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Quality features and immunomodulatory activity of transglutaminase-modified bovine-soy milk fermented by *Lactiplantibacillus plantarum* AHQ-14

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ABSTRACT: Fermented dairy products are gradually gaining more attention as functional foods with potential immune-related benefits, but mixed protein systems still face challenges in maintaining stable structure and biological activity. In this study, *Lactiplantibacillus plantarum* AHQ-14, a probiotic with good proteolytic ability, and transglutaminase (TG) were used to ferment and modify bovine-soy milk, and their effect on the texture properties and potential immunomodulatory function of the corresponding product were evaluated. The results indicated that TG modification compensated for the quality shortcomings of the fermented milk produced by single probiotic. In *vitro* experiments demonstrated that the fermented bovine-soy milk prepared by *L. plantarum* AHQ-14 combined with TG (AT group) modulated macrophage responses and increased the production of IL-1β, TNF-α, and NO. Furthermore, in *vivo* experiments with cyclophosphamide-induced immunosuppressed mice showed that the AT group alleviated immune organ damage, promoted splenic lymphocyte proliferation, partially restored serum cytokine and immunoglobulin levels, and rebalanced the intestinal microbiota composition. These findings suggest that the fermented bovine-soy milk prepared with *L. plantarum* AHQ-14 and TG may serve as a candidate functional fermented product with immunomodulatory potential.

Keywords: *Lactiplantibacillus plantarum*, transglutaminase, quality characteristics, immunomodulatory effect

1. Introduction

Recently, fermented milk products, particularly those containing probiotics, have attracted considerable attention due to their potential immunomodulatory properties and gut health-promoting effects. Fermented dairy products serve as the most effective vehicle for delivering probiotics. A sufficient intake of probiotics contributes to maintaining body health[1].

Lactiplantibacillus plantarum is a well-known probiotic recognized for its ability to survive in the gastrointestinal tract and modulate immune responses. Fang et al.[2] reported that *L. plantarum* CCFM8610 enhanced antioxidant enzyme activity, suppressed oxidative stress responses, modulated the release of inflammatory factors such as TNF-α, IL-1β, and IL-6 in the gut, reduced intestinal damage, repaired the intestinal barrier, and improved gut health across multiple dimensions. Zhao et al.[3] demonstrated that

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maternal rats consuming *L. plantarum* S58 during the perinatal period exhibited reduced inflammatory cytokine levels and a lower incidence of immune-related diseases in their offspring.

It is worth pointing out that an increasing number of researchers are dedicated to producing compound protein foods that integrate desirable nutritional and functional characteristics. For example, cheese powders prepared from soy and bovine milk and modified with flavorzyme, peptidase R, and lipase MER exhibited a potential antihypertensive effect[4]. The soy-cow milk was treated with commercially lyophilized LAB starter culture and microbial transglutaminase, effectively reducing the antigenicity of β-lactoglobulin[5]. Lactic acid bacteria (*Streptococcus thermophilus* ST5, *Bifidobacterium longum* R0175, and *Lactobacillus helveticus* R0052) fermented soy and cow compound milk, which demonstrated that the frozen fermented products significantly reduced TNF-α-induced secretion of the proinflammatory cytokine (IL-8) by intestinal epithelial cells, thereby exerting immunomodulatory effects[6]. *Lactobacillus* and *Bifidobacterium* (*L. rhamnosus* ATCC 7469, *L. casei* ATCC 393, *L. plantarum* ATCC 14917, *L. acidophilus* ATCC 4356, *B. longum* DSM 20219, and *B. lactis* DSM 10140) fermented soy milk regulated intestinal immune responses and alleviated symptoms of colitis[7]. As mentioned above, the compound proteins of soy and bovine milk exhibit excellent processing characteristics and high nutritional value.

Additionally, the gel structure of conventional fermented milk relies primarily on relatively weak non-covalent interactions (electrostatic interactions, hydrogen bonding, and hydrophobic bonds), which are regarded as a major cause of textural defects such as low viscosity and hardness[8]. Transglutaminase (TG) can cross-link many protein molecules, thereby enhancing texture and stability, synergism, emulsification properties, gelation, and water-holding capacity of food products without altering their pH, color, taste, or nutritional quality[9]. In our previous study, we found that direct-vat-set starter (DVS) fermented bovine-soy milk with TG exhibited better texture, viscosity, gel structure stability, and improved sensory attributes[10].

Moreover, we also isolated *L. plantarum* AHQ-14 from traditional dairy products in the Xinjiang district of China. It exhibited low allergenicity to β-lactoglobulin and possessed excellent probiotic properties and protein-hydrolyzing capabilities[11, 12]. Furthermore, co-fermentation with *L. plantarum* AHQ-14 and DVS markedly reduced the antigenicity and IgE-binding ability[13]. As mentioned above, two key questions also remain to be explored: whether *L. plantarum* AHQ-14 can be effectively applied in bovine-soy milk fermentation and how TG influences its texture. More importantly, the immunomodulatory activity of the resulting product also needs further comprehensive evaluation.

Therefore, this study explores the effects of *L. plantarum* AHQ-14 fermented bovine-soy milk with TG modified on the quality properties. Furthermore, the effect of *L. plantarum* AHQ-14 fermentation combined with TG treatment on the immune-related biological activities of the fermented bovine-soy milk was investigated *in vitro* and *in vivo* by a combination of macrophage model and immunosuppressed mice model.

2. Materials and methods

2.1 Preparation of microorganism

L. plantarum AHQ-14 used in this study was isolated from traditional dairy products in the Xinjiang district, China. It is stored at the School of Food Science and Technology, Dalian Polytechnic University (Dalian, China). The direct-vat-set (DVS) starter cultures contained *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp. *Bulgaricus* (Hirschholm, Denmark). The *L. plantarum* AHQ-14 was added to sterile MRS medium (Solarbio Science & Technology Co., Ltd., Wuhan, China) at 4% (v/v) and incubated at 37℃ for 24 h. The *L. plantarum* AHQ-14 was activated for two generations and prepared for use.

2.2 Preparation of the fermented bovine-soy milk

According to the method of a previous study with some modifications to prepare the fermented bovine-soy milk[14]. Briefly, the soybeans were soaked in distilled water for 12 h and ground together with 10 times the volume of distilled water in a blender, then filtered to produce the soy milk and finally boiled for 20 min to remove the beany flavor. Next, soy milk and bovine milk were mixed at a ratio of 1:1 (v/v) and heated at 95℃ for 5 min to sterilize.

After the compound milk was cooled, the compound milk was divided equally into five groups. The first group (UM) was unfermented compound milk; the second group (A) was compound milk inoculated with *L. plantarum* AHQ-14 at a ratio of 4% (v/v); the third group (AT) was compound milk inoculated with *L. plantarum* AHQ-14 at 4% (v/v) combined with TG addition (1 U/g protein); the fourth group (F) was compound milk inoculated with DVS at a ratio of 0.2 U/L according to the manufacturer's instructions; and the fifth group (FT) was compound milk inoculated with 0.2 U/L DVS combined with TG addition (1 U/g protein)[13]. For the TG-modified groups, TG was first added to the cooled compound milk at 1 U/g protein. After TG treatment, the samples were heated at 85℃ for 5 minutes to inactivate TG, then cooled to inoculate with *L. plantarum* AHQ-14 or DVS. All compound milk samples were cultured at 42℃ until the pH reached 4.6 and stored at 4℃ for 48 h before further analysis.

2.3 Quality properties of the fermented bovine-soy milk

2.3.1 Texture properties

The texture profile analysis (TPA) of the fermented bovine-soy milk samples was conducted using the texture analyzer (TA.XT PLUS, Stable Micro Systems, Surrey, UK). Referring to the experimental parameters of Wang et al.[15] with made appropriate modifications. The height (60 mm) was calibrated before testing. Test conditions: cylindrical probe (P/0.5); test speed of 5 mm/s; test depth of 10 mm. The samples of hardness (g), consistency (g·s), cohesiveness (g), and viscosity index (g·s) were recorded. Results were presented as the average of three measurements.

2.3.2 Rheological properties

Rheological properties of the fermented bovine-soy milk samples were measured by a KINEXUS rotational rheometer (Malvern Instruments, Shanghai, China). Steady shear tests were conducted over a

range of shear rates from 1 s^{-1} to 1000 s^{-1} using the method of Carboni et al.[16], recording the variation of shear viscosity with shear rate for the samples. According to Fan et al.[17], the variations of elastic modulus (G') and viscous modulus (G") within the range of 0.1-10 Hz were recorded. All measurements were conducted at 25℃.

2.3.3 Water holding capacity (WHC)

According to Li et al.[18], with some appropriate modifications. First, the mass of the fermented bovine-soy milk samples was recorded. Subsequently, the fermented milk sample was centrifuged at 1700×g for 5 min. Finally, the supernatant was discarded, and the mass of the residue was recorded. WHC was calculated using the following formula:

$$WHC=\frac{M_1}{M_2}\times 100\%$$

Where M_1 is the mass of the residue after centrifugation and discarding of the supernatant, and M_2 is the mass of the fermented bovine-soy milk sample before centrifugation.

2.3.4 Sensory Evaluation

The sensory evaluation panel consists of ten trained assessors. Each assessor evaluated the colour (10 points), flavour (20 points), texture (20 points), and overall acceptability (50 points) of fermented compound milk using the sensory evaluation method described by Lin et al.[10]. Each panel member independently assessed the provided samples. Before tasting each sample, assessors cleansed their mouths with purified water to minimize the impact of sensory carryover.

2.4 Immunomodulatory activity in vitro

2.4.1 The proliferative activity of RAW 264.7 cells

RAW 264.7 cells in the logarithmic growth phase were collected and then seeded at a density of 2×10^5 cells/well in a 96-well plate. The supernatant of the fermented milk was collected by centrifugation, sterilized by filtration through a 0.22 μm film, and then lyophilized. The cells were then assigned to different groups and treated as follows. The control group (NC, cultured in complete medium only), the positive control group (LPS, stimulated with 1 μg/mL lipopolysaccharide), and the experimental groups (UM, A, AT, F, and FT) treated by lyophilized supernatant powder at 250 μg/mL and 500 μg/mL diluted with DMEM medium, respectively. Then, all groups were added to a 96-well plate at 100 μL/well. After continuing culture for 24 h, 10 μL of cell counting kit-8 (CCK-8, Beijing Solaibao Technology Co., Ltd., Beijing, China) solution was added to each well, and the absorbance of macrophages was detected at 450 nm. The proliferation rate of the cells was calculated by the following equation[19]:

$$\text{Macrophage proliferation rate}(\%)=\frac{\text{Treatment group}_{OD}}{\text{Blank group}_{OD}}\times 100\%$$

2.4.2 Determination of cytokine secretion in RAW 264.7 cells

Referring to the method of Yang et al.[20] with some modifications. In short, RAW 264.7 cells were seeded at 5×10^5 cells/well and incubated at 37℃ in a 5% CO_2 incubator for 24 h. The cells were separately treated with the supernatants of UM, A, AT, F, and FT groups at 250 μg/mL. LPS group was treated with 1 μg/mL LPS, and NC group was treated with an equal volume of cell culture medium. ELISA kit (Langton Biotechnology Co., Ltd., Shanghai, China) was used to detect the expression levels of IL-1β, TNF-α, and NO.

2.5 Immunomodulatory activity in vivo

2.5.1 Animals and experimental design

Eighty male BALB/c mice, which were pathogen-free and had an average body weight of 15 to 18 g, were selected for the experiment. The experimental mice had free access to food and water during the 7-day adaptation period. The experimental mice were subsequently randomly divided into eight groups (n=10) using a random number table. An immunosuppressed mouse model was established according to Jiang et al.[21] and Yu et al.[22]. The next 3-day were the molding period, all groups except the control group (NC) were intraperitoneally injected with cyclophosphamide (Cy, 80 mg/kg·BW), whereas the NC group received an equal volume of normal saline. During the last 7-day of the experiment, the control group (NC) and the model group (M) were gavaged with an equal volume of normal saline; the positive control groups (PC) was gavaged levamisole hydrochloride at 40 mg/kg·BW; and the five experimental groups (UM, A, AT, F, and FT) were gavaged the fermented bovine-soy milk at 80 mg/kg·BW.

The body weights of the mice were recorded every two days until the end of the experiment. On the 11th day, all mice were weighed and euthanized, and their organs, including spleen and thymus, were excised and weighed.

All animals were provided by Liaoning Changsheng Biotechnology Co., Ltd. (Benxi, China). Mice were conducted in accordance with the National Guidelines for Experimental Animal Care and Use, and all animal procedures were approved by the Animal Ethics Committee of Dalian Polytechnic University (Animal Experiment License DLPU2023123). Animals were monitored and fed daily throughout the experiment, and no accidental deaths occurred.

2.5.2 Immune organ index and histomorphology of the spleen

The immune organ index was calculated according to the method of Zhao et al.[23], which was calculated as organ weight/body weight (mg/g). The spleens were fixed and sectioned according to the method of Chen et al.[24], and then stained using the H&E method to observe the histopathological changes.

2.5.3 Spleen lymphocyte proliferation in vitro

The spleen was cut into small pieces and added to the erythrocyte lysate after gently squeezing on a 200-mesh stainless steel sieve. After being incubated and centrifuged at room temperature, the cells were resuspended in RPMI 1640. The resuspended splenocytes were seeded in a 96-well plate at a density of 1×10^5 cells/well, and the final concentration of 5 μg/L Concanavalin A (Con A) was added. The proliferation of splenocytes was detected by CCK-8 kit (Beijing Solaibao Technology Co., Ltd., Beijing, China) at 450 nm after incubation at 37℃ for 48 h [25].

2.5.4 Determination of splenocyte apoptosis

Splenocytes, as described in Section 2.5.3, were collected and stained with Annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) using a commercial kit (Nanjing Novozyme Medical Technology Co., Ltd., Nanjing, China). Cells were analyzed on NovoExpress flow cytometer (Agilent Technologies, Hangzhou, China) [26] equipped with a 488 nm argon laser. Splenocytes were gated on FSC-A/SSC-A (FSC = 98, SSC = 58). FITC (green) was detected in the FL1 channel, and PI (red) was detected in the FL3 channel. For each sample, 10,000 events were acquired. Apoptosis was assessed by Annexin V-FITC vs PI dot plots using FlowJo software.

2.5.5 Assay of cytokines and immune globulins in serum

ELISA kit (Langton Biotechnology Co., Ltd., Shanghai, China) was used to determine the tumor necrosis factor (TNF-α), interleukin (IL-1β), and immunoglobulins (IgA, IgE, and IgM) in the serum.

2.5.6 Analysis of gut microbiome

Microbial total genomic DNA from the feces was extracted using a TIANamp Stool DNA kit. The V3-V4 region of the bacterial 16S rRNA gene was amplified by PCR using a forward primer 338F (5′-ACTCCTACGGGAGGCAGCAG-3′) and a reverse primer 806R (5′-GGACTACHVGGGTWTCTAAT-3′), and sequencing was performed on the Illumina MiSeq-PE250 platform (Illumina, USA). Raw sequencing data were processed on the Majorbio Cloud Platform (Meiji Biomedical Technology Co., Ltd., Shanghai, China). Alpha-diversity was evaluated using the Chao and Shannon indices. Beta-diversity was assessed by principal coordinates analysis (PCoA) based on the OTU-level distance matrix.

2.6 Statistical analysis

The fermented bovine-soy milk was prepared in three independent batches, and the physicochemical analysis and cell experiments were independently repeated three times. All data was expressed as mean ± standard deviation. SPSS 22.0 was used for statistical analysis. The statistical significance of differences among groups was assessed by one-way analysis of variance (ANOVA), followed by Duncan's multiple range test. P-value < 0.05 was considered significant.

3. Results and discussion

3.1 Quality characteristics of the fermented bovine-soy milk

3.1.1 Texture properties

TPA is commonly used to evaluate the textural characteristics of food. However, texture is not only influenced by food composition but also plays a crucial role in food quality evaluation[27]. As shown in Table 1, A group (A) exhibited relatively weak texture compared to F group (F) did, which may be influenced by microbial species. However, TG addition (AT and FT) notably enhanced their textural strength ($P < 0.05$). FT group demonstrated the highest hardness and viscosity index among all groups, reflecting the strong gel-forming ability of commercial starter when combined with TG. But AT still showed significant improvement compared to A ($P < 0.05$), with its hardness and index of viscosity increasing by 2.8- and 3.2-fold. It indicated that microbial acidification and proteolytic action alone were insufficient to form a protein network with high mechanical strength. TG might play a key role in strengthening the protein matrix. Temiz et al.[8] reported that microbial TG improved the firmness of probiotic fermented milk produced from a mixture of cow milk and soy drinks, suggesting that TG promoted protein cross-linking and formation of a denser gel network. García-Gómez et al.[28] prepared yogurt from skim milk treated with microbial transglutaminase and found that both hardness and viscosity increased significantly during extended storage time. Zhang et al.[29] used hydrogel nanoparticles cross-linked with highly catalytic TG and observed the cohesiveness strength was enhanced in the hydrogels. These findings were consistent with the results of our study.

Briefly, TG generally enhanced the texture of fermented bovine-soy milk, and AT group demonstrated a superiority over F group in hardness and viscosity index ($P < 0.05$), exhibited a moderate texture.

Table 1. Texture properties of compound fermented bovine-soy milk

Sample	Firmness/g	Consistency/(g·s)	Cohesiveness/g	Index of Viscosity/(g·s)
A	63.94±1.58^{d}	101.34±3.42^{c}	-18.54±2.89^{a}	-14.58±3.27^{a}
AT	242.84±8.84^{b}	305.49±25.50^{b}	-38.22±10.43^{b}	-61.47±5.87^{b}
F	162.48±1.29^{c}	280.79±4.75^{b}	-37.93±1.53^{b}	-21.02±8.74^{a}
FT	402.96±32.85^{a}	617.21±35.22^{a}	-46.39±6.71^{b}	-164.54±11.81^{c}

Values are given as the mean ± standard deviation. Values with the different lowercases within the same column represent significant differences ($P < 0.05$). (A: the fermented bovine-soy milk prepared by *L. plantarum* AHQ-14; AT: the fermented bovine-soy milk prepared by *L. plantarum* AHQ-14 combined with transglutaminase (TG); F: the fermented bovine-soy milk prepared by direct-vat-set (DVS) starter; FT: the fermented bovine-soy milk prepared by DVS combined with TG.)

3.1.2 Rheological properties

The changes in shear viscosity of the fermented bovine-soy milk samples were shown in Fig. 1A. All samples exhibited typical non-Newtonian shear-thinning behavior, in which the shear viscosity decreased with increasing shear rate. During shearing, the molecular chains gradually untangle and align along the flow direction, forming an oriented structure that reduces internal friction and consequently leads to a decrease in shear viscosity[30]. Interestingly, at low shear rates (< 10 s^{-1}), *L. plantarum* AHQ-14 fermented milk (A) exhibited higher apparent viscosity than the DVS fermented milk (F), whereas at higher shear rates (> 10 s^{-1}) the trend was reversed. A formed network structure was sensitive to the shear, whereas F formed a dense gel

network structure, exhibited higher shear stability, and maintained higher viscosity under intense deformation. TG addition markedly modified the rheological response in both systems. Notably, the fermented bovine-soy milk prepared by *L. plantarum* AHQ-14 in combination with TG (AT) exhibited a sudden drop at approximately 10 s^{-1}. This phenomenon indicated a partial network disruption or structural transition within this shear range. The fermented bovine-soy milk prepared by DVS in combination with TG (FT) maintained high viscosity and shear resistance over the entire shear range.

The elastic modulus (G') and viscous modulus (G") represent the elastic and viscous characteristics of the fermented bovine-soy milk[31]. As clearly shown in Fig. 1B, the G' and G" values of the samples increase with enhanced frequency, and the G' values of each group were greater than the G" values. This demonstrated that all samples exhibited a solid-like characteristic, with the network structure of the fermented bovine-soy milk being more stable[32]. A and F groups exhibited the low G' and G" values, indicating the most fragile gel network structure. However, TG addition improved this behavior. It indicated that TG effectively complemented the textural deficiencies of the bovine-soy milk fermented by *L. plantarum* AHQ-14 or DVS alone. This result was consistent with the findings of the texture profile analysis. Overall, these results revealed that TG facilitates the formation of a more robust and stable three-dimensional network structure by catalyzing covalent cross-linking between protein molecules. Masiá et al.[33] discovered that TG enhanced the rheological characteristics of the emulsifying gel produced from pea protein during fermentation. Similarly, Li et al.[34] also observed comparable results in their study on the application of TG in yogurt. These rheological results were consistent with the texture profile analysis, further supporting that TG contributed to the formation of a stronger and more stable protein network in fermented bovine-soy milk.

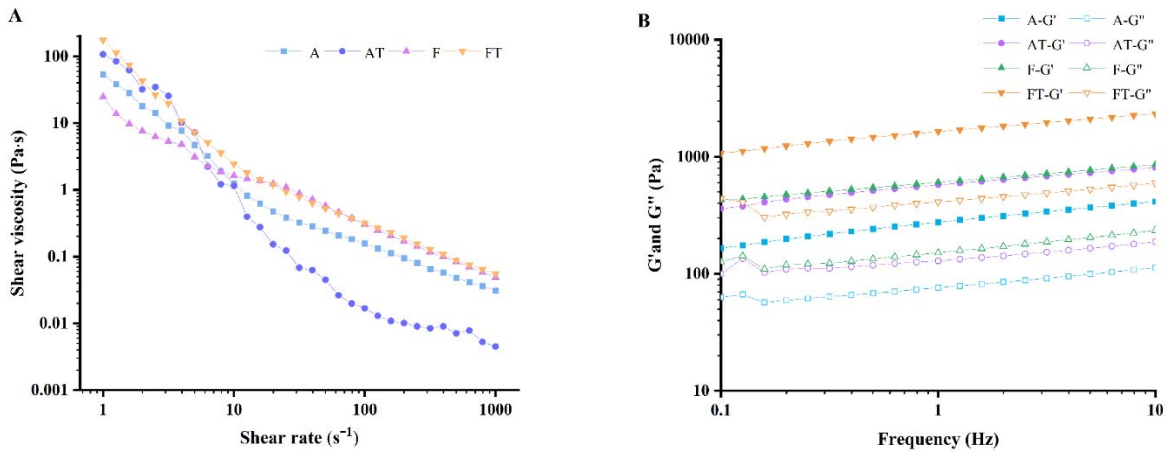


Fig. 1. The effect of the fermented bovine-soy milk on rheological properties. Shear viscosity (A). Frequency sweep (B).

3.1.3 Water holding capacity

The WHC of fermented milk directly depends on its gel network structure formed by protein acidification, while the crosslinking density within this network determines its ultimate water retention ability[35]. Fig. 2 showed WHC in different fermented milk samples. The bovine-soy milk fermented by *L. plantarum* AHQ-14 (A) exhibited significantly lower WHC than DVS (F) ($P < 0.05$), suggesting its gel

network was less effective at the capacity of holding water. TG addition significantly enhanced WHC in both fermentation systems. The AT group showed a marked increase relative to A group, it might be inferred that TG significantly strengthens and refines the microstructure of the gel network by catalyzing covalent cross-linking reactions between protein molecules[36]. This process effectively locks water within the protein matrix, thereby reducing whey separation and significantly enhancing WHC. The above results are in accordance with the results demonstrated by the rheological properties. Although DVS and TG co-fermented bovine-soy milk reached the highest WHC, the effects of the fermented bovine-soy milk prepared with *L. plantarum* AHQ-14 and TG-modified were comparable to those of the former.

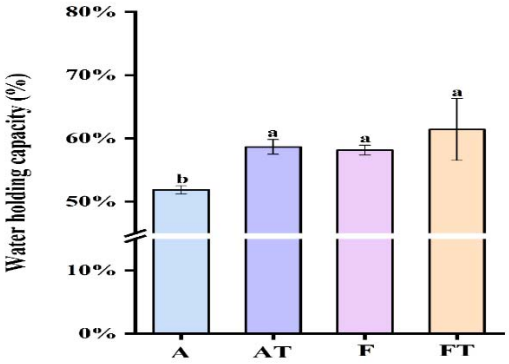


Fig. 2. The effect of the fermented bovine-soy milk on water holding capacity. Values represent mean±standard deviation and different lowercase letters indicated significant differences ($P < 0.05$).

3.1.4 Sensory evaluation

Color is the primary sensory attribute that consumers discern when they bought food. As demonstrated in Fig. 3, *L. plantarum* AHQ-14 fermented milk (A) exhibited weaker performance in both color and texture evaluations when compared with the DVS fermented milk (F). However, no significant differences were observed in flavor and overall acceptability ($P \geq 0.05$). The score of AT was improved compared with A in both texture and flavor, indicating that TG-modified cross-linking compensates for the fragile gel network formed by single-strain fermentation. This result was highly consistent with the aforementioned results of increased hardness, consistency, and G' values. Abdul-Wahed et al.[37] treated meat and dairy products with glutaminase and discovered that glutaminase kept food fresh by greatly improving the taste and smell of dairy products. In contrast, TG was added to DVS fermented milk did not further enhance overall sensory acceptance. Although FT maintained an acceptable flavor and odour, its texture was slightly lower than that of F, indicating that excessive structural fortification may reduce sensory smoothness and palatability.

In conclusion, the fermented bovine-soy milk prepared by *L. plantarum* AHQ-14 and TG (AT) was preferred by sensory assessors in terms of sensory characteristics.

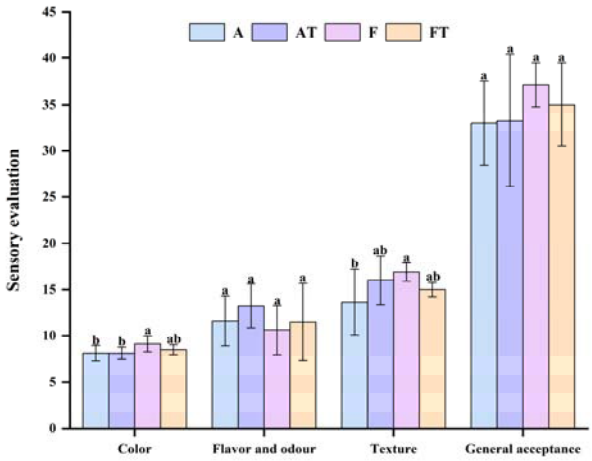


Fig. 3. The effect of the fermented bovine-soy milk on sensory evaluation. Values represent mean±standard deviation and different lowercase letters indicated significant differences among different samples under the same sensory score ($P < 0.05$).

3.2 In vitro immunomodulatory activity of the fermented bovine-soy milk

Macrophages can eliminate a broad spectrum of pathogens and release various chemoattractants and cytokines to enhance the host's resistance to infection[38]. Fig. 4A showed the effect of the fermented bovine-soy milk on RAW 264.7 cell proliferation. RAW 264.7 cell was treated by fermented compound milk samples, the cell proliferation activity was higher than 100%, indicating that the fermented compound milk samples had no toxic effect on the RAW 264.7 cells. At a concentration of 250 μg/mL, except for UM group, the supernatant from the other four fermented groups promoted the proliferation of RAW264.7 macrophages compared with the control group. Notably, AT group showed the strongest promotional effect on macrophage proliferative, reaching 124.17 ± 3.75%.

In addition, the effects of the fermented bovine-soy milk on inflammatory mediator production in RAW 264.7 macrophages were further evaluated. As shown in Fig. 4B-D, compared with the control group, RAW264.7 macrophages stimulated with fermented bovine-soy milk significantly increased the production of IL-1β, TNF-α, and NO, suggesting that the fermented milk modulated macrophage responses. Previous studies have shown that cytokines can not only stimulate macrophage inflammatory responses but also contribute to the resolution of inflammation and tissue repair[39]. Therefore, inflammation may play a dual role in host defense: moderate inflammatory activation may contribute to immune responsiveness, whereas excessive inflammation may be detrimental to tissue stability. Without TG, *L. plantarum* AHQ-14 fermented milk group (A) induced higher IL-1β and NO levels than unfermented milk group (UM) did, suggesting that microbial fermentation may generate bioactive components associated with macrophage activation. Notably, cytokine levels were higher in TG-modified groups (AT and FT) than in their corresponding unmodified counterparts (A and F). Specifically, AT group exhibited the highest levels of IL-1β, TNF-α, and NO, reaching 6.30 ± 0.13 ng/L, 184.56 ± 16.93 ng/mL, and 25.25 ± 0.35 μmol/L, respectively. *L. plantarum* AHQ-14 may have promoted partial proteolysis of proteins and exposed functional peptide sequences potentially related to

immunomodulatory activity[13]. Additionally, TG promoted protein cross-linking[40], which may alter the protein matrix and thereby influence the generation or release of fermentation-derived metabolites and active peptides during co-fermentation. These changes may be associated with the stronger macrophage response observed in the AT group, although the underlying mechanism requires further investigation.

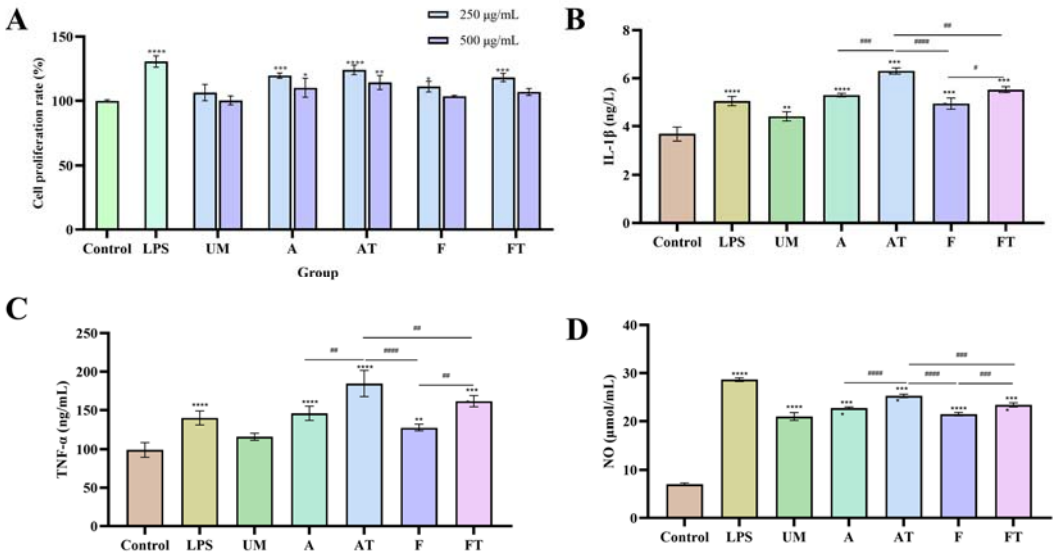


Fig. 4. The effect of the fermented bovine-soy milk on proliferation rate (A), the expression of cytokines (B: IL-1β, C: TNF-α) and the expression of NO (D) in RAW264.7 macrophages. (*, $P < 0.05$ versus control group; **, $P < 0.01$ versus control group; ***, $P < 0.001$ versus control group; ****, $P < 0.0001$ versus control group; significant differences between the experimental groups: #, $P < 0.05$; ##, $P < 0.01$; ###, $P < 0.001$; ####, $P < 0.0001$).

3.3 In vivo immunomodulatory activity of the fermented bovine-soy milk

3.3.1 The effects of the fermented bovine-soy milk on body weight and immune organ indices of immunosuppressed mice

Currently, Cy is commonly used as an antitumor and immunosuppressive agent, and its administration reliably induces immune dysfunction in mice. Therefore, after verifying the *in vitro* immunomodulatory activity, a mouse model of immunosuppression induced by Cy was built to investigate the *in vivo* immunomodulatory activity of the fermented bovine-soy milk and its influence on the intestinal flora in the mice. The animal experimental protocol and group assignments are shown in Fig. 5.

As shown in Fig. 6A, after the treatment of Cy, each group exhibited a remarkable reduction in body weight relative to the NC group, which indicated that the Cy-induced immunosuppressed mouse model was successfully established. After the intake of the fermented bovine-soy milk, the body weight of mice in each group increased more obviously than the model did. The thymus and spleen are critical lymphoid organs reflecting overall immune status[41]. As presented in Fig. 6B-C, compared with the model group, both spleen and thymus indices of fermented groups increased after gavage fermented bovine-soy milk, particularly the spleen index. The spleen and thymus indices in A and F were higher than those in UM, indicating that immune-active components were probably produced during the fermentation process, thereby stimulating the development of lymphoid organs. TG addition further modulated immune organ responses. Compared with A, AT showed increased spleen and thymus indices, while FT exhibited a similar enhancement relative to F. The

improvement in the spleen was obvious in the AT group, which was 2.97-fold higher than that of the model group. Similar immunoprotective effects of bioactive peptides extracted from the skin protein hydrolysate of *Nibea japonica* on splenic and thymic indices were also reported in other Cy-induced immunosuppression models[42]. Research also indicated that hydrolyzed food protein products or peptides played a crucial role in counteracting Cy-induced suppression of the spleen and thymus[43]. The changes in body weight and immune organ indices of immunosuppressed mice revealed that the fermented bovine-soy milk effectively alleviated Cy-induced weight loss and immune organ atrophy in mice.

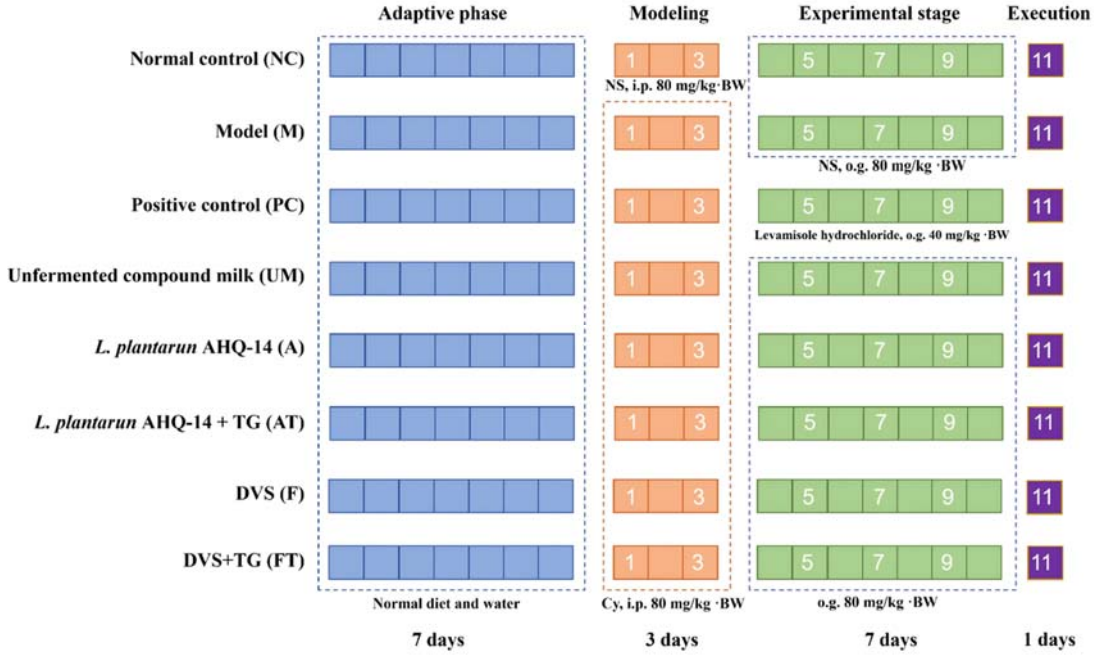


Fig. 5. Animal experimental protocol used in this study. (i.p., intraperitoneal injection; o.g., oral gavage; NS, normal saline; Cy, Cyclophosphamide.)

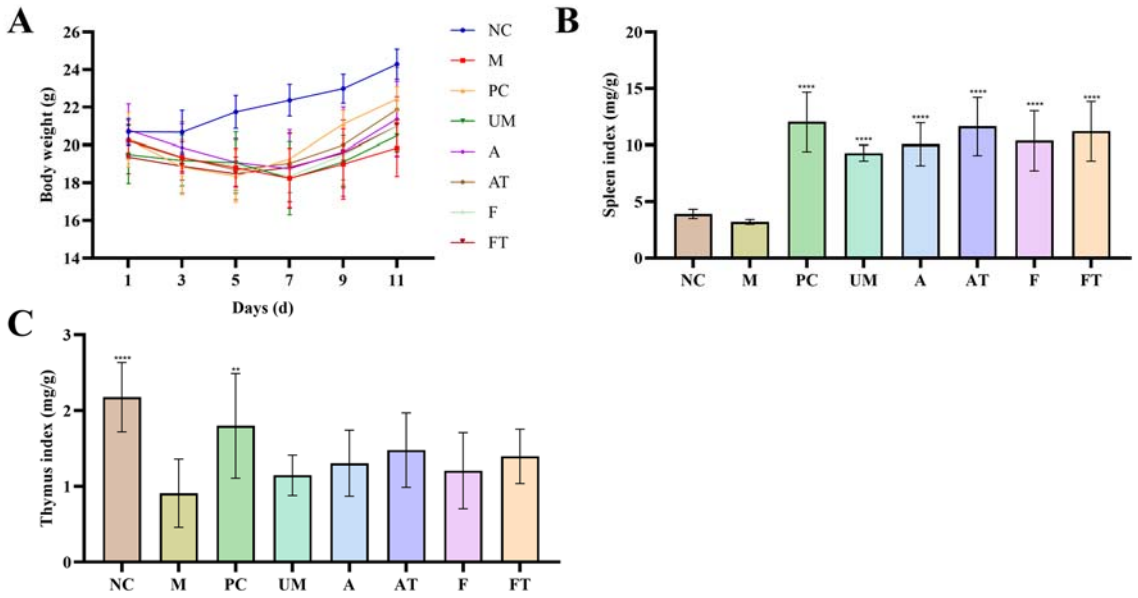


Fig. 6. The effects of the fermented bovine-soy milk on body weight (A), immune organ index (B: Spleen index, C: Thymus index) in Cy-induced immunosuppressed mice. (**, $P < 0.01$ versus model group; ****, $P < 0.0001$ versus model group).

3.3.2 The effects of the fermented bovine-soy milk on splenic histopathology, lymphocyte proliferation, and apoptosis of immunosuppressed mice

The histopathological changes in the mouse spleen induced by the fermented bovine-soy milk were shown in Fig. 7A. Normal group exhibited a homogeneous population of splenocytes with normal morphology. In contrast, the model group displayed disorganization with loosely arranged cells and obvious cell rupture. After gavage of the fermented bovine-soy milk, the number of intact splenocytes increased, intercellular gaps were markedly reduced, and tissue morphology was comparable to that of normal group. Meanwhile, it was found that the morphology of the spleen tissues in AT group was restored to a level more similar to that of the NC and PC groups. To determine whether the structural abnormalities observed in the histopathological analysis were associated with impaired cellular function and survival, splenocyte proliferation and apoptosis were evaluated. The proliferative activity of splenic lymphocytes serves as an important indicator of the activity of the immune response[44]. After the induction of Cy, the splenic lymphocytes in model group showed significant impairment (Fig. 7B). Both A and F significantly promoted splenocyte proliferation compared with UM, suggesting that it might be associated with the production of low molecular weight peptides or other metabolites during fermentation. Although splenic lymphocyte proliferation activity increased after TG addition, the difference was not statistically significant ($P \geq 0.05$). Compared with the model group, the proliferative activity of splenic lymphocytes in A, AT, F, and FT groups was increased by 47.71%, 56.50%, 33.72%, and 44.49%, respectively. Among these, the greatest increase in splenic lymphocyte proliferation was observed in AT group. Thus, the fermented bovine-soy milk prepared by *L. plantarum* AHQ-14 and TG restored splenic lymphocyte proliferation in Cy-induced immunosuppressed mice, thereby alleviating the immunosuppression.

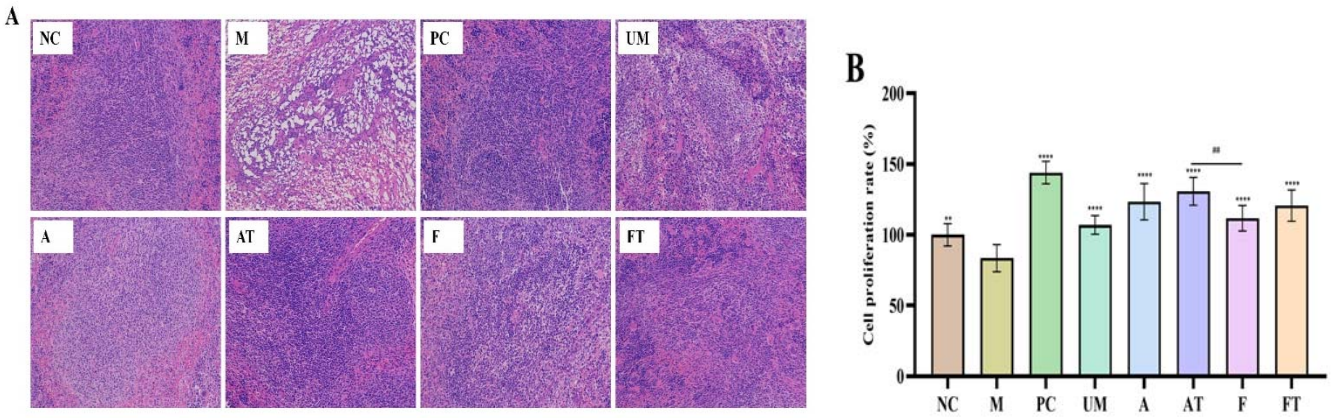


Fig. 7. A: Histopathological evaluation of the effect of the fermented bovine-soy milk on the spleen of immunosuppressed mice (200 × magnification); B: The proliferative activity of the effect of fermented bovine-soy milk on spleen lymphocyte in immunosuppressed mice. (**, $P < 0.01$ versus model group; ****, $P < 0.0001$ versus model group; significant differences between the experimental groups: ##, $P < 0.01$).

The apoptotic rate of splenic lymphocytes is the percentage of immune cells undergoing programmed cell death in the spleen. Excessive apoptosis can impair immune function and lead to immunodeficiency. Therefore, the apoptotic rate of splenic lymphocytes can serve as an indicator of immune competence in

mice[45]. In the scatter plot, Q1-Q4 represented necrotic cells, late apoptotic cells, early apoptotic cells, and normal cells, respectively (Fig. 8A). The apoptosis rate of splenocytes in model group was significantly upgraded to 34.17±1.99% compared with normal group, indicating that Cy induced excessive apoptosis of splenic lymphocytes (Fig. 8B). The gavage of the fermented bovine-soy milk markedly reduced apoptosis. A and F already alleviated apoptosis compared with UM, confirming fermentation processes'

immune protection. Both A and F were modified by TG, further reducing apoptosis compared to A and F, with AT exhibiting the lowest rate of 5.54 ± 0.38%.

Therefore, the fermented bovine-soy milk prepared with *L. plantarum* AHQ-14 and TG effectively protected lymphocytes from Cy-induced apoptosis, thereby preserving immune organ function and alleviating immunosuppression in mice.

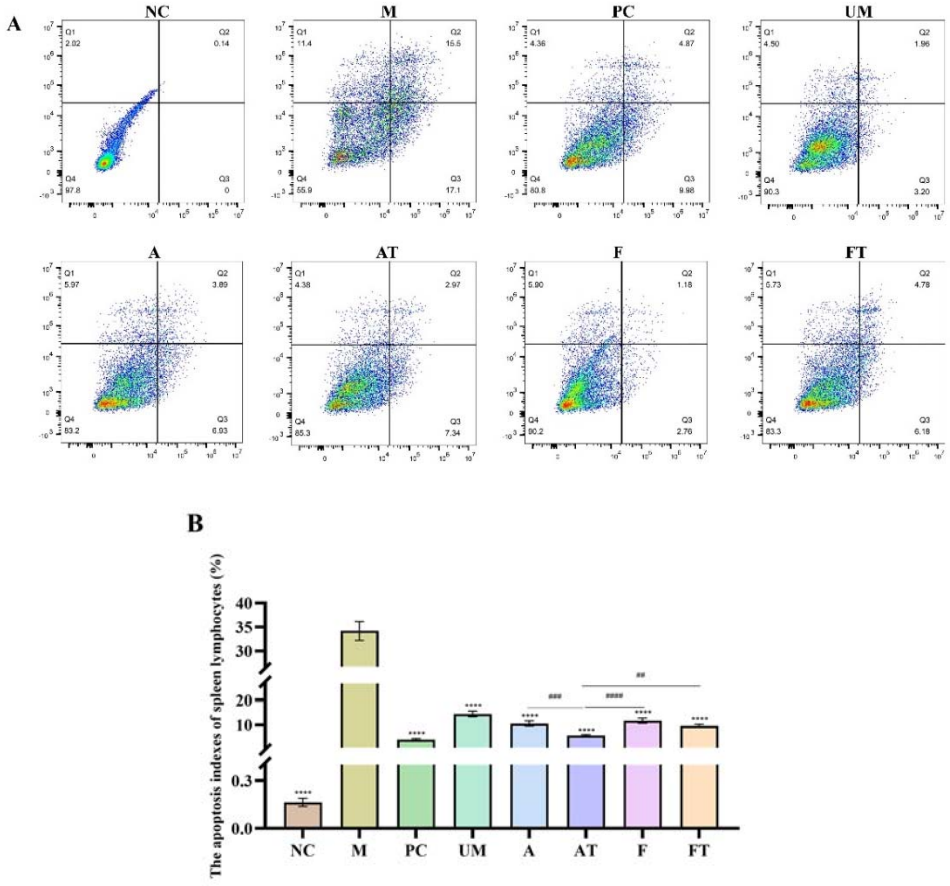


Fig. 8. A: Flow diagram of apoptosis activity of immunosuppressed mice spleen cells. (Q1: necrotic cells, Q2: later apoptotic cells, Q3: early apoptotic cells, Q4: normal cells) B: The effects of the fermented bovine-soy milk on the apoptotic rate of splenic lymphocytes (****, $P < 0.0001$ versus model group; significant differences between the experimental groups: ##, $P < 0.01$; ###, $P < 0.001$; ####, $P < 0.0001$).

3.3.4 The effects of the fermented bovine-soy milk on the expression of serum cytokines in immunosuppressed mice

Cytokines such as IL-1β and TNF-α are key mediators of immune regulation. IL-1β promotes inflammatory responses and immune cell recruitment, while TNF-α acts as a major host defense molecule with broad effects on immune and non-immune cells[46]. Compared with model group, the fermented groups

significantly elevated the serum levels of IL-1β and TNF-α, indicating recovery of immune responsiveness (Fig. 9). A and F not modified by TG improved the expression of IL-1β and TNF-α, especially *L. plantarum* AHQ-14 fermented milk (A). This result was consistent with He et al.[47] research that following treatment with *L. fermentum* F6, there was a significant increase in cytokines, including IL-1β and TNF-α, within a dextran sulfate sodium-induced colitis model group. Meanwhile, AT and FT showed higher IL-1β and TNF-α levels than A and F. It was particularly noticeable that the expression of IL-1β and TNF-α in serum increased to 43.37 ± 3.36 ng/L and 207.41 ± 65.79 ng/L in the AT group, which were 1.48 and 3.51 times over model group, respectively. Similar immunostimulatory trends were reported by Mao et al.[25], who observed elevated serum cytokines in immunosuppressed mice treated with bioactive oligopeptides.

In summary, the fermented bovine-soy milk prepared with *L. plantarum* AHQ-14 and TG relatively restored serum cytokine expression in Cy-induced immunosuppressed mice, possibly by promoting the generation of bioactive peptides and metabolites that stimulate the synthesis of key inflammatory mediators.

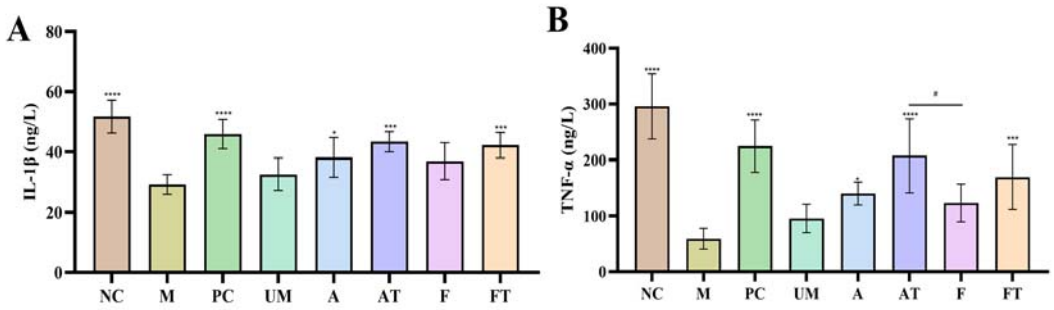


Fig. 9. The effect of the fermented bovine-soy milk on the expression of cytokines in the serum of immunosuppressed mice (*, $P < 0.05$ versus model group; ***, $P < 0.001$ versus model group; ****, $P < 0.0001$ versus model group; significant differences between the experimental groups: #, $P < 0.05$).

3.3.5 The effect of the fermented bovine-soy milk on immunoglobulins in immunosuppressed mice

Immunoglobulins are essential biomarkers of humoral immunity. Among them, IgA serves as the first line of defense of human tissues against pathogens, IgM is the primary antibody in the initial phase of the immune response, and IgE is mainly associated with allergies or parasitic infections. The level of immunoglobulins is a good indicator of the immune activity of the body[48]. As shown in Fig. 10, similar to the changes in serum cytokines, the levels of IgA, IgE, and IgM significantly increased in treatment groups compared with model group. TG modification further elevated IgA, IgE, and IgM in AT compared with A, whereas FT showed only limited improvement over F. The levels of IgA, IgE, and IgM in AT group were significantly increased to 0.62 ± 0.08 mg/mL, 19.29 ± 1.91 U/mL, and 1061.16 ± 163.09 μg/mL, representing 2.21-, 1.28-, and 1.87-fold increases over those in the M group, respectively. This might be due to the proteolytic activity of *L. plantarum* AHQ-14 producing bioactive peptides and molecules or other immunologically active substances[11]. Thus, the fermented bovine-soy milk obtained by the combination of *L. plantarum* AHQ-14 with TG was more effective in enhancing serum immunoglobulin levels in the Cy-induced immunosuppressed mice.

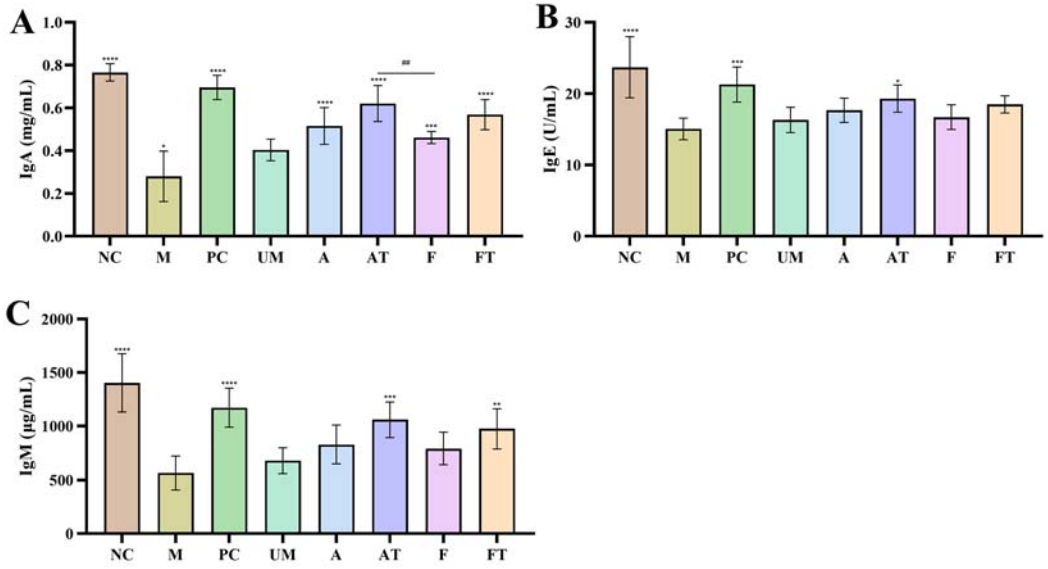


Fig. 10. The effect of the fermented bovine-soy milk on the expression of immunoglobulins in immunosuppressed mice. (*, $P < 0.05$ versus model group; **, $P < 0.01$ versus model group; ***, $P < 0.001$ versus model group; ****, $P < 0.0001$ versus model group; significant differences between the experimental groups: ##, $P < 0.01$).

3.3.6 The effect of the fermented bovine-soy milk on intestinal microbiota in immunosuppressed mice

The colonization of the intestinal microbiota influences the development of the immune system. Cy was known to alter the composition of the intestinal microbiota and increase the permeability of the intestine and impair intestinal mucosal immunity, thereby promoting inflammation[49]. Therefore, this study further explored the changes in gut microbiota composition following fermented milk intervention in Cy-induced immunosuppressed mice.

Alpha-diversity was first assessed using the Chao and Shannon indices (Fig. 11A-B). Overall, Cy treatment altered richness and diversity of the intestinal microbiota, whereas the intervention with fermented bovine-soy milk, particularly AT and FT groups, showed a tendency to restore microbial diversity. These results suggested that the fermented milk partially alleviated the microbiota disturbance induced by Cy.

Beta-diversity was further analyzed by principal coordinates analysis (PCoA) based on the OTU level. As shown in Fig. 12A, PC1 and PC2 explained 17.74% and 9.62% of the total variation. The normal and model groups were clearly separated, indicating that Cy markedly changed the overall microbial community structure. Notably, AT was closer to normal group compared to the other groups in the PC1 distribution (Fig. 12B), suggesting that the fermented milk prepared with TG and *L. plantarum* AHQ-14 may be associated with a partial restoration of the Cy-disrupted gut microbiota structure.

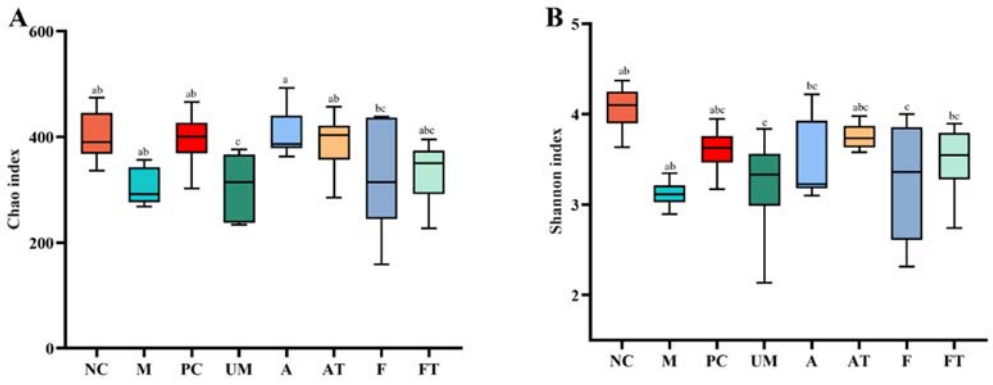


Fig. 11. The effects of the fermented bovine-soy milk on the gut microbiota in immunosuppressed mice. Alpha diversity estimation for gut microbiota from (A) Chao, (B) Shannon of gut microbiota. Different lowercases stand for significant differences ($P < 0.05$).

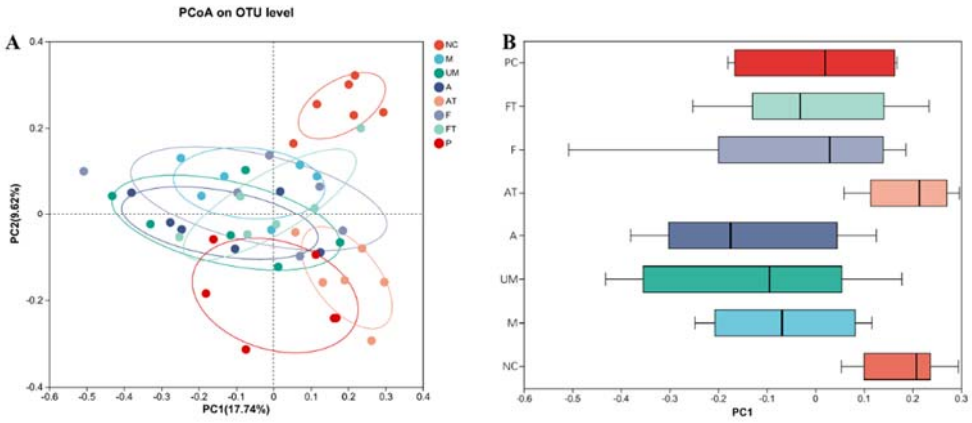


Fig. 12. Beta-diversity estimation for gut microbiota from PCoA (A) and Principal Component 1 (B) of gut microbiota.

To further characterize these community shifts, taxonomic compositions were analyzed at phylum, family, and genus levels. As illustrated in Fig. 13A, the *Firmicutes* and *Bacteroidota* were dominant in all groups. Both *Firmicutes* and *Bacteroidota* can produce short-chain fatty acids by fermentation of dietary fibers, which regulate immune signaling through G-protein-coupled receptors to modulate the immune response[50]. The ratio of *Firmicutes* and *Bacteroidota* (F/B) is commonly used as an indicator of gut microbial balance. In the present study, the F/B ratio was significantly raised in model group after being induced by Cy, suggesting a disturbance in gut microbial structure. Previous studies have reported that alterations in the F/B ratio are associated with disorders such as obesity[51], colitis[52] and immunodeficiency[53]. Notably, the F/B ratio of AT group was lower than that of model group, indicating that AT intervention partially restored Cy-induced gut microbial imbalance. This trend aligns with the observation by Zhao et al.[23], who observed a decreased F/B ratio in healthy mice following treatment with crude polysaccharides from enoki mushrooms.

At the family level (Fig. 13B), Cy treatment reduced the relative abundance of *Muribaculaceae*, *Saccharimonadaceae*, *Bacteroidaceae*, and *Prevotellaceae*, while increasing *Lactobacillaceae*, *Marinifilaceae*, *Tannerellaceae*, *Enterobacteriaceae*, *Lachnospiraceae*, *Oscillospiraceae*, and *Erysipelatoclostridiaceae*. Compared with A, AT showed higher relative abundances of *Muribaculaceae* and *Bacteroidaceae* and a more evident restoration of *Prevotellaceae*. *Muribaculaceae* is positively correlated

with immune response. Specifically, Liu et al.[54] demonstrated that the abundance of *Muribaculaceae* (S24-7) was closely associated with hepatic immune function during liver regeneration, further supporting a positive correlation between this family and host immune response. *Bacteroidaceae* can ameliorate metabolic and immune disorders and can be recognized by TLR4[55], while *Prevotellaceae* is an intestinal commensal bacterium involved in mucus formation and the production of short-chain fatty acids in the mucosal layer of the intestine[56]. These findings are consistent with our observation that AT intervention which improved Cy-induced immunosuppression. In contrast, the relative abundance of *Eggerthellaceae* was lower in the fermented bovine-soy milk intervention groups than the model group did. It has been reported that *Eggerthellaceae* is associated with immune dysregulation[57], this decrease may reflect an improvement in gut microbial balance.

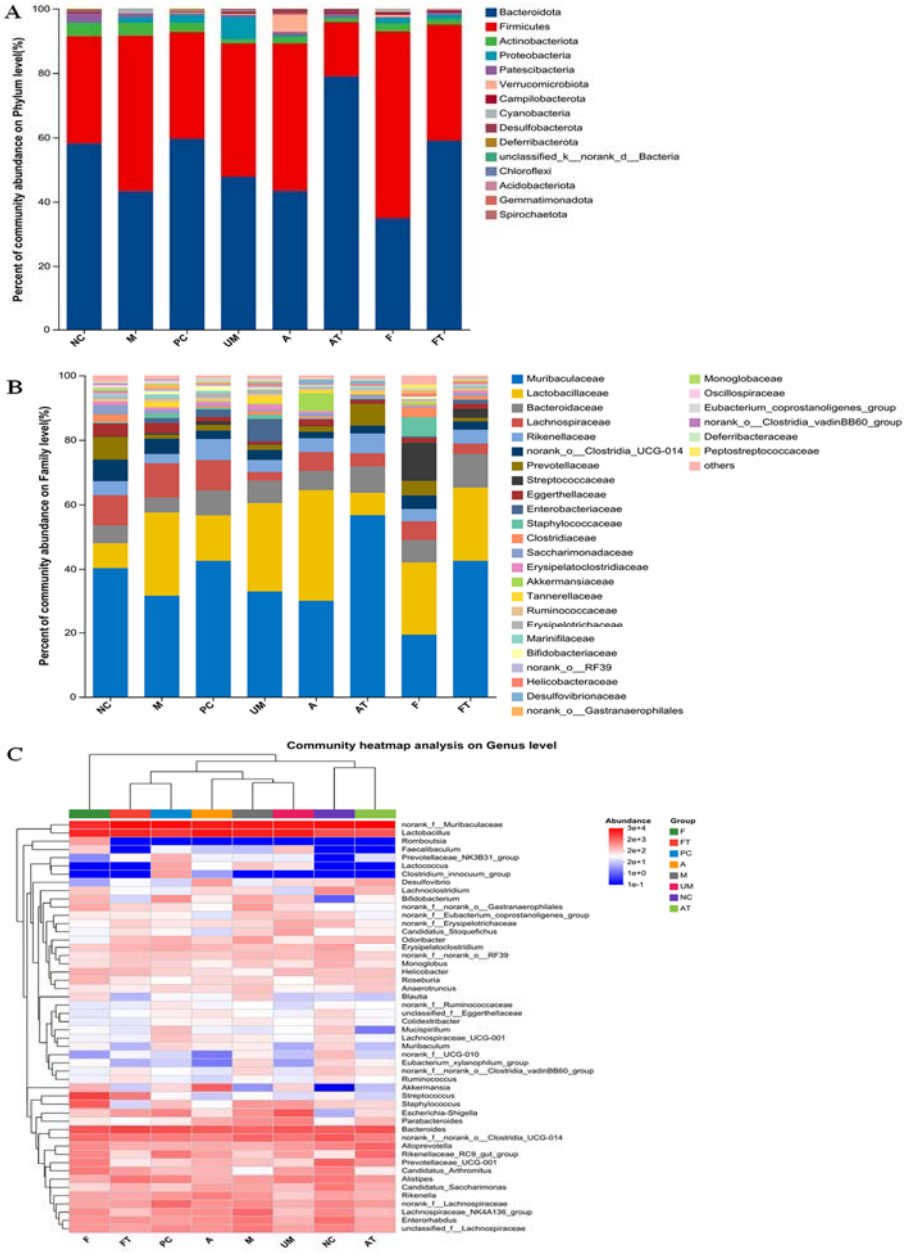


Fig. 13. A: Microbial community stack column plot at the phylum level; B: Microbial community stack column plot at the family level; C: The heatmap of the top 50 dominant genera in each group. (*, $P < 0.05$ versus NC group; #, $P < 0.05$ versus M group; ##, $P < 0.01$ versus M group; ####, $P < 0.0001$ versus M group).

Moreover, the heatmap in Fig. 13C presents the genus-level composition of the gut microbiota. Each column represented a different group, and the color intensity reflected the relative abundance of microbial genera. Compared with model group, AT group showed higher relative abundances of genera such as *Alloprevotella* and *Akkermansia*, together with a lower abundance of *Escherichia-Shigella*. *Alloprevotella* utilizes carbohydrates to ferment and produce short-chain fatty acids to maintain intestinal microbial stability[58], whereas *Escherichia-Shigella* is commonly associated with intestinal dysbiosis. Thus, the microbial changes in the AT group suggested a shift toward a more balanced intestinal microbial community.

Importantly, *L. plantarum* AHQ-14 and TG-modified bovine-soy fermented milk exhibit immune-related effects in Cy-induced immunosuppressed mice, including enhancing splenic lymphocyte proliferation, reducing splenocyte apoptosis, and increasing serum cytokine and immunoglobulin levels. Meanwhile, AT intervention was accompanied by gut microbiota remodeling. Specifically, it increased the abundance of taxa related to short-chain fatty acid production, intestinal homeostasis, and mucosal barrier maintenance, while decreasing taxa associated with gut microbial dysbiosis. These findings suggest that the microbiota shifts observed in the AT group were consistent with the improvement of immune-related parameters. However, current research can only indicate that there is a connection between changes in the microbiota and immune regulation. The specific mechanism of action still requires further research to clarify.

4. Conclusion

This study investigated the feasibility of using *L. plantarum* AHQ-14 combined with TG to develop fermented bovine-soy milk with suitable product quality and immunomodulatory potential. The co-fermented bovine-soy milk prepared with probiotic AHQ-14 and TG may enhance product quality and show immune-related effects in *vitro* and in *vivo*. Furthermore, the immune-related responses observed in *vivo* were accompanied by changes in gut microbiota composition. These findings provide preliminary support for the development of fermented bovine-soy milk as a candidate functional product with immunomodulatory potential. Nevertheless, the present study did not explore the underlying molecular mechanisms nor assess anti-inflammatory cytokines such as IL-10, which limits the interpretation of the immune response. Further research will focus on identifying bioactive metabolites and elucidating their molecular signaling pathways in immune regulation.

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Conflicts of Interest

All authors declare that there is no conflict of interest.

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