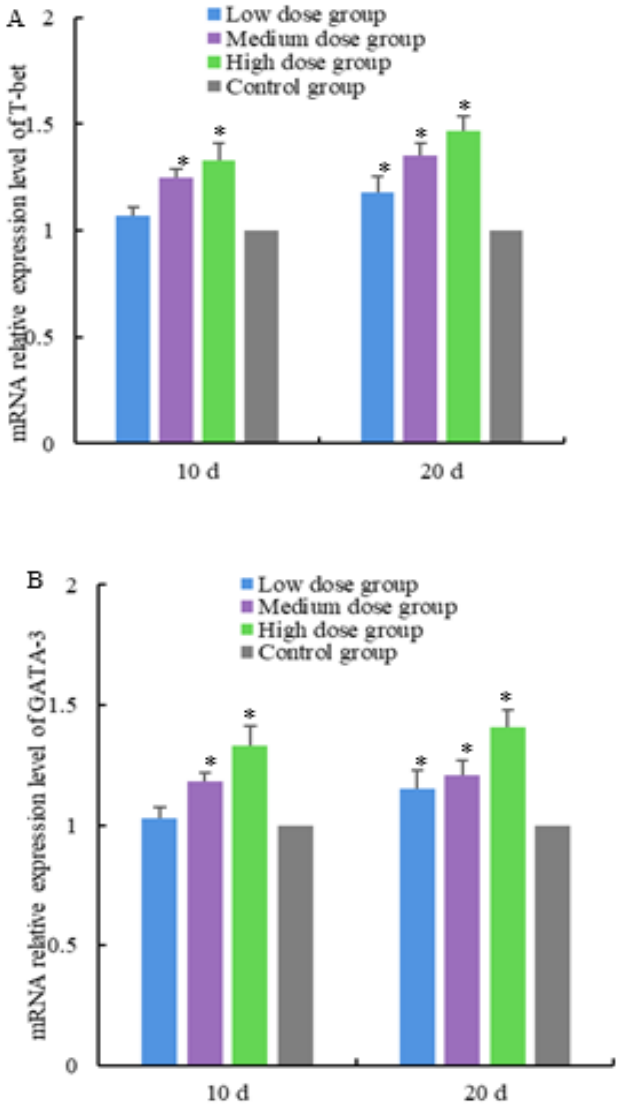

Fig. 5: mRNA relative expression level of (**A**) T-bet and (**B**) GATA-3 in splenic lymphocytes of mice. * Significant difference compared with the control group (P<0.05) and ** Extremely significant difference compared with the control group (P<0.01)

Discussion

L. plantarum is one of the common probiotics. The extracellular polysaccharides secreted during its growth have a variety of health functions and have attracted extensive attention from researchers in the food field. The present study explored the immunomodulatory function of *L. plantarum* EPS in BALB/c mice, thereby providing a theoretical basis for elucidating its immune-enhancing mechanism.

The immune organ index and the DTH response serve as robust indicators for evaluating overall immunocompetence. The thymus is an essential central immune organ of the animal body and the place where T lymphocytes differentiate and mature (Xing *et al*., 2020). At the same time, the thymus produces thymosin, a key orchestrator of cellular immunity. As the body's largest peripheral immune organ, the spleen is densely populated with lymphocytes, macrophages, and numerous B cells, all of which are critically involved in the humoral immune response. The weight and function of the thymus and spleen are closely linked to their immune cell populations. Therefore, the thymus and spleen indices can serve as indicators of lymphocyte proliferation levels, reflecting the body's immune status to a certain extent. DTH is a cell-mediated immune response driven by TDTH cells. This response activates macrophages through cytokines secreted by TDTH cells, enabling them to function as effector cells. These activated macrophages can directly kill target cells or induce their death indirectly by releasing inflammatory mediators such as TNF-α. Furthermore, the DTH response helps confine infections to specific sites by forming chronic granulomatous inflammation with infiltrating cells, thereby controlling local spread. Simultaneously, during a DTH response, $CD8^+$ T cells can proliferate and differentiate into cytotoxic T (Tc) cells upon antigen stimulation. These Tc cells subsequently induce the lysis and apoptosis of target cells (Srinoulprasert, 2021; Gray and Gardner, 2024). Therefore, the DTH response plays an essential role in the immune response. It can not only activate effector cells to kill target cells directly or indirectly, but also control infection through specific killing mechanisms and protect the body from pathogens. The results of this experiment showed that the intake of different doses of *L. plantarum* EPS increased the spleen and thymus indices of mice and promoted the DTH response, which preliminarily indicated that it had the effect of enhancing the immune function of mice.

Macrophages play a key role in the immune system. They can phagocytize and deal with foreign substances such as pathogens and cell fragments, thus maintaining the stability of the body's internal environment. The determination of the phagocytic ability of peritoneal macrophages is helpful in revealing the functional state of macrophages and reflecting the immune state of the body (Pandey *et al*., 2022). In this study, we assessed the phagocytic index and percentage of peritoneal macrophages in all experimental groups at the conclusion of the dosing regimen. Our results showed that with the increase of the ingested dose, its promotion effect on the phagocytic ability of mouse macrophage was gradually enhanced, indicating that *L. plantarum* EPS has a particular promotion effect on the non-specific immune response of mice.

Serum hemolysin concentration is correlated with the

capacity of B cells to proliferate, differentiate, and secrete specific antibodies. It is one of the indices often used to evaluate the humoral immune response (Znazen *et al*., 2006). The present study showed that the serum hemolysin levels of mice in all dose groups were significantly higher than those of the control group, suggesting that *L. plantarum* EPS can enhance the antibody response in mice. The observed elevation in serum hemolysin levels across all EPS administered groups, compared with the controls, points to an enhanced functional capacity of B lymphocytes, indicating that *L. plantarum* EPS can potentiate the antibody response in mice.

Complementing humoral immunity, the cellular immune response orchestrated by T lymphocytes constitutes a fundamental component of specific immunity. The lymphocyte proliferation test is a common index to detect the function of the cellular immune response (Ganesan *et al*., 2023). Initial T cells can differentiate into $CD4^+$ and $CD8^+$ T lymphocytes. $CD8^+$ T cells have functions such as assisting in antigen recognition, participating in signal transduction, killing target cells, and playing a role in immunomodulation. $CD4^+$ T cells can differentiate into Th cells, such as Th1 and Th2 subsets. Th1 cells can secrete cytokines such as IL-2 and IFN-γ, which mainly mediate the immune response related to cytotoxicity and local inflammation, participate in cellular immunity and the DTH response, and play an essential role in the fight against intracellular pathogen infection. Th2 cells primarily secrete cytokines like IL-4 and IL-10. These cytokines directly stimulate B cell proliferation, leading to antibody secretion and a prominent role in the humoral immune response. Therefore, the content of Th1 and Th2 type cytokines can be used to evaluate the level of a specific immune response (Liu *et al*., 2023). Splenocyte transcription factors T-bet and GATA-3 can maintain the dynamic balance of Th1 immunity and Th2 immunity by regulating the differentiation of Th1 and Th2 subsets. In addition, the gene expression of cytokines is also regulated by T-bet and GATA-3 transcription factors (Neely *et al*., 2022). Moreover, serum IgG subclasses are also closely associated with Th1 and Th2 type immune responses. In mice, the Th1 type of immune response produces antibodies to the IgG2a subtype, while the Th2 type produces antibodies to the IgG1 subtype. In this study, we measured not only the proliferation levels of splenic lymphocytes and the serum concentrations of the above four cytokines in each group of mice, but also the mRNA expression levels of T-bet and GATA-3, the content of serum IgG1 and IgG2a, as well as the content of $CD4^+$ and $CD8^+$ T-lymphocytes in the spleens of mice. The results showed that all doses of *L. plantarum* EPS could improve these immunity indexes in the gavaged mice, especially in the high dose group.

The present study demonstrates that *L. plantarum* EPS significantly boosts immune function in mice. The observed effects included enhanced development of immune organs, elicited DTH response, improved macrophage phagocytosis, and increased levels of serum hemolysin and splenic lymphocyte proliferation. Mechanistically, EPS administration dose dependently upregulated serum levels of IgG1, IgG2a, IL-2, IFN-γ, IL-4, and IL-10, increased the population of splenic $CD4^+$ and $CD8^+$ T cells, and elevated expression of the key transcription factors T-bet and GATA-3, consistent with a modulated Th1/Th2 response. This work lays the groundwork for the potential use of *L. plantarum* EPS in medicine and veterinary science. Future studies will focus on purifying and characterizing the EPS structure to further decipher its molecular immunoregulatory pathways.

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Conflict of interest

None of the authors has any other financial or personal relationships that could inappropriately influence or bias the content of the paper.

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