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Immunomodulatory effects of *Lactiplantibacillus plantarum* CCFM8661 + stachyose on cyclophosphamide-induced immunosuppression mice

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Introduction: The increasing stress of modern life has led to a decline in immunity, sparking widespread interest in new strategies to boost immune function. *Lactiplantibacillus plantarum* and stachyose have gained attention for their immune-regulating effects, but the mechanisms of their combined application remain unclear. This study aims to investigate the immunoregulatory effects of *Lactiplantibacillus plantarum* CCFM8661 combined with stachyose in cyclophosphamide-induced immunocompromised mice.
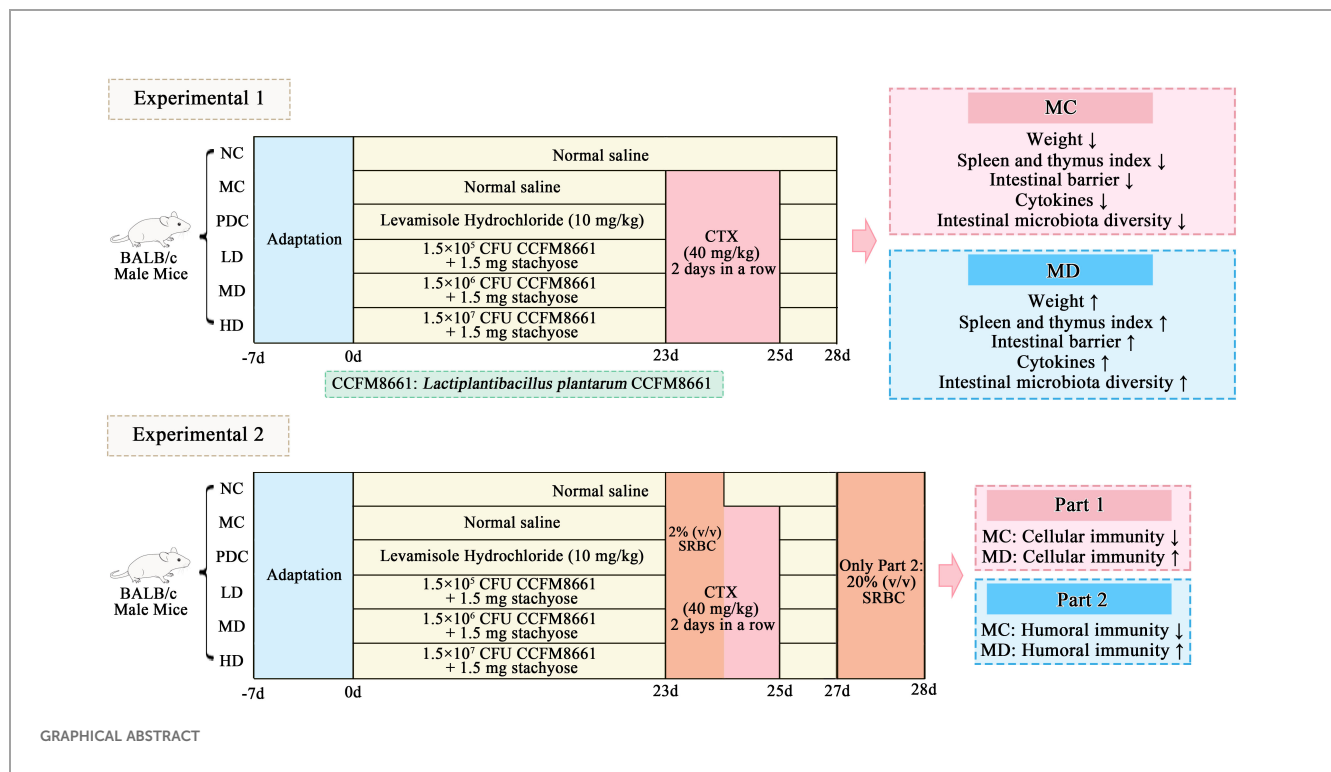
Methods: Mice were divided into the normal control group, model control group (normal saline), positive drug control group (levamisole hydrochloride, 10 mg/kg), and low, medium, and high-dose groups (1.5×10^5, 1.5×10^6, and 1.5×10^7 CFU of *Lactiplantibacillus plantarum* CCFM8661 + 1.5 mg stachyose). Each treatment group received continuous oral gavage administration for 28 days. On days 23 and 24, except for the normal control group, all other groups were intraperitoneally injected with cyclophosphamide (40 mg/kg) to establish an immunosuppressed model. On day 28, 30 minutes after the final administration, the mice were euthanized to collect the thymus, spleen, serum, ileum, and feces for subsequent analysis of immune indicators, intestinal barrier function, serum cytokines, and intestinal microbiota.

Results: The combination significantly improved immune organ atrophy, restored intestinal morphology, and normalized cytokine levels in immunosuppressed mice, indicating enhanced immune function. Additionally, it restored intestinal microbiota diversity by increasing the abundance of *Muribaculaceae* while reducing *Lachnospiraceae*, potentially promoting intestinal homeostasis.

Discussion: The combination of *Lactiplantibacillus plantarum* CCFM8661 and stachyose has immune-enhancing effects, potentially achieved by regulating inflammation levels and maintaining intestinal homeostasis.

KEYWORDS

immunoregulation, probiotics, *Lactiplantibacillus plantarum*, cyclophosphamide, intestinal microbiota

Experimental 1

NC		Normal saline
MC		Normal saline
PDC	Adaptation	Levamisole Hydrochloride (10 mg/kg)
LD		1.5×10^5 CFU CCFM8661 + 1.5 mg stachyose
MD		1.5×10^6 CFU CCFM8661 + 1.5 mg stachyose
HD		1.5×10^7 CFU CCFM8661 + 1.5 mg stachyose

CTX (40 mg/kg) 2 days in a row

-7d 0d 23d 25d 28d

CCFM8661: *Lactiplantibacillus plantarum* CCFM8661

BALB/c Male Mice

MC
Weight ↓
Spleen and thymus index ↓
Intestinal barrier ↓
Cytokines ↓
Intestinal microbiota diversity ↓

MD
Weight ↑
Spleen and thymus index ↑
Intestinal barrier ↑
Cytokines ↑
Intestinal microbiota diversity ↑

Experimental 2

NC		Normal saline
MC		Normal saline
PDC	Adaptation	Levamisole Hydrochloride (10 mg/kg)
LD		1.5×10^5 CFU CCFM8661 + 1.5 mg stachyose
MD		1.5×10^6 CFU CCFM8661 + 1.5 mg stachyose
HD		1.5×10^7 CFU CCFM8661 + 1.5 mg stachyose

2% (v/v) SRBC

CTX (40 mg/kg) 2 days in a row

Only Part 2: 20% (v/v) SRBC

-7d 0d 23d 25d 27d 28d

BALB/c Male Mice

Part 1
MC: Cellular immunity ↓
MD: Cellular immunity ↑

Part 2
MC: Humoral immunity ↓
MD: Humoral immunity ↑

GRAPHICAL ABSTRACT

1 Introduction

The accelerating pace of modern life has led to increased stress, contributing to a notable decline in population immunity (1, 2). The COVID-19 pandemic, which emerged in 2019, resulted in hundreds of millions of infections and substantial mortality, underscoring the critical need for enhanced immune health (3, 4). Throughout this crisis, many individuals displayed diminished immunity (5, 6). This reality has prompted widespread interest in strategies to boost immune function and prevent disease. While traditional chemical immunomodulators have shown limited effectiveness against immunosuppressive disorders and often come with significant side effects, alternative therapeutic agents are gaining prominence (7). These include traditional Chinese medicine extracts (8, 9), nano-formulations (10), probiotics (11, 12), etc. Probiotics, as active microbial agents, enhance disease resistance by stimulating the immune system and improving immune responses (13). Additionally, they produce beneficial metabolites and help maintain the ecological balance of intestinal microbiota (14, 15).

The World Health Organization defines probiotics as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (16). Probiotic strains play a crucial role in immune regulation in various diseases, which may be related to their ability to modulate intestinal microbiota (17–19). In addition, the functions of probiotics in regulating cellular immune factors (20–22) and repairing the intestinal barrier (23) are also closely related to their role in enhancing immunity. In recent years, many strains of *Lactiplantibacillus plantarum* have been found to possess immune-regulating effects (23, 24). Ervina (25) et al. believe that *Lactiplantibacillus* sp. plays a crucial role in immune

maintenance. Lactoferrin combined with *Lactiplantibacillus* can significantly enhance the immune response in kittens, increase immunoglobulin levels, and regulate inflammatory factors (26). Goya-Jorge (27) et al. revealed through the TripleSHIME® system that Heyndrickxia coagulans combined with *Lacticaseibacillus casei* enhances the body's immunity by regulating the intestinal microbiota, protecting the intestinal barrier, and other mechanisms.

Lactiplantibacillus plantarum CCFM8661 has previously been shown to mitigate toxicity induced by benzo (a) pyrene and effectively address the metabolic damage caused by heavy metals (28–30). Li (31) et al. suggested that a combination of probiotics containing *Lactiplantibacillus plantarum* CCFM8661 can alleviate antibiotic-associated diarrhea induced by ampicillin in mice. Our laboratory's previous research has shown that *Lactiplantibacillus plantarum* CCFM8661 can effectively enhance both humoral and cellular immunity in cyclophosphamide-induced immunosuppressed mice. The underlying mechanisms for its immune-boosting effects may involve the regulation of cytokines, improvement of the intestinal barrier, and modulation of the intestinal microbiota.

Stachyose, a novel functional water-soluble oligosaccharide naturally present in plants, is noted for its low sweetness, low caloric content, and remarkable stability, which makes it ideal for improving food quality and developing functional foods (32). Previous studies have demonstrated that stachyose exhibits anti-inflammatory properties (33), alleviates intestinal colitis (34, 35), improving intestinal microbiota (36) and other effects. When combined with isoflavones, stachyose enhances bioavailability, thus improving hyperlipidemia and hyperglycemia (37), and it also promotes the absorption of tea polyphenols while protecting

the liver (38). Wang (39) et al. found that stachyose combined with Fu brick tea polysaccharides and sheep whey protein could improve the immunity of immunocompromised mice. However, the effects of the combined application of *Lactiplantibacillus plantarum* CCFM8661 and stachyose on immune function have not been previously studied.

Cyclophosphamide (CTX) is a cytotoxic chemotherapy agent known to induce immune deficiency by inhibiting both humoral and cellular immunity in animals. It is widely utilized in the creation of animal models for immunosuppression (40). In this study, CTX was employed to establish an immunocompromised mouse model to investigate the effects of varying doses of *Lactiplantibacillus plantarum* CCFM8661 combined with water-soluble oligosaccharide on the immune function of CTX-induced immunocompromised mice, aiming to provide a further theoretical basis for the development and utilization of probiotics.

2 Materials and methods

2.1 Experimental materials

2.1.1 Bacterial strain and culture

Lactiplantibacillus plantarum CCFM8661 was acquired from Hebei Yiran Biotechnology Co., Ltd., while stachyose was sourced from Anhui Zhongxinkang Pharmaceutical Co., Ltd. Prior to the experiment, both *Lactiplantibacillus plantarum* CCFM8661 and stachyose were kept in a - 80°C refrigerator. Our experimental design included low, medium, and high dose groups, consisting of 1.5×10^5, 1.5×10^6, and 1.5×10^7 CFU of probiotics (*Lactiplantibacillus plantarum* CCFM8661) and 1.5mg stachyose, all prepared with physiological saline and activated prior to use.

2.1.2 Animals

SPF-grade, BALB/c male mice aged 6 weeks were obtained from Liaoning Changsheng Biotechnology Co., Ltd. (Shenyang, China). The animals were maintained in a barrier environment within a temperature-controlled room (20 to 23°C) and humidity-controlled setting (30 to 60%), maintained on a 12:12-hour light-dark cycle, with free access to food and water. Total animal experiments stuck to the regulations outlined in the Management of Experimental Animals of Heilongjiang University of Traditional Chinese Medicine and received approval from the Animal Ethics Committee of Heilongjiang University of Traditional Chinese Medicine (ethical approval code: 2024032915).

2.2 Experimental methods

2.2.1 Experimental 1 design

Following a 7-day acclimatization period, BALB/c mice were randomly allocated into various groups: the normal control group (NC), model control group (MC), positive drug control group (PDC), and groups receiving low, medium, and high doses of a combination of *Lactiplantibacillus plantarum* CCFM8661 and stachyose (LD, MD, HD), each consisting of 10 mice. The PDC

group received levamisole hydrochloride (10 mg/kg, Taiyuan in Shanxi Province Pharmaceutical Co., Ltd., Shanghai, China) via gavage, while the LD, MD, and HD groups were administered *Lactiplantibacillus plantarum* CCFM8661 (1.5×10^5, 1.5×10^6, or 1.5×10^7 CFU) combined with 1.5 mg stachyose by gavage. Mice in the NC and MC groups administered an equal volume of physiological saline daily via gavage for a duration of 28 days. Except for the NC group, all other groups received intraperitoneal injections of cyclophosphamide (40 mg/kg, Jiangsu Hengrui Medicine Co., Ltd., Shanghai, China) once daily on the 23rd and 24th day of intragastric administration of *Lactiplantibacillus plantarum* CCFM8661 + stachyose to establish the immunosuppressive model. Mice in the NC groups were administered an intraperitoneal injection of the same volume of normal saline. At the conclusion of the experiment, the immune organs of the mice were weighed for analysis, blood samples were collected and keptd at −80°C, ileal tissues were harvested for histopathological examination, and fresh feces were harvested in sterile conditions and keptd at −80°C for analysis of intestinal microbiota.

2.2.2 Experimental 2 design

The experimental animals were divided into two groups, each containing 10 mice. On the 23rd day of administration, each mice was intraperitoneally injected with 0.2mL 2% (v/v) SRBC (R22395, Shanghai Yuanye Co., LTD., Shanghai, China.) for sensitization. Four days after immunization with 2% (v/v) SRBC, the first group of mice was injected subcutaneously with 20 µL of 20% (v/v) SRBC at the left hind toe. 24h later, the animals were sacrificed at the end of the last measurement. Mice in the second group were immunized with 2% (v/v) SRBC for 5 days and sacrificed after blood collection.

2.2.3 Trends in weight gain

The starting body weight of the mice in all groups was measured at 8:30 AM on the first day of the experiment, after which the corresponding treatments were administered. The weights of the mice in all groups were then measured and documented at 9:00 AM every two days.

2.2.4 Determination of immune organ index

The thymus and spleen of the experimental animals were dissected and weighed, after which the indices for both the thymus and spleen were calculated. The organ index was calculated as follows:

$$\text{Thymus or spleen index (mg/g)} = \frac{\text{Thymus or spleen mass (mg)}}{\text{Animal body weight (g)}}$$

2.2.5 Delayed type hypersensitivity determination

The degree of delayed-type hypersensitivity was measured by plantar thickening method. On day 23, each mouse was intraperitoneally injected with 2% (v/v) SRBC 0.2 mL for sensitization. 4 days later, the thickness of the left hind toe was assessed, followed by a subcutaneous injection of 20 µL of 20% (v/v) SRBC at the same site (approximately 1×10^8 SRBC per mouse).

After 24 hours, the thickness of the left hind toe was assessed again, and the average of three measurements at the same site was calculated. The difference in toe thickness before and after injection was used to indicate the degree of DTH response.

2.2.6 Determination of serum hemolysin level

All mice immunized with 0.2 mL of 2% (v/v) SRBC via intraperitoneal injection on the 23rd day after oral administration. After 5 days, corresponding to a total of 28 days of gastric gavage, blood was collected from the eyes into a centrifuge tube. The samples were allowed to sit for about 1 hour, then centrifuged at 2,000 r/min for 10 minutes to separate serum, which was diluted with normal saline at a 1:100 ratio. The diluted serum was placed into a 96-well plate, followed by the sequential addition of 10% (v/v) SRBC and complemented guinea pig serum. After incubating the plate in a water bath maintained at 37°C for 30 minutes, the supernatant was centrifuged and transferred to a separate plate. Join Drabkin's solution (R22785, Shanghai Yuanye Co., LTD., Shanghai, China.) and set the half hemolysis hole. Finally, the optical density of the mixed solution in each well was assessed by an automatic microplate reader. The amount of hemolysin was expressed as the half hemolytic value (HC_{50}), which was calculated as follows:

$$Sample \ HC_{50} = \frac{Sample \ optical \ density \ value \ \times \ dilution}{Optical \ density \ of \ SRBC \ at \ half \ hemolysis}$$

2.2.7 Histopathological analysis of ileum

Ileal tissues were preserved in 4% paraformaldehyde and kept 48 hours, before being embedded in paraffin (JB-P5, Wuhan Junjie Electronics Co., Ltd, Wuhan, China) and sectioned into 4-μm slices (RM2016, Leica, Shanghai, China). The sections were stained with hematoxylin-eosin after dewaxing, followed by photography under light and examination using a microscope (Nikon Eclipse Tzu Hsiang L, 100x magnification; Nikon, Tokyo, Japan).

2.2.8 Serum level testing

After the final administration, the blood of the experimental animals was centrifuged (2000r/min) for serum extraction and frozen in a −80°C refrigerator for determination. The levels of serum IgA (MM-0055M1), IgG (MM-0057M1), IFN-γ (MM-0182M1), TNF-α (MM-0132M1), IL-2 (MM-0105M1), IL-6 (MM-0163M1) and IL-12 (MM-0701M1) were detected by ELISA kits (Jiangsu Meimian Industrial Co., Ltd., Yancheng, China).

2.2.9 Metagenomic analysis

Total microbial DNA was extracted from the samples using the MagPure Soil DNA KF kit (D6356-F-96-SH, Magen, Shanghai, China). DNA concentration determination and integrity analysis were performed using a NanoDrop2000 spectrophotometer (Thermo Fisher Scientific, MA, USA) and agarose gel electrophoresis. Subsequently, DNA fragmentation and purification were conducted. The Qubit dsDNA HS Quantification Kit (Q32851, LifeTechnologies, California, USA)

was used to detect concentrations, and the quality of library fragments was checked using an Agilent 2100 system (Agilent, California, USA). Library construction, sequencing, and data analysis were carried out by Shanghai Ouyi Biomedical Technology Co., Ltd.

2.2.10 Statistical analysis

All data were analyzed using SPSS 26.0 statistical software, with results presented as mean ± SD. Differences between groups were assessed using one-way analysis of variance (ANOVA). $P<0.05$ was considered to indicate a statistically significant difference.

3 Results

3.1 Effect of probiotics on body weight in mice

Figure 1A illustrates that the difference in body weight before and after the experiment was statistically significant across the various administration groups ($P<0.01$). In comparison to the NC group, the weight gain in the MC group was markedly reduced ($P<0.001$), while the PDC, MD, and HD groups exhibited significantly increased weight gain compared to the MC group ($P<0.01$). These findings suggest that *Lactiplantibacillus plantarum* CCFM8661 + stachyose effectively mitigated weight loss induced by cyclophosphamide.

3.2 Effect of probiotics on thymus and spleen indices in mice

Compared to the NC group, the thymus index (Figure 1B) and spleen index (Figure 1C) in the MC group showed significant differences, with both indices decreasing markedly ($P<0.001$). The PDC group exhibited a significant increase in the thymus index ($P<0.001$), while the MD and HD groups also showed significant increases ($P<0.01$), with the high-dose group demonstrating a marked difference ($P<0.001$), when compared to the MC group. Cyclophosphamide-induced immunodeficiency can result in the atrophy of immune organs, whereas *Lactiplantibacillus plantarum* CCFM8661 + stachyose appears to mitigate the damage to these organs.

3.3 Effects of probiotics on cellular and humoral immunity in mice

As shown in Figure 1D, the foot thickness in the MC group was significantly reduced compared to the NC group ($P<0.001$). In comparison to the MC group, the PDC group exhibited a significant increase in foot thickness ($P<0.001$), while the MD and HD groups also showed significant increases ($P<0.01$), with the high-dose group demonstrating a marked difference ($P<0.001$). Additionally,
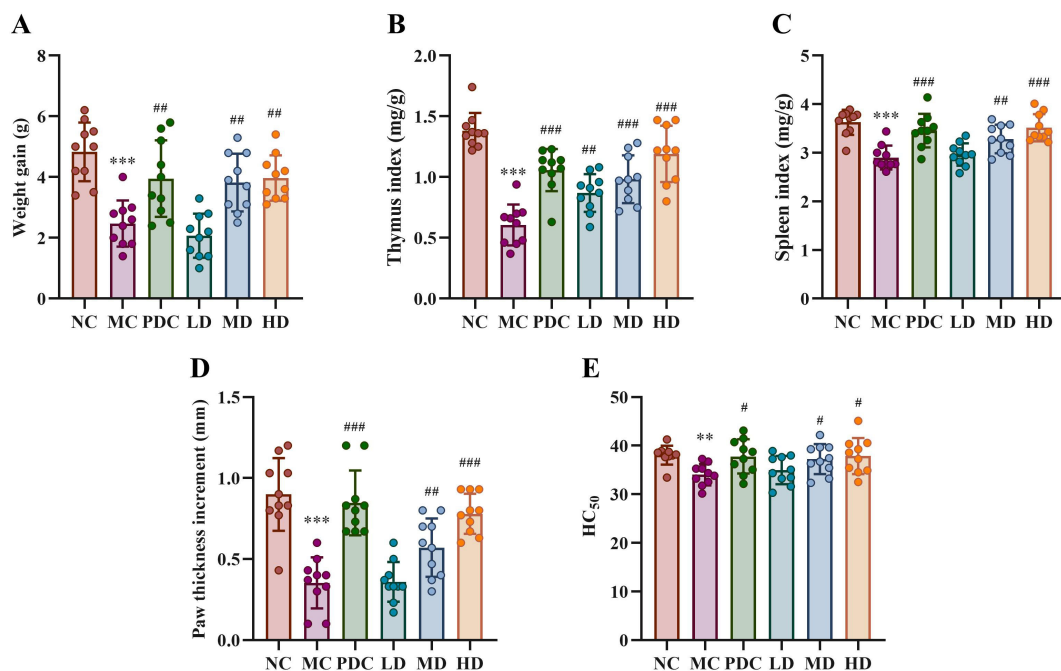
FIGURE 1
Effect of *Lactiplantibacillus plantarum* CCFM8661 + stachyose on immune-related indexes in immunocompromised mice model induced by cyclophosphamide. **(A)** Weight gain before and after the experiment in different drug administration groups; **(B, C)** Thymus index and spleen index; **(D)** Determination of cellular immune function by delayed type hypersensitivity; **(E)** Measurement of humoral immune function by serum hemolysin level. Normal control group: NC, model control group: MC, positive drug control group: PDC, *Lactiplantibacillus plantarum* CCFM8661 + stachyose-low dose groups: LD, *Lactiplantibacillus plantarum* CCFM8661 + stachyose-medium dose groups: MD, *Lactiplantibacillus plantarum* CCFM8661 + stachyose-high dose groups: HD. Data are presented as the mean $\pm$ SD. MC vs NC, * $P<0.05$, ** $P<0.01$, *** $P<0.001$; PDC, LD, MD. HD vs MC, # $P<0.05$, ## $P<0.01$, ### $P<0.001$, n=10.

regarding humoral immune function (Figure 1E), serum hemolysin levels in the MC group were lower compared to the NC group ($P<0.01$), while serum hemolysin levels in the PDC, MD, and HD groups increased following administration of the positive drug and adequate doses of *Lactiplantibacillus plantarum* CCFM8661 combined with stachyose ($P<0.05$). This indicated that *Lactiplantibacillus plantarum* CCFM8661+ stachyose was helpful in both cellular and humoral immunity of mice.

3.4 Effect of probiotics on intestinal barrier disruption

The HE staining results of ileal tissues of mice in all categories are shown in Figure 2A. The small intestinal villi were tightly arranged and of uniform thickness in the NC group, while the MC group displayed atrophy and sparse villi. The PDC, MD, and HD groups showed alleviation of small intestinal villus lesions, with the PDC and HD groups demonstrating superior improvement compared to the MD group; the LD group had minimal effects. Villus height-to-crypt depth ratio (VH/CD ratio) was statistically considerably lower in the MC group by comparison with the NC group ($P<0.001$). Inversely, this ratio was increased in the PDC, MD, and HD groups in comparison with the MC group ($P<0.05$), with VH/CD ratio found to be higher in the HD group than in the

MD group (Figure 2B). In Furthermore, the volume of goblet cells per the length of the unit was dramatically reduced in the MC group with respect to the NC group ($P<0.001$), whereas the volume of goblet cells was increased in the PDC group and the HD group with respect to the MC group ($P<0.05$) (Figure 2C). Altogether, these observations indicate that *Lactiplantibacillus plantarum* CCFM8661 + stachyose can effectively mitigate intestinal damage inflicted by cyclophosphamide in immune-impaired mice.

3.5 Effect of probiotics on serum cytokine levels in mice

Figure 3 illustrates that the levels of IgG, TNF-α, IFN-γ, IL-2, IL-6, and IL-12 were obviously lower in the MC group versus the NC group ($P<0.05$), with IgA showing a marked decrease ($P<0.001$). In contrast, the PDC group exhibited increased levels of IgG, TNF-α, IFN-γ, IL-2, IL-6, and IL-12 compared to the MC group ($P<0.05$), and IgA levels were also obviously elevated ($P<0.001$). The LD group showed no appreciable change by comparison with the MC group, whereas the level of IgA, IFN-γ, IL-2, IL-6, and IL-12 were augmented in the MD group ($P<0.05$). The HD group demonstrated substantially increased levels of IgG, TNF-α, IFN-γ, IL-2, IL-6, and IL-12 in comparison with the MC group ($P<0.05$), as were the levels of IgA ($P<0.001$).
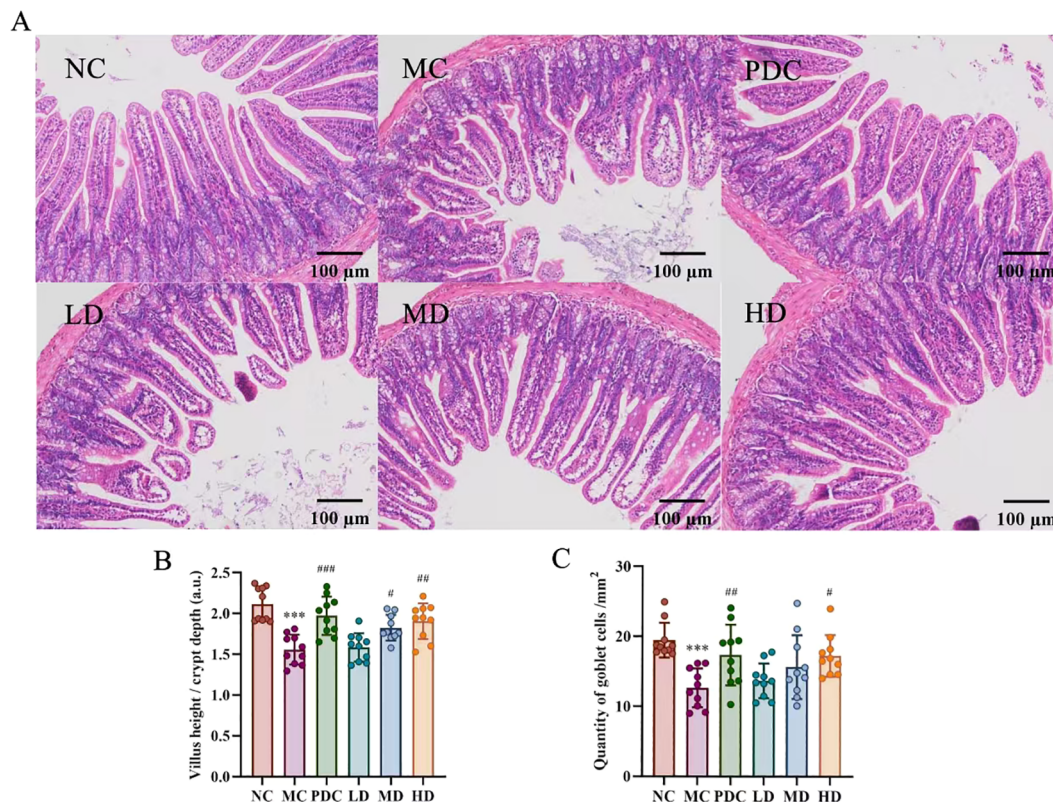
FIGURE 2

Lactiplantibacillus plantarum CCFM8661 + stachyose effect on the intestinal tract of immunocompromised mice induced by cyclophosphamide. **(A)** HE staining of mouse ileum sections (× 100); **(B)** Villus height/crypt depth; **(C)** The quantity of goblet cells in mice ileum per unit length. Normal control group: NC, model control group: MC, positive drug control group: PDC, *Lactiplantibacillus plantarum* CCFM8661 + stachyose-low dose groups: LD, *Lactiplantibacillus plantarum* CCFM8661 + stachyose-medium dose groups: MD, *Lactiplantibacillus plantarum* CCFM8661 + stachyose-high dose groups: HD. Data are presented as the mean $\pm$ SD. MC vs NC, * $P<0.05$, ** $P<0.01$, *** $P<0.001$; PDC, LD, MD. HD vs MC, # $P<0.05$, ## $P<0.01$, ### $P<0.001$, n=10.
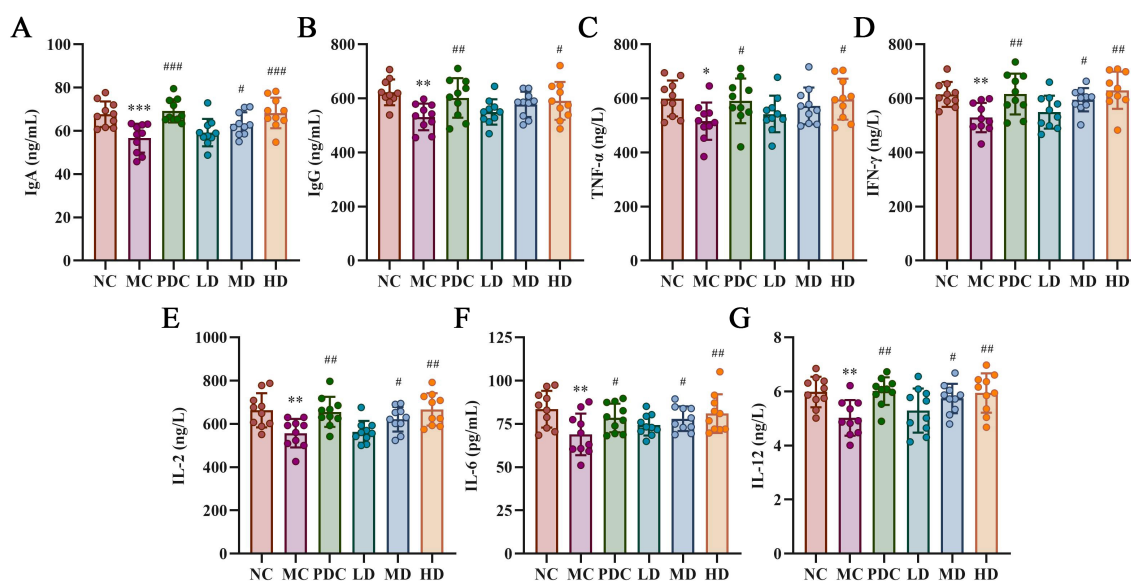
FIGURE 3

Lactiplantibacillus plantarum CCFM8661 + stachyose effect on cytokine levels in serum of immunocompromised mice induced by cyclophosphamide. **(A)** IgA; **(B)** IgG; **(C)** TNF-α; **(D)** IFN-γ; **(E)** IL-2; **(F)** IL-6; **(G)** IL-12. Normal control group: NC, model control group: MC, positive drug control group: PDC, *Lactiplantibacillus plantarum* CCFM8661 + stachyose-low dose groups: LD, *Lactiplantibacillus plantarum* CCFM8661 + stachyose-medium dose groups: MD, *Lactiplantibacillus plantarum* CCFM8661 + stachyose-high dose groups: HD. Data are presented as the mean $\pm$ SD. MC vs NC, * $P<0.05$, ** $P<0.01$, *** $P<0.001$; PDC, LD, MD. HD vs MC, # $P<0.05$, ## $P<0.01$, ### $P<0.001$, n=10.

3.6 Effects of probiotics on intestinal microbiota in mice

3.6.1 Alpha diversity analysis

Alpha diversity analysis assessed the multiplicity of intestinal microbiota across groups of mice, employing the Simpson index for evaluation, where a higher index signifies greater community diversity. No marked variation was observed between the NC and MC groups; however, when compared to the PDC group and various dose groups, the Simpson index significantly increased after administration (*P*<0.05). These results indicate that *Lactiplantibacillus plantarum* CCFM8661 + stachyose effectively enhance the multiplicity of intestinal microbiota in mice (Figure 4A).

3.6.2 Beta diversity analysis

Alpha diversity reflects both the richness and uniformity of species within a sample. Beta diversity, on the other hand, is used to analyze striking discrepancies in the structure of microbial communities between different samples (or groups). In the NMDS analysis based on Bray-Curtis (Figure 4B), distinct characteristics of intestinal microbiota distribution were observed among the different mouse groups, with a stress value of 0.1063. Intestinal microbiota of the NC and MC groups differed significantly, with the LD group being more similar to the MC group, while the Group PDC, MD, and HD had microbial communities closer to the Group NC and away from Group MC. These results suggested that *Lactiplantibacillus plantarum* CCFM8661 + stachyose at medium and high doses could effectively modulate the composition of the immunodeficient mice intestinal microbiota to be closer to that of normal mice.
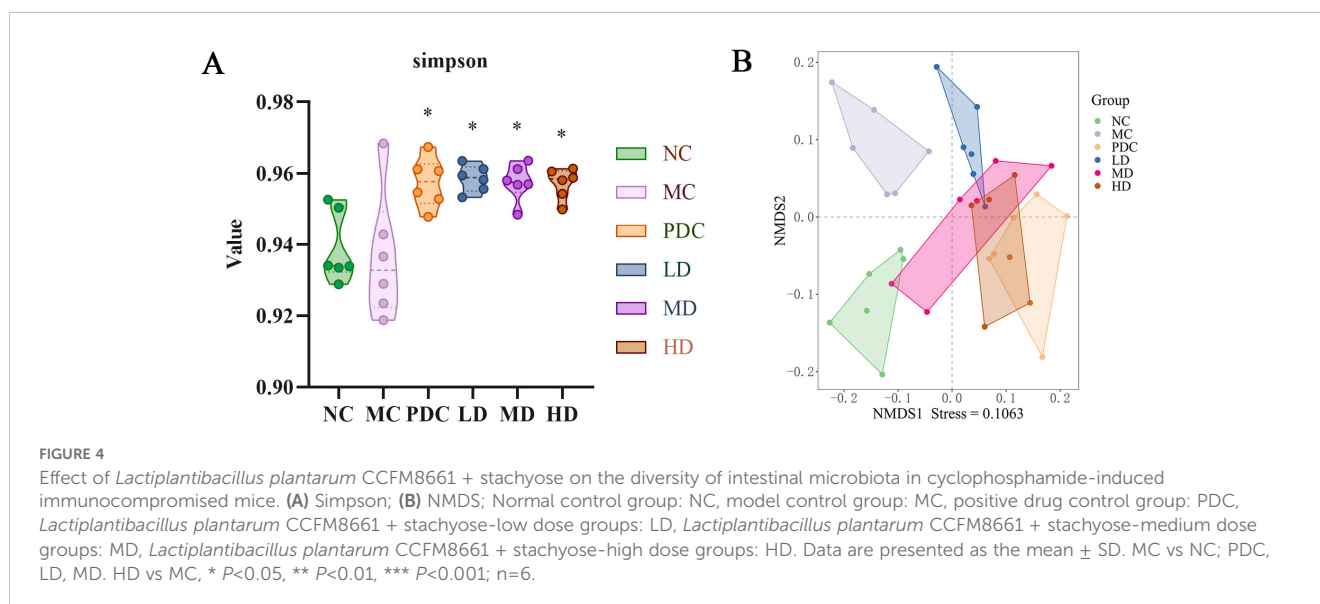
3.6.3 Effect of probiotics on intestinal microbiota composition in mice

Based on species richness data, cumulative bar charts depicting the comparative richness of species at the phylum and species level

were generated to visualize the diversity of different species across the groups at various taxonomic levels.

Within the phylum level, *Bacteroidota* and *Bacillota* emerged as the predominant species across all groups, collectively accounting for over 90% of the total species. The relative abundance of *Bacteroidota* decreased by 19.57% (*P*<0.05) in the MC group over the NC group, while the relative abundance of *Bacillota* increased by 19.17% (*P*<0.05). Our results indicate a shift in the composition of the intestinal microbiota in immunocompromised mice induced by cyclophosphamide, characterized by an excessive increase in *Bacillota* and a corresponding inhibition of *Bacteroidota*, resulting in an elevated *Bacillota/Bacteroidota* ratio, which was consistent with the composition changes of intestinal microbiota in immunocompromised mice previously and was a manifestation of intestinal microbiota imbalance in mice (41). Notably, the relative abundance of *Bacteroidota* in the PDC, MD, and HD groups increased by 24.68% (*P*<0.05), 22.58% (*P*<0.05), and 25.62% (*P*<0.05), respectively, with respect to the MC group. Conversely, the relative abundance of *Bacillota* decreased by 22.14% (*P*<0.05), 19.83% (*P*<0.05), and 23.19% (*P*<0.01), leading to a reduced *Bacillota/Bacteroidota* ratio (Figures 5A–C). These results suggest that the combination of *Lactiplantibacillus plantarum* CCFM8661 and stachyose can significantly alter the relative abundance of phyla in the intestinal microbiota and mitigate the effects of cyclophosphamide.

At the species level, the organization of the groups of microorganisms consisted mainly included *Muribaculaceae bacterium*, *Lachnospiraceae bacterium*, *Palleniella intestinalis*, *Bacteroidales bacterium*, *Bacteroides acidifaciens*, *Clostridia bacterium*, *Oscillospiraceae bacterium* and *Bacteroides* sp. Versus the NC group, *Palleniella intestinalis*, *Bacteroidales bacterium*, *Bacteroides acidifaciens*, and *Bacteroides* sp. in the Group MC decreased. The relative abundance of *Muribaculaceae bacterium* was diminished by 4.84% (*P*<0.05). In Group PDC, MD, and HD, the relative abundance of *Muribaculaceae bacterium*, *Muribaculum* sp., *Bacteroides acidifaciens* and *Bacteroides* sp. enhanced compared

FIGURE 4
Effect of *Lactiplantibacillus plantarum* CCFM8661 + stachyose on the diversity of intestinal microbiota in cyclophosphamide-induced immunocompromised mice. **(A)** Simpson; **(B)** NMDS; Normal control group: NC, model control group: MC, positive drug control group: PDC, *Lactiplantibacillus plantarum* CCFM8661 + stachyose-low dose groups: LD, *Lactiplantibacillus plantarum* CCFM8661 + stachyose-medium dose groups: MD, *Lactiplantibacillus plantarum* CCFM8661 + stachyose-high dose groups: HD. Data are presented as the mean ± SD. MC vs NC; PDC, LD, MD. HD vs MC, * *P*<0.05, ** *P*<0.01, *** *P*<0.001; n=6.

to Group MC. The relative abundance of *Muribaculaceae bacterium* was elevated by 4.86% (*P*<0.05), 4.53% (*P*<0.05), and 5.05% (*P*<0.05), respectively. Within MC group, the relative abundance of *Lachnospiraceae bacterium*, *Clostridia bacterium*, and *Oscillospiraceae bacterium* was elevated compared to the NC group. The relative abundance of *Lachnospiraceae bacterium* increased by 6.67%. In the PDC, MD, and HD groups, the relative abundance of *Lachnospiraceae bacterium*, *Palleniella intestinalis*, *Clostridia bacterium*, and *Oscillospiraceae bacterium* decreased with respect to the MC group. The relative abundance of *Lachnospiraceae bacterium* decreased by 11.01% (*P*<0.05), 9.62% (*P*<0.05), and 11.51% (*P*<0.05), respectively (Figures 5D–F). The combination of *Lactiplantibacillus plantarum* CCFM8661 and fructose significantly altered the relative abundance of each species among different groups of mice, as demonstrated by these results.

3.6.4 Correlation analysis of dominant flora at the species level with immune-related indicators, intestinal barrier-related indicators and cytokines

Spearman's method was utilized to analyze the association of immune-related indices, intestinal barrier-related indices, cytokines with the expression of dominant species of intestinal microbiota at the species level, to explore the role of intestinal microbiota in the treatment process mediated by *Lactiplantibacillus plantarum* CCFM8661 + stachyose. The relative abundance of *Muribaculaceae bacterium* was dramatically and positively associated with HC50 and cytokines (IgA, TNF-α), and positively correlated with other immune indicators and intestinal barrier-related indicators, but not significantly. *Bacteroidales bacterium* was positively correlated with weight gain, immune organ index, and IFN-γ. *Oscillospiraceae bacterium* was significantly negatively correlated with weight gain, immune organ index, cellular
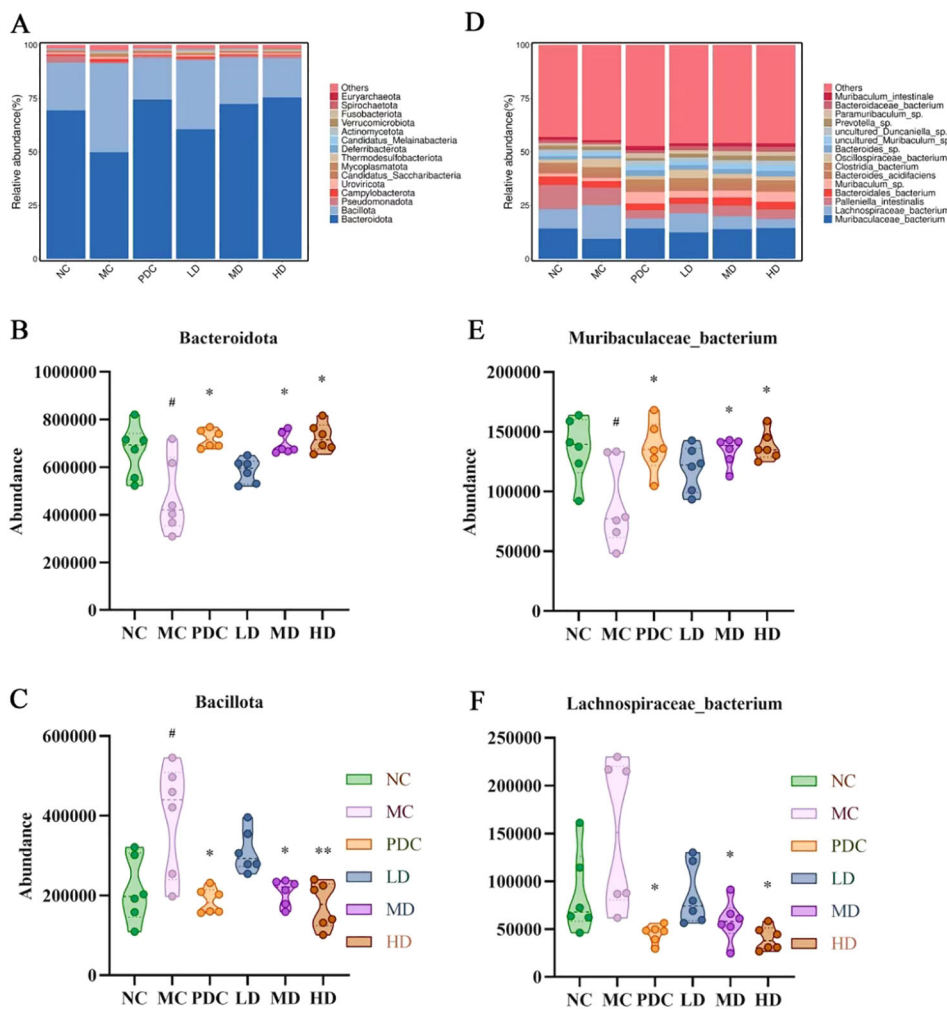
FIGURE 5
Lactiplantibacillus plantarum CCFM8661 + stachyose effect on the intestinal microbiota composition in immunocompromised mice model induced by cyclophosphamide. **(A)** Phylum level composition of intestinal microbiota; **(B)** Species level composition of intestinal microbiota; **(C)** *Bacteroidota*; **(D)** *Bacillota*; **(E)** *Muribaculaceae bacterium*; **(F)** *Lachnospiraceae bacterium*; Normal control group: NC, model control group: MC, positive drug control group: PDC, *Lactiplantibacillus plantarum* CCFM8661 + stachyose-low dose groups: LD, *Lactiplantibacillus plantarum* CCFM8661 + stachyose-medium dose groups: MD, *Lactiplantibacillus plantarum* CCFM8661 + stachyose-high dose groups: HD. Data are presented as the mean ± SD. MC vs NC; PDC, LD, MD. HD vs MC, * *P*<0.05, ** *P*<0.01, *** *P*<0.001; n=6.

immunity index, foot swelling degree, intestinal barrier-related indicators, and cytokines (IgG, IL2, IL-6, and IL-12). *Clostridia bacterium* was negatively correlated with immune organ index and IFN-γ (Figure 6).

4 Discussion

The study demonstrates that the combination of *Lactiplantibacillus plantarum* CCFM8661 and stachyose exhibits significant immunoregulatory potential. This combination effectively alleviates immune dysfunction by reducing immune organ atrophy, enhancing humoral and cellular immunity, restoring intestinal barrier integrity, and rebalancing the intestinal microbiota. The positive control drug, levamisole hydrochloride, exhibits significant immunomodulatory and immune-enhancing effects (42–44). The immunomodulatory capacity demonstrated by the combination of *Lactiplantibacillus plantarum* CCFM8661 and stachyose is comparable to that of levamisole hydrochloride. The findings highlight the ability of the innovative combination of *Lactiplantibacillus plantarum* CCFM8661 and stachyose to boost immune function and promote intestinal health.

As key immune organs, the thymus and spleen play critical roles in regulating the body's immune function, and the thymus index and spleen index have become widely recognized visual indicators of immune status (45). The combination of *Lactiplantibacillus plantarum* CCFM8661 and stachyose can significantly alleviate the atrophy of the thymus and spleen in immunosuppressed mice. Notably, this study found that the thymus is more responsive to the dosage of this combination compared to the spleen, as improvements in thymic atrophy were observed even at low doses. Consistent with this study, certain *Lactiplantibacillus plantarum* strains have been shown to enhance the function of the thymus and spleen (46, 47). Zhang (48) et al. found that a

combination of multiple prebiotics, including stachyose, and *Limosilactobacillus fermentum* DALI02 could restore immune organ indices in a CTX-induced immunosuppression model. It is worth noting that You (49) et al. reported that *Lactiplantibacillus plantarum* P101 had limited effects on reducing weight loss and immune organ atrophy. This discrepancy may be attributed to the irreversible damage caused by prolonged CTX injection to immune organs, making it difficult for *Lactiplantibacillus plantarum* to restore them.

Cellular immunity and humoral immunity are the two main defense mechanisms of the immune system. Immune function regulation can be assessed via DTH for cellular immunity (50) and serum hemolysin levels for humoral immunity (51). The combination of *Lactiplantibacillus plantarum* CCFM8661 and stachyose can significantly improve the specific immune function of immunosuppressed animals and exhibits a certain dose-dependent effect. Research by Zhang (52) et al. demonstrated that a specific dose of *Lactiplantibacillus plantarum* BF_15 could protect mice from a reduction in paw swelling and serum hemolysin antibody levels, thereby enhancing cellular and humoral immune function in mice. Zeng (53) et al. found that oral administration of *Lactiplantibacillus plantarum* expressing aCD11c can regulate cellular and humoral immunity, as well as enhance mucosal immunity. Studies have also shown that *Lactiplantibacillus plantarum* enhances cellular and humoral immunity in Paralichthys olivaceus (54, 55).

The integrity of the intestinal barrier is essential for maintaining overall health and is influenced by several key factors (56).Goblet cells maintain the mucosal barrier by producing mucin (57), while a higher villus height (VH) to crypt depth (CD) ratio indicates enhanced epithelial renewal, nutrient absorption, and barrier function (58, 59). The combination of *Lactiplantibacillus plantarum* CCFM8661 and stachyose can significantly counteract the damage to intestinal barrier integrity caused by
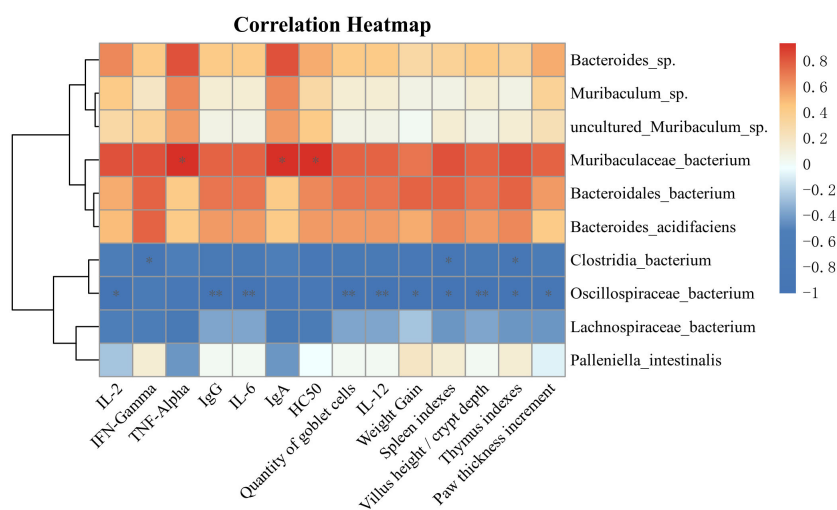

FIGURE 6
The Spearman method was employed to examine the correlations among immune-related indicators, intestinal barrier metrics, cytokines, and the expression of predominant bacteria within the intestinal microbiota. *, ** and *** (*P*<0.05, *P*<0.01 and *P*<0.001, respectively) indicate significant associations.

immunosuppressants. Rawling (60) et al. found that a probiotic combination of *Lactiplantibacillus helveticus* and *Lactiplantibacillus helveticus* enhanced the intestinal barrier function and innate immunity of healthy zebrafish, with its mechanism linked to the growth of intestinal villi. Furthermore, Ren (61) et al. found that the combination of stachyose and *Lactiplantibacillus rhamnosus* GG improved intestinal barrier dysfunction caused by acute hypoxic hypoxia by mitigating inflammatory responses and oxidative stress. Zeng (62) et al. suggested that *Lactiplantibacillus plantarum* enhances intestinal immune function by regulating the intestinal barrier in an immunosuppressed mouse model, as evidenced by an increased VH/CD ratio and a higher number of goblet cells.

Immunoglobulins (IgA and IgG) are key markers of humoral immunity, reflecting B cell function (50, 63). Cytokines such as IL-2, IL-6, and IL-12 play vital roles in immune regulation and are associated with the secretion of IFN-γ and TNF-α (63–68). The combination of *Lactiplantibacillus plantarum* CCFM8661 and stachyose can counteract the reduction of cytokines caused by immunosuppressants. Cheng (69) et al. reported that cyclophosphamide disrupts cytokine secretion, a conclusion that was also supported by Xie et al. (70). Research by Meng (71) et al. revealed that *Lactiplantibacillus plantarum* KLDS1.0318 significantly upregulated the levels of various cytokines, effectively restoring abnormal cytokine levels in CTX-induced immunosuppressed mice. Similarly, Kim (47) et al. found that *Lactiplantibacillus plantarum* 200655 exhibited a similar trend in regulating IL-6 and TNF-α. Research has also demonstrated that the combination of stachyose, sheep whey protein, and Fu brick tea polysaccharides significantly upregulated the levels of IL-2, IL-6, and IgG (39). However, it has also been shown that *Lactiplantibacillus plantarum* can alleviate dextran sulfate sodium (DSS)-induced colitis in mouse models by reducing the release of pro-inflammatory cytokines (72, 73). These findings suggest that the regulatory effect of *Lactiplantibacillus plantarum* on cytokines is not a simple increase or decrease but rather a modulation of cytokine levels to restore them to a normal physiological state.

Previous studies have found that intestinal microbiota can induce the activation of immune cells, thereby influencing cytokine production, while immune cells can also mediate the neurogenic response of the intestinal microbiota through cytokines (74, 75). The combination of *Lactiplantibacillus plantarum* CCFM8661 and stachyose significantly improved the reduction in intestinal microbiota species diversity in immunocompromised mice. It regulated the composition and abundance of intestinal microbiota at various levels, thereby restoring intestinal ecological balance. At the phylum level, an imbalance between *Bacillota* and *Bacteroidota* was evident, with an increased *Bacillota/Bacteroidota* ratio, a pattern commonly linked to cyclophosphamide-induced immunodeficiency (76). Restoring the balance between these phyla may be a key indicator of improved immune function. *Lactiplantibacillus plantarum* CCFM8661 and stachyose restored the imbalance of the *Bacillota/Bacteroidota* ratio. At the species level, changes in various bacterial strains were observed in the immunosuppressed model, notably a significant decrease in *Muribaculaceae bacterium* and an increase in *Lachnospiraceae bacterium*. The combination of *Lactiplantibacillus*

plantarum CCFM8661 and stachyose modulated the abundance of these bacteria, gradually restoring them to normal levels in a dose-dependent manner. Consistent with previous studies, *Muribaculaceae bacterium* and *Lachnospiraceae bacterium* play important roles in immune function. Chen (77) et al. found that *Muribaculaceae bacterium* was significantly down-regulated after the imbalance of immune-driven flora in immunodeficient mice. Previous studies have shown that sea cucumber tendon polysaccharide (SCTPII) can enhance immunity by regulating the diversity of intestinal microbiota. In the process of regulation, the homeostasis of intestinal microbiota is improved by reducing the relative abundance of *Lachnospiraceae* NK4A136 group (78).

However, this study still has some limitations. First, the potential effects of stachyose on the growth, viability, and metabolism of *Lactiplantibacillus plantarum* CCFM8661 were not thoroughly explored, which may influence their pharmacological effects under different combination conditions. Future research should further investigate these aspects, especially the specific effects of stachyose on the biological characteristics of probiotics. Second, there are certain physiological differences between the intestinal microbiota and immune system of mouse models and humans, which may reduce the general applicability and extrapolation of the experimental results. Studies should validate the practical application of this combination based on the human intestinal microecological environment and immune system.

5 Conclusion

The combination of *Lactiplantibacillus plantarum* CCFM8661 and stachyose may enhance immunity through multiple pathways, including mitigating immunosuppressant-induced atrophy of immune organs, intestinal barrier damage, cytokine imbalance, and intestinal microbiota dysbiosis. With the increasing use of probiotics, this study provides valuable insights into the immune-regulating mechanisms of *Lactiplantibacillus plantarum* CCFM8661 combined with stachyose. This research could lead to further optimization and promotion of this therapeutic approach as a novel strategy in the field of immunomodulation. Additionally, this combination may also hold significant potential in anti-inflammatory and intestinal health applications.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: PRJNA1168472 (SRA).

Ethics statement

The animal study was approved by Animal Ethics Committee of Heilongjiang University of Traditional Chinese Medicine (ethical approval code: 2024032915). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

WM: Data curation, Funding acquisition, Writing – original draft. HS: Data curation, Writing – original draft. LL: Formal analysis, Writing – review & editing. LG: Formal analysis, Writing – review & editing. YW: Formal analysis, Writing – review & editing. LH: Formal analysis, Funding acquisition, Supervision, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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