# Co-fermented cow milk protein by *Lactobacillus helveticus* KLDS 1.8701 and *Lactobacillus plantarum* KLDS 1.0386 attenuates its allergic immune response in Balb/c mice

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## ABSTRACT

Milk protein is one of the major food allergens. As an effective processing method, fermentation may reduce the potential allergenicity of allergens. This study aimed to evaluate the therapeutic potential of co-fermented milk protein using *Lactobacillus helveticus* KLDS 1.8701 and *Lactobacillus plantarum* KLDS 1.0386 in cow milk protein allergy (CMPA) management. This study determined the secondary and tertiary structures of the fermented versus unfermented proteins by Fourier-transform infrared spectroscopy and surface hydrophobicity to evaluate its conformational changes. Our results showed that different fermentation methods have significantly altered the conformational structures of the cow milk protein, especially the tertiary structure. Further, the potential allergenicity of the fermented cow milk protein was assessed in Balb/c mice, and mice treated with the unfermented milk and phosphate-buffered saline were used as a control. We observed a significant reduction in allergenicity via the results of the spleen index, serum total IgE, specific IgE, histamine, and mouse mast cell protease 1 in the mice treated with the co-fermented milk protein. In addition, we analyzed the cytokines and transcription factors expression levels of spleen and jejunum and confirmed that co-fermentation could effectively reduce the sensitization of cow milk protein by regulating the imbalance of T helper (Th1/Th2 and Treg/Th17). This study suggested that changes of conformational structure could reduce the potential sensitization of cow milk protein; thus, fermentation may be a promising strategy for developing a method of hypoallergenic dairy products.

**Key words:** cow milk allergy, fermentation, *Lactobacillus*, Balb/c mice, protein structure

## INTRODUCTION

Cow milk protein is a nutrient-rich food due to its good functional properties, a widely used processing raw material in the food industry (Kim et al., 2007). However, milk contains antigenic proteins (casein, whey protein) that can induce allergic reactions, including eczema, pulmonary infiltrates, rhinitis, and diarrhea, and is considered one of 8 major allergens by the Food and Agriculture Organization of the United Nations and the World Health Organization (Villa et al., 2018). The incidence of allergies to cow milk is close to 3.6% in Morocco, with an increased incidence compared with recent years (Azdad et al., 2017). So far, eliminating allergens is the most effective way to prevent allergies, but it can be challenging to achieve. However, effective processing methods can reduce sensitization by modifying the immunoreactive components in the cow milk protein.

Several reports based on cow milk protein allergy (**CMPA**) have concluded that CMPA is mainly an IgE immune-mediated reaction based on the IgE binding epitopes of the antigen (Ma et al., 2021). When the allergen enters the body, CD4^{+} T helper (**Th**) cells are activated and induce B cells to produce IgE by secretion of inflammatory factors. Then IgE specifically binds to a high-affinity Fc receptor on the surface of mast cells or basophils that binds to allergen epitopes and triggers the release of inflammatory mediators such as histamine and MCPT-1 (Yang et al., 2009). However, the effect of any food processing method on

sensitization depends on the allergen structure and the nature of the processing method used (Zhang et al., 2016; Gupta et al., 2017). These processing methods inactivate IgE binding epitopes by altering the protein structure. Furthermore, studies have demonstrated that this protein's secondary and tertiary structures are essential for the immune system to recognize epitopes (Gupta et al., 2017; Xue et al., 2019). Shi et al. (2014) reported that a large number of allergic epitopes were cleaved during the fermentation and reduced the IgE binding capacity of α-CN, β-LG, and α-LA to reduce immunoreactivities in vitro. According to Rui et al. (2019), fermentation reduced the IgE reactivity of the soy protein isolates, likely due to changes in the primary and higher structure of the protein. In addition, an in vivo study demonstrated that fermented milk can regulate the Th2/Th1 balance in sensitized mice, thereby stimulating the tolerance process (Wróblewska et al., 2020).

In our previous work, we reported that combined fermentation could utilize protein cooperatively using the complex proteolytic system of lactic acid bacteria to reduce the antigenicity of cow milk protein by destroying the linear epitopes in vitro (Zhao et al., 2021). Therefore, in the present work, we monitored the changes of fermented milk protein advanced structures, including the secondary and tertiary structures, to illustrate the relationship between the conformational epitope changes and hypoallergenic of the fermented cow milk protein. Furthermore, Balb/c mice were administered with the control, unfermented and fermented proteins in separated groups, respectively, and evaluated the potential allergenicity of fermented versus unfermented proteins in vivo. Taken together, this study provides a new strategy for developing a method of hypoallergenic dairy products and a theoretical basis for clinical research.

## MATERIALS AND METHODS

### Fermentation of Skim Milk Protein

Reconstituted skim milk (**RSM**) fermentation was prepared as previously described (Zhao et al., 2021). The groups were given one of the following treatments: (1) the normal control group (**NC**; 0.1 $M$ PBS, pH 7.4); (2) unfermented RSM (**NFRSM**); (3) the fermented RSM by *L. plantarum* KLDS 1.0386 (**FRSM-LAB1**); (4) the fermented RSM by *L. helveticus* KLDS 1.8701 (**FRSM-LAB2**); (5) the co-fermented RSM by *L. helveticus* KLDS 1.8701 and *L. plantarum* KLDS 1.0386 (**FRSM-Mix**).

### Conformational Structures Analysis

*Determination of the Secondary Structure.* Before and after fermentation, the secondary structure of the freeze-dried RSM was determined using the Fourier-transform infrared spectrometer (**FTIR**, Nicolet iS50, Thermo Scientific). Then, the samples were mixed with KBr and pressed into chip (Tian et al., 2020). The spectrum of the protein was selected in the range of 1,700 to 1,600 cm$^{-1}$. Each spectrum was corrected and analyzed by peakfit software (4.12, SeaSolve Software) to calculate the content of the α-helix, β-sheet, β-turn, and random coil.

*Tertiary Structure Determination.* Surface hydrophobicity is important for stabilizing the protein structure within the molecular structure (Lampart-Szczapa et al., 2006), which is determined using fluorescence probe 8-anilino-1-naphthalene sulfonic acid (**ANS**, 8 mmol/L) to monitor the tertiary structure of proteins (Luo et al., 2014; Wang et al., 2014). The freeze-dried RSM before and after fermentation was diluted with PBS (0.01 $M$, pH 7.0) to obtain concentrations ranging from 0.05 to 0.25 mg/mL (0.05, 0.1, 0.15, 0.2, and 0.25 mg/mL). Diluted samples (1 mL) were added with 5 μL of ANS working solution. The mixture was incubated for 15 min in the dark. The sample solution's fluorescence intensity (**FI**) was measured by a fluorescent spectrophotometer (F-7100; Hitachi Ltd.). The excitation and emission wavelengths are 390 nm and 470 nm, respectively. The initial slope of FI was used as the surface hydrophobicity ($R^2 > 0.99$).

### Animal Model Analysis

*Experimental Protocols.* The specific-pathogen-free female Balb/c mice (5–6 wk old) were purchased from the Vital River Laboratory Animal Technology Company. All mice were housed and bred in pathogen-free environments with a 12-h light-dark cycle and given access to cow milk protein-free mouse chow and water. The temperature and relative humidity were maintained at 24 ± 2°C and 50%, respectively.

The experiment protocol was adjusted according to the previous reports (van Esch et al., 2011; Lozano-Ojalvo et al., 2017). Forty mice were randomly divided into 5 groups (n = 8/group) and treated via oral gavage after acclimation for one week. Briefly, on d 0, 7, 14, and 21, the mice were sensitized with the mixture (200 μL) of 20 mg of RSM with 10 μg of cholera toxin (**CT**) as a Th2-polarizing adjuvant (Tianjin Alpha Biotechnology Co., Ltd.; Pablos-Tanarro et al., 2018) as an adjuvant. Control mice were sensitized with the same
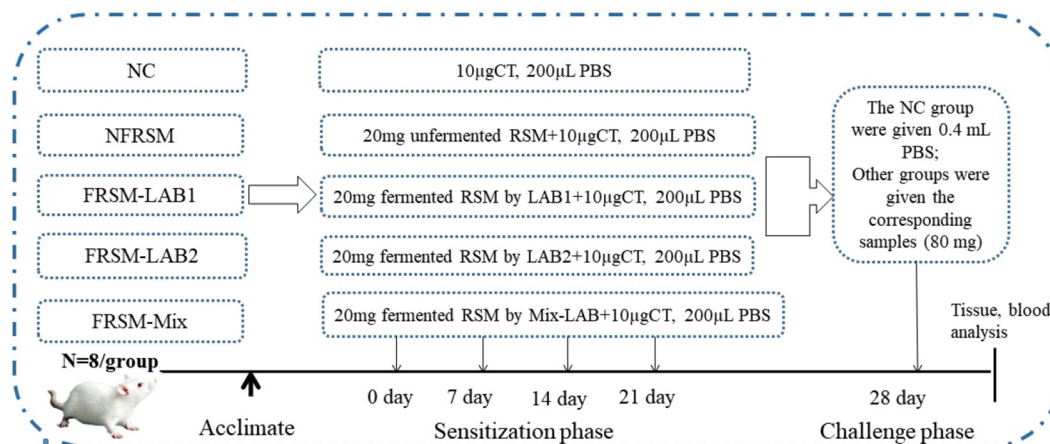
**Figure 1.** Experimental design. CT = cholera toxin; RSM = reconstituted skim milk; LAB1 = *Lactobacillus plantarum* KLDS 1.0386; LAB2 = *Lactobacillus helveticus* KLDS 1.8701; Mix-LAB = *L. helveticus* KLDS 1.8701 and *L. plantarum* KLDS 1.0386.

dose of PBS (including 10 μg of CT). On d 28, the mice were orally challenged with 0.4 mL of 80 mg RSM, and controls were given 0.4 mL of PBS (Figure 1). Then, 30 min after oral challenge, the anaphylactic symptoms in mice were determined by scoring clinical signs (Table 1; Pablos-Tanarro et al., 2018; Zhang et al., 2020). Tissue and blood were collected for measuring the following biomarkers. The blood samples were centrifuged at $2,500 \times g$ for 10 min and stored at $-20°C$ until further experiments.

The Ethical Committee Northeast Agricultural University approved all animal protocols (permit number: NEAUEC20210476).

***Immune Organ Indices.*** At the end of the experiment, the spleens were collected. Then, the weight of the spleens of the mice was accurately weighed. The indices were calculated according to the following formula (Zhang et al., 2016):

$$\text{Organ index (mg/g)} = \frac{\text{organ mass (mg)}}{\text{animal body mass (g)}}.$$

***Total and Specific IgE Antibodies Levels.*** The total IgE levels for serum in different groups of mice were measured using mouse IgE ELISA kit (Kenuodi) following the manufacturer instructions. The results

were expressed in terms of concentration. The specific IgE levels were determined using the indirect ELISA method described previously (Gupta et al., 2017). The 96-well microplates were coated with skim milk protein (concentration: 2 μg/100 μL; volume: 100 μL) and left overnight at 4°C. After incubation, all wells were washed 3 times with PBS containing 0.05% Tween-20 (**PBST**) and blocked with 1.5% gelatin for 1 h at 37°C. Then all wells were washed 3 times with PBST, incubated overnight with diluted sera (1:20) at 4°C, and washed 3 times. Horseradish peroxidase (**HRP**, 100 μL) labeled rabbit antimouse IgE solutions were added to all wells and incubated at 37°C for 2 h. The reaction was developed by adding 100 μL of 3,3′,5,5′-tetramethylbenzidine to each well and incubating at 37°C for 30 min under dark conditions. Then the stop solution (50 μL of 4 $M$ $H_2SO_4$) was added to all wells, and the absorbance was measured at 450 nm using spectraMax reg iD3 (Molecular Device). The results were expressed in terms of the optical density values among the serum.

***Measurement of Serum mMCP-1.*** The mMCP-1 levels for serum in different groups of mice were measured using a mouse mMCP-1 ELISA kit (Kenuodi) following the manufacturer instructions. The results were expressed in terms of concentration.

**Table 1.** Anaphylactic symptom scoring

| Score | Clinical symptom |
|-------|------------------|
| 0 | No symptoms |
| 1 | Scratching and rubbing nose and mouth frequently |
| 2 | Puffiness around the eyes and mouth, increased breathing rate, reduced activity |
| 3 | Wheezing and labored respiration, rash around mouth and tail, diarrhea |
| 4 | No activity after stimulation with loss of consciousness, trembling, and convulsions |
| 5 | Shock or death |

**Table 2.** Primer sequences used in reverse-transcription quantitative PCR assays in proximal jejunum tissue

| Gene | Primer sequence $(5' \rightarrow 3')^1$ |
|------|------------------------|
| Foxp3 | F: TTACTCGCATGTTCGCCTACTTCAG |
| | R: CTCGCTCTCCACTCGCACAAAG |
| RORγt | F: ACAGCCACTGCATTCCCAGTTT |
| | R: TCTCGGAAGGACTTGCAGACAT |
| T-bet | F: ATCACTAAGCAAGGACGGCGAATG |
| | R: ACCAAGACCACATCCACAAACATCC |
| GATA-3 | F: TCTGGAGGAGGAACGCTAATGGG |
| | R: CGGGTCTGGATGCCTTCTTTCTTC |
| GAPDH | F: GACAGCCGCATCTTCTTGTG |
| | R: AATCCGTTCACACCGACCTT |

[1]F = forward, R = reverse.

***Measurement of Plasma Mouse Histamine.*** The histamine levels for plasma in different groups of mice were measured using a mouse histamine ELISA kit (Kenuodi) following the manufacturer instructions. The results were expressed in terms of concentration.

***Histological Analysis.*** Tissue samples were taken for histological analysis as previously described (Azdad et al., 2017). Each group's proximal jejunum and lungs were taken, washed with PBS, and placed in formaldehyde (4%, vol/vol). They were fixed at room temperature (**RT**) for 24 h. Subsequently, the tissue samples were embedded with paraffin, thick sections (5 μm) were made, and stained with hematoxylin-eosin. The tissue morphology was observed by microscopy, and the images were photographed and collected by an automatic imaging system.

***Cytokine Determination Using Spleen Cell Culture.*** Mice spleens in each group were isolated under sterile conditions, and the splenocytes were obtained according to previous studies (Yang et al., 2009). The spleen cells were eventually resuspended in the complete medium (RPMI 1640 medium supplemented with 10% fetal calf serum and 1% antibiotics) and cultured in a 24-well microplate ($2 \times 10^6$ cells/mL). Then the spleen cells were stimulated with 4 mg/mL of skim milk protein, fermented skim milk protein and PBS separately. The microplate was incubated at 37°C for 72 h in a 5% $CO_2$ and 95% air incubator. The spleen cells were centrifuged at $250 \times g$ for 5 min at 4°C. The supernatants from all groups were harvested and stored at −80°C until analysis. The content of IL-4, IL-5, IL-10, IL-17, TGF-β, and IFN-γ in mouse splenocytes were measured using a commercial ELISA kit (Kenuodi) according to the manufacturer instructions.

***Measurement of Proximal Jejunum Cytokines by Real-Time PCR.*** The relative gene expressions of Foxp3, RORγt, T-bet, and GATA-3 were determined by reverse-transcription quantitative PCR (**RT-qPCR**), and *GAPDH* was used as the internal reference gene.

Briefly, according to the manufacturer instructions, total RNA in intestines of all groups was extracted using a FastPure Tissue Total RNA Isolation Kit (Vazyme Biotech Co. Ltd.). Then the cDNA synthesis was performed using the GoScript Reverse Transcription Mix Kit (Promega Biotech Co. Ltd.). The RT-q PCR was performed using Go Taq SYBR-Green qPCR Master Mix (Promega Biotech Co. Ltd.) on the QuantStudio 3 Real-Time PCR System (Thermo Fisher). The relative expressions of target genes in different groups were analyzed by the $2^{-\Delta\Delta CT}$ method. The primers were designed and synthesized by Sangon Biotech Co. Ltd. (Table 2).

### Statistical Analysis

All data are presented as the mean ± standard deviation and analyzed by one-way ANOVA and Duncan's multiple comparison test using SPSS 22.0 software (SPSS Inc.). The graphs were conducted using Graph-Pad Prism 8.0 software (GraphPad Inc.). In all results, $P$-values $<0.05$ were considered to be significant.

## RESULTS

### Effects of Fermentation on the Secondary and Tertiary RSM Structure

We used FTIR and fluorescence spectrum to monitor the fermentation-induced conformational structure alterations of RSM. Deconvoluted FTIR interferogram of RSM and fermented RSM are shown in Figure 2A. Moreover, the corresponding secondary structure of proteins was calculated and presented in Figure 2B. However, compared with the NFRSM group, the content of β-sheet significantly increased in all fermented RSM samples ($P < 0.05$). At the same time, the content of β-turns significantly increased in all fermented RSM samples ($P < 0.05$), and the random coil slightly changed. However, the amount of α-helix decreased significantly in all fermented RSM samples ($P < 0.05$). The fermentation process can convert the α-helix into β-sheet and β-turn. We employed the alterations of the surface hydrophobicity using fluorescence probe ANS to evaluate the change of tertiary structure of RSM, and the results are shown in Figure 2C. We noticed that fermentation could decrease the hydrophobicity ($P < 0.05$) significantly, but the changes among the fermented samples were not significant.

### Anaphylactic Shock Score

Symptoms such as anaphylactic shock and diarrhea were observed in the NFRSM group after gavage (Fig-

ure 3A). However, these symptoms are less severe in the NC and FRSM-Mix groups than in the NFRSM group, and there was no significant difference between the 2 groups.

### Immune Organ Indices

As shown in Figure 3B, the spleen indices of the NFRSM group ($4.64 \pm 0.103$ mg/g) were significantly higher than those of the NC group ($3.484 \pm 0.093$ mg/g; $P < 0.05$). However, fermented-protein groups showed a significant decrease in the spleen indices compared with the NFRSM group. Remarkably, the spleen indexes of the FRSM-Mix group ($3.515 \pm 0.119$ mg/g) were higher than that of the FRSM-LAB1 ($3.776 \pm$

$0.127$ mg/g) and FRSM-LAB2 group ($4.222 \pm 0.171$ mg/g; $P < 0.05$).

### Total and Specific IgE Antibodies Levels

Total and specific IgE antibodies levels were evaluated in the serum of allergic mice, as shown in Figure 3C and D. The results found that both total IgE and specific IgE antibody levels increased significantly in the NFRSM group's serum compared with the NC group ($P < 0.05$). However, significantly reduced levels of total IgE and bovine milk protein-specific IgE were observed in the sera of fermented cow milk protein-treated mice ($P < 0.05$). More importantly, serum total IgE and specific IgE levels in the FRSM-Mix group
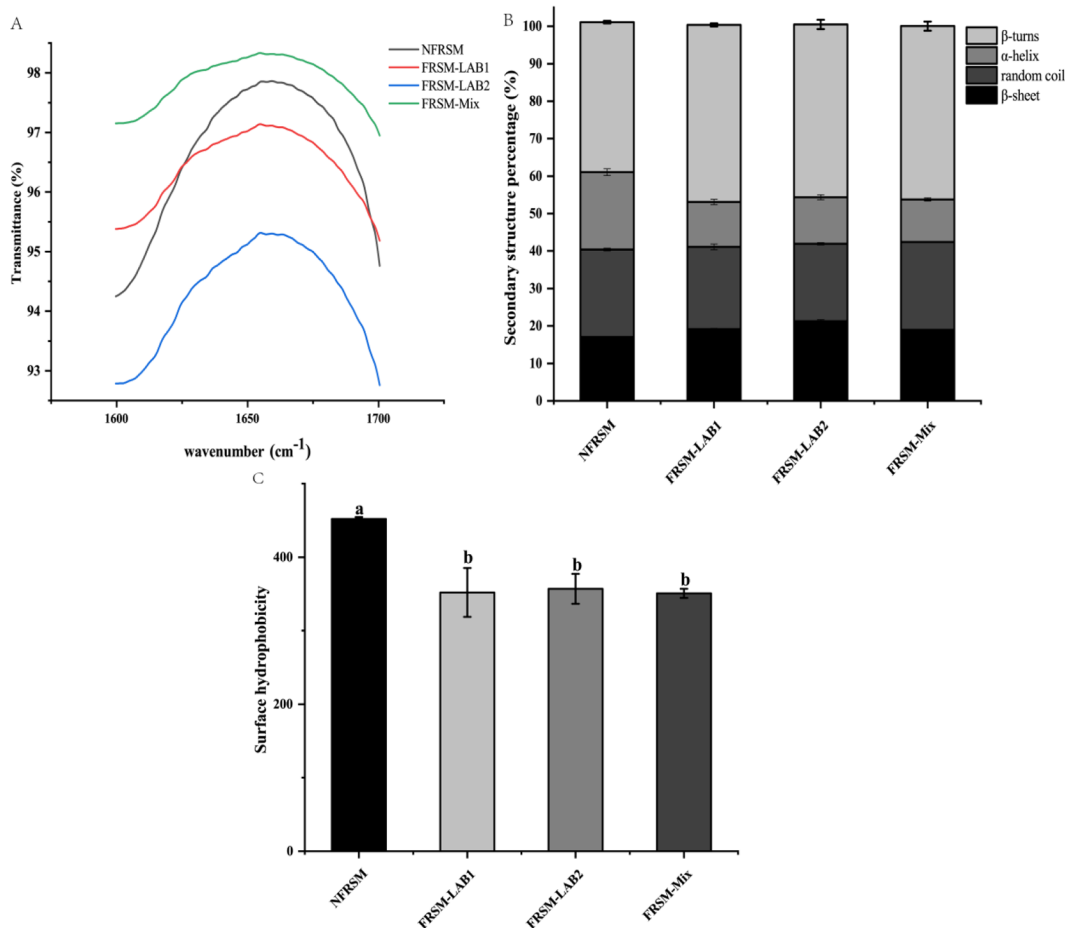


**Figure 2.** Conformational structures analysis. (A) The deconvoluted Fourier-transform infrared spectrometer (FTIR) interferograms of the reconstituted skim milk (RSM) and fermented RSM. (B) The corresponding secondary structure of the RSM and fermented RSM as determined by FTIR. (C) The surface hydrophobicity of the RSM and fermented RSM. All data are presented as the mean $\pm$ SD (n = 3). Different letters indicate significant differences among groups ($P < 0.05$; Duncan's multiple comparison test). NFRSM = unfermented RSM; FRSM-LAB1 = RSM fermented by *Lactobacillus plantarum* KLDS 1.0386; FRSM-LAB2 = RSM fermented by *Lactobacillus helveticus* KLDS 1.8701; FRSM-Mix = RSM co-fermented by *L. helveticus* KLDS 1.8701 and *L. plantarum* KLDS 1.0386.
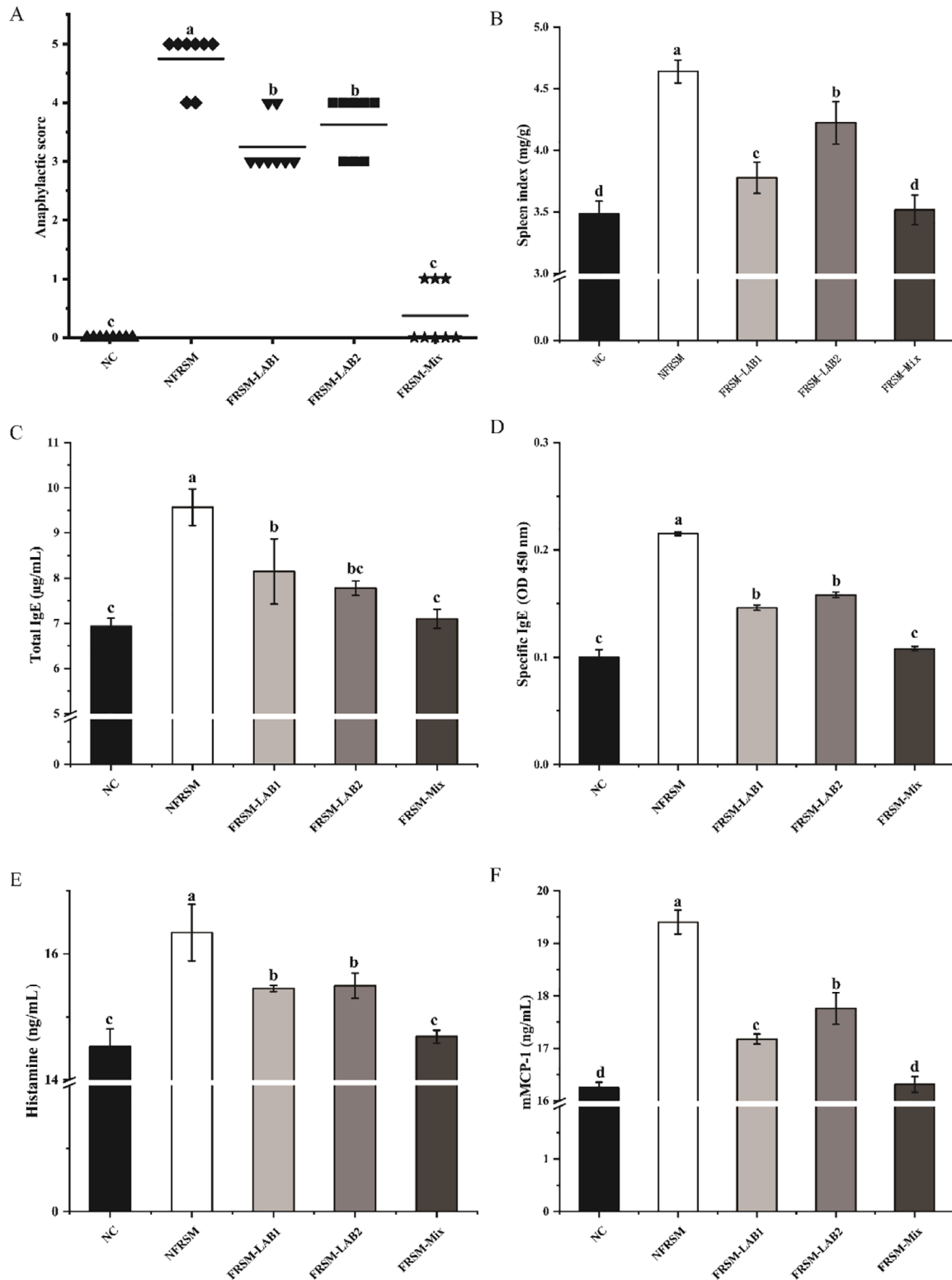
**Figure 3.** Allergenicity of the fermented versus unfermented reconstituted skim milk (RSM) in Balb/c mice. (A) Anaphylactic score. (B) Index of spleen. (C) Levels of total IgE in serum. (D) Levels of specific IgE in serum. (E) Levels of histamine in serum. (F) Levels of mMCP-1 in serum. All data are presented as the mean ± SD (A: n = 8; B–F: n = 3). Different letters indicate significant differences among groups ($P$ < 0.05; Duncan's multiple comparison test). OD = optical density; NC = normal control group; NFRSM = unfermented RSM; FRSM-LAB1 = RSM fermented by *Lactobacillus plantarum* KLDS 1.0386; FRSM-LAB2 = RSM fermented by *Lactobacillus helveticus* KLDS 1.8701; FRSM-Mix = RSM co-fermented by *L. helveticus* KLDS 1.8701 and *L. plantarum* KLDS 1.0386.
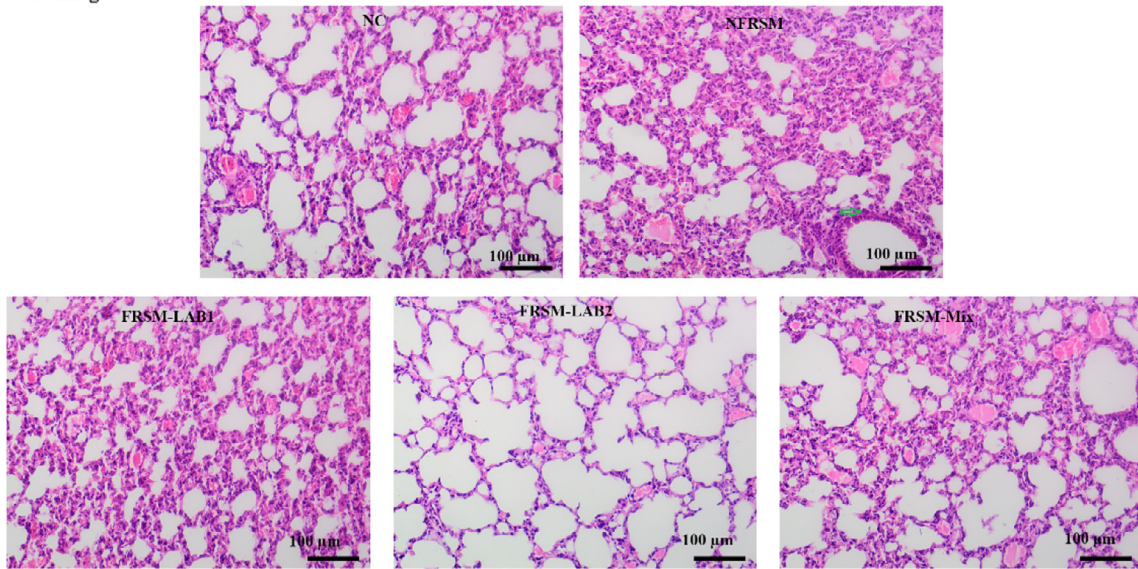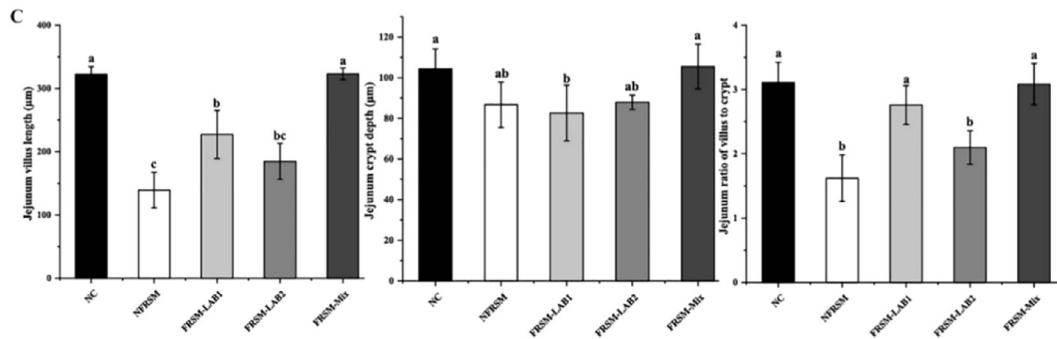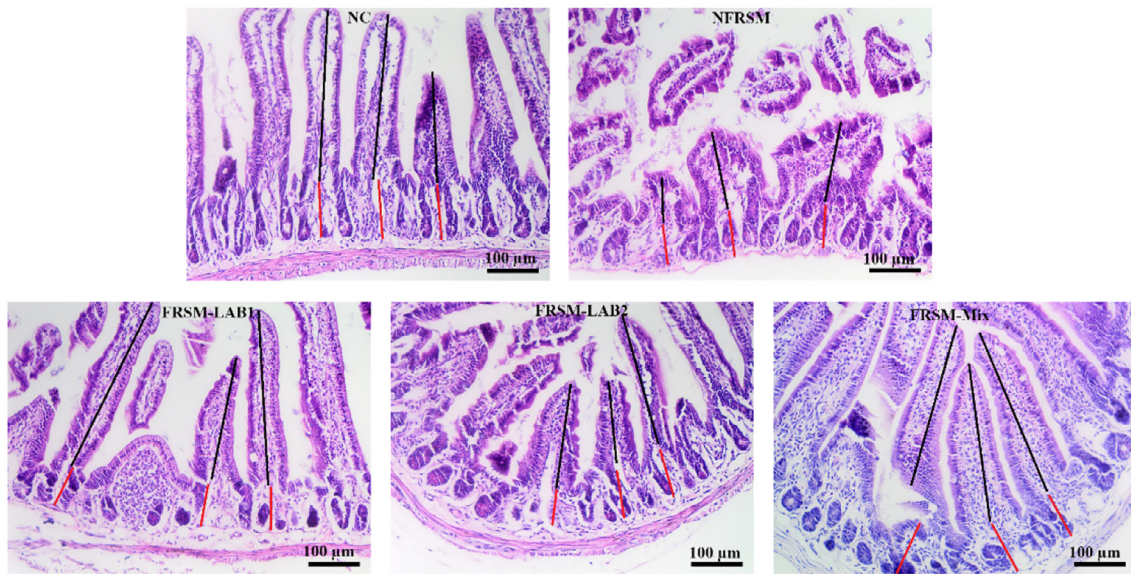
**Figure 4.** Histological analysis. (A) Histopathological observation of the lung. (B) Histopathological observation of the jejunum. (C) The analysis of villus length, crypt depth, and the ratio of villus to the crypt. All data are presented as the mean ± SD (n = 3). Different letters indicate significant differences among groups ($P < 0.05$; Duncan's multiple comparison test). NC = normal control group; NFRSM = unfermented RSM; FRSM-LAB1 = RSM fermented by *Lactobacillus plantarum* KLDS 1.0386; FRSM-LAB2 = RSM fermented by *Lactobacillus helveticus* KLDS 1.8701; FRSM-Mix = RSM co-fermented by *L. helveticus* KLDS 1.8701 and *L. plantarum* KLDS 1.0386.
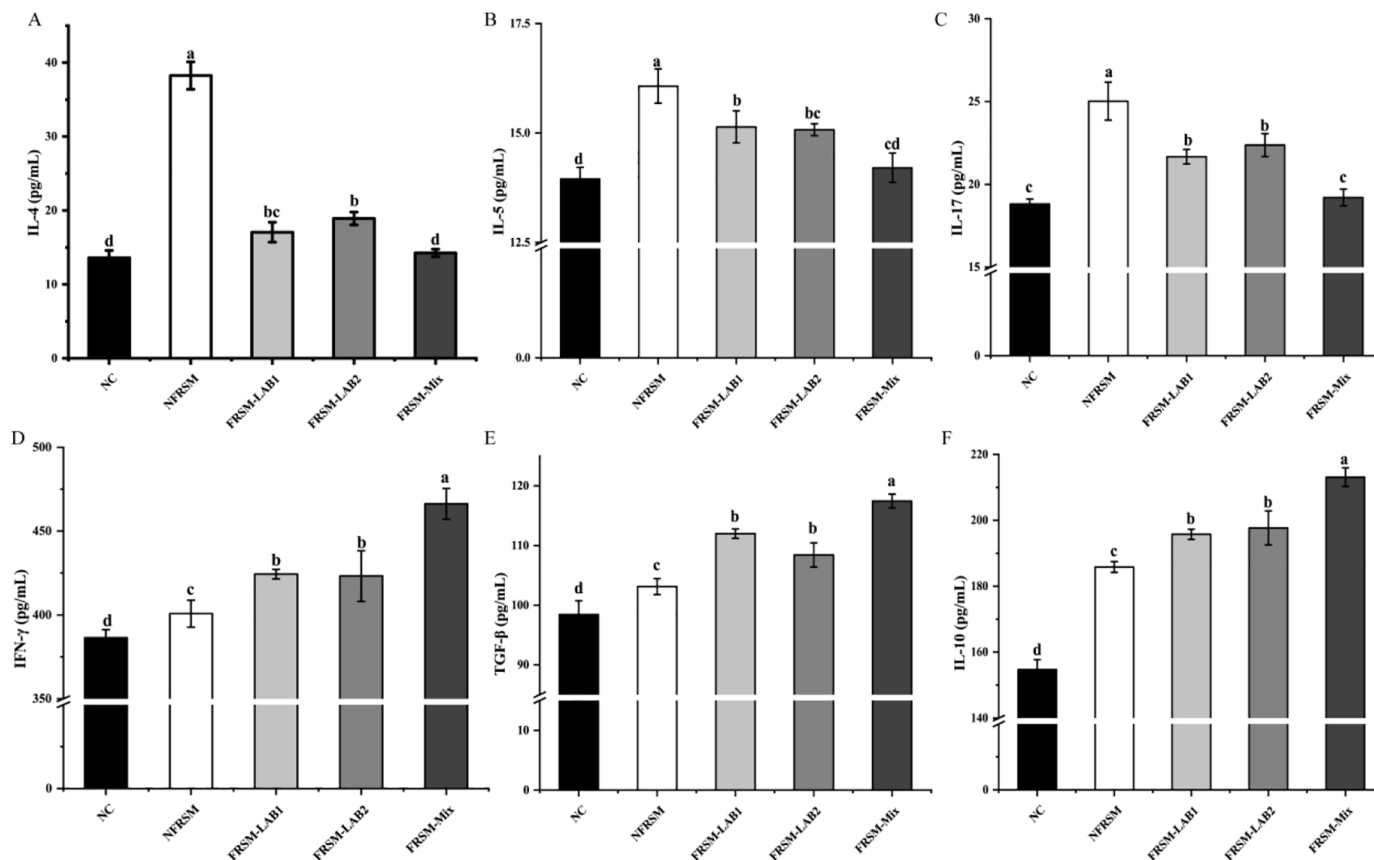
**Figure 5.** Cytokine determination using spleen cell culture. (A) Levels of IL-4. (B) Levels of IL-5. (C) Levels of IL-17. (D) Levels of IFN-γ. (E) Levels of TGF-β. (F) Levels of IL-10. All data are presented as the mean ± SD (n = 3). Different letters indicate significant differences among groups ($P < 0.05$; Duncan's multiple comparison test). NC = normal control group; NFRSM = unfermented RSM; FRSM-LAB1 = RSM fermented by *Lactobacillus plantarum* KLDS 1.0386; FRSM-LAB2 = RSM fermented by *Lactobacillus helveticus* KLDS 1.8701; FRSM-Mix = RSM co-fermented by *L. helveticus* KLDS 1.8701 and *L. plantarum* KLDS 1.0386.

were not significantly different from those in the NC group.

### Measurement of Plasma Mouse Histamine

Histamine levels in the plasma of allergic mice are shown in Figure 3E. Significantly elevated histamine levels were observed in the mice of the NFRSM group compared with the NC group, whereas in all fermented RSM groups, the levels of histamine were significantly lower than the NFRSM group ($P < 0.05$). In particular, the histamine levels in the FRSM-Mix group were not significantly different from those in the NC group.

### Measurement of Serum mMCP-1

In this study, mMCP-1 levels in the serum of all mice were determined. As shown in Figure 3F, the mMCP-1 levels also increased markedly in the NFRSM group compared with the NC group ($P < 0.05$). However,

compared with the NFRSM group, the mMCP-1 levels of all fermented RSM groups significantly decreased, especially in the FRSM-Mix group ($P < 0.05$).

### Histological Analysis

The lungs and jejunum histopathological symptoms of each group are shown in Figure 4A. In the NC group, alveoli were structurally intact without obvious pathological symptoms. However, the mice in the NFRSM group showed aggregation of inflammatory cells around the lung bronchi, exfoliation of epithelium and disruption of alveolar structure. It is crucial that FRSM-LAB1, FRSM-LAB2, and FRSM-Mix groups markedly alleviated the histopathological symptoms of the lungs, especially the FRSM-Mix group, as compared with the NC group. The lungs of mice in the FRSM-Mix group showed normal alveolar structure without inflammatory cells infiltrating and were similar to that of the NC group. Similarly, the jejunum villi in the NC group
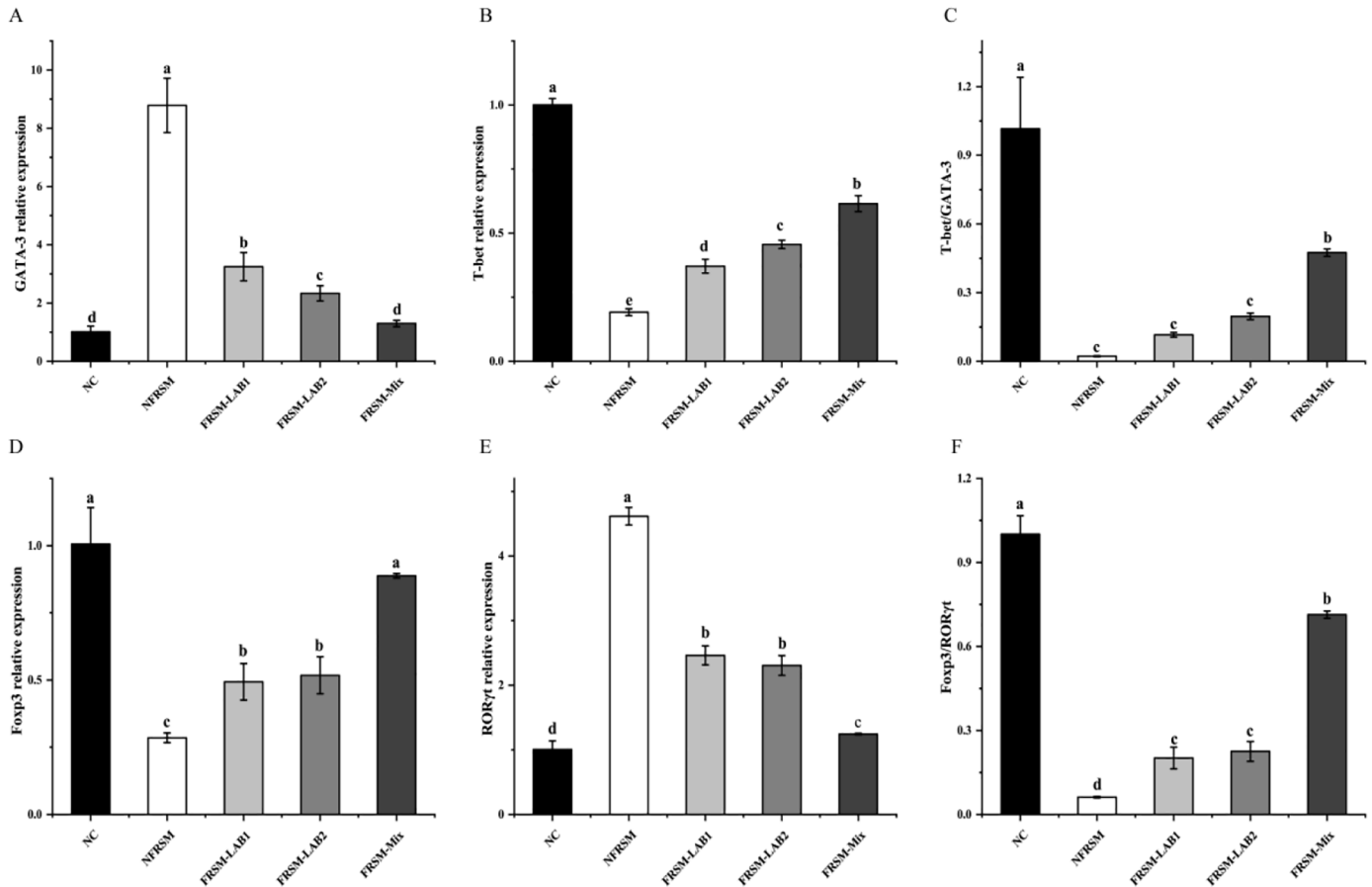
**Figure 6.** Measurement of jejunum transcription factors: (A) GATA-3, (B) T-bet, (C) T-bet/GATA-3, (D) Foxp3, (E) RORγt, and (F) FOXp3/RORγt. All data are presented as the mean ± SD (n = 3). Different letters indicate significant differences among groups ($P < 0.05$; Duncan's multiple comparison test). NC = normal control group; NFRSM = unfermented RSM; FRSM-LAB1 = RSM fermented by *Lactobacillus plantarum* KLDS 1.0386; FRSM-LAB2 = RSM fermented by *Lactobacillus helveticus* KLDS 1.8701; FRSM-Mix = RSM co-fermented by *L. helveticus* KLDS 1.8701 and *L. plantarum* KLDS 1.0386.

are regularly arranged without obvious pathological symptoms (Figure 4B). However, the villi of the jejunum structure in the NFRSM group are irregular severely damaged with inflammatory cell infiltration. In contrast, after fermentation treatment, intestinal allergic symptoms of mice in all groups were significantly improved without prominent edema and inflammatory cell infiltration, especially the FRSM-Mix group. Moreover, we analyzed the jejunum villus length, jejunum crypt depth and villi-to-crypt ratio of mice stimulated by different samples (Figure 4C). Compared with the NC group, the jejunum villus length and the ratio of villi to the crypt of the NFRSM group was significantly decreased, whereas its crypt depth was not significantly changed, resulting in lower villi-to-crypt ratio. It is important to note that the jejunum villus length and the villi-to-crypt ratio in the FRSM-Mix group was similar to that of the NC group.

## Cytokine Determination Using Spleen Cell Culture

In vitro, RSM protein and fermented RSM protein-induced cytokines production from splenocytes (IL-4, IL-5, IL-17, IL-10, TGF-β, and IFN-γ) were measured to assess the strength of the immune response. As shown in Figure 5A-F, the levels of IL-4, IL-5, and IL-17 in the splenocyte supernatant were significantly increased ($P < 0.05$) in the NFRSM group compared with the NC group. However, their production in the splenocyte supernatant of all fermented RSM groups was significantly reduced ($P < 0.05$) compared with the NFRSM group. Notably, there was no significant difference between the FRSM-Mix and NC groups. On the contrary, in the splenocyte supernatant of the fermented RSM group, production of IFN-γ, TGF-β, and IL-10 was significantly higher ($P < 0.05$) than those of the NC and NFRSM groups.

### Measurement of Jejunum Transcription Factors

The present study analyzed the relative gene expressions of Foxp3, RORγt, T-bet, and GATA-3 in all the groups using the RT-qPCR technique, as shown in Figure 6A-F. The results showed that the mRNA levels of GATA-3 and RORγt were significantly downregulated in all fermented RSM groups compared with the NFRSM group, and the relative expression level of GATA-3 was no significant difference between the FRSM-Mix group and the NC group. Nevertheless, significantly upregulated expressions of T-bet and Foxp3 were observed in the intestine of all fermented RSM groups compared with the NFRSM group, whereas no significant differences in the mRNA levels of Foxp3 were found observed between the FRSM-Mix group with the NC group. More importantly, the FRSM-Mix group enhanced the ratio of T-bet/GATA-3 and Foxp3/RORγt as compared with the NFRSM group, whereas they were approximately 21.5 and 4.5 times upregulated in the FRSM-Mix group, respectively.

## DISCUSSION

The fermentation process may be advantageous over other food processing methods to decrease the antigenicity and allergenicity of milk proteins. Previously, we reported that the combined fermentation could reduce the antigenicity of cow milk protein by destroying the linear epitopes in vitro (Zhao et al., 2021). In the present study, we monitored the structural characteristics to analyze the conformational changes in protein by fermentation. We evaluated that the combined fermentation attenuates the allergic immune response of cow milk protein allergen in the Balb/c mice model, which provided the pathological and biological features in vivo.

Fermentation results in structural changes of milk protein to varying degrees, which reduces allergenicity (Rui et al., 2019). The changes of shape and position of the peak in the amide I band ($1,600$–$1,700$ cm$^{-1}$) were considered indicators to assess the secondary structure (Zhou et al., 2021). In the amide I region, 4 primary peaks were monitored, namely β-sheet, random coil, α-helix, and β-turns (Byler et al., 1986). Previous studies have found that fermentation may significantly affect the modification of the β structure in protein and lead to the unfolding and opening up of the secondary and tertiary structure (Jiang et al., 2015; Rui et al., 2019). Our study also confirmed that the α-helix converted into β-sheet and β-turn during fermentation. This suggests that the conformation of the protein was changed to cleave the conformational epitopes, and it became looser, which may make the allergic linear epi-

topes of the antigen protein more easily exposed to be hydrolyzed by proteases during the fermentation.

Our results also showed that the surface hydrophobicity was decreased. This finding may be due to the formation of new hydrophilic peptides during the fermentation by enzymatic hydrolysis, thus reducing hydrophobicity (Qla et al., 2021). This also means that allergy epitopes are more easily degraded. This may also be because hydrophobic AA may also be buried by oxidation of free SH into disulfide bond to obtain a similar network structure, and the protein was degraded into short peptides to reduce hydrophobic binding sites (Bao et al., 2017; Duan et al., 2017). The result demonstrated that the protein's tertiary structure is altered, which might destroy or mask some conformation epitopes (Rahaman et al., 2015). That further decreased the antigenicity of protein.

The spleen is one of the most crucial immune organs, which can make immune substances and produce immune responses. When antigens appear in the blood, lymphocytes and macrophages in the spleen will produce immune responses to recognize and phagocytize antigens, resulting in splenomegaly (Wen et al., 2020; Ma et al., 2021). Therefore, the spleen index is usually used to evaluate the degree of sensitization. In our study, the spleen index in the combined fermentation group significantly reduced that in the sensitized group. There was no significant difference with the unsensitized mice, suggesting that the cow milk protein after fermentation treatment can effectively reduce or eliminate the allergic characterization of the body.

Several reports based on CMPA have concluded that CMPA is mainly IgE immune-mediated reaction (Ma et al., 2021). When the epitopes of the antigen are bound to the IgE on mast cells and basophils release various inflammatory mediators, such as histamine and mMCP-1, to induce sensitization (Kumar et al., 2012; Ma et al., 2021). Thus, concentrations of mMCP-1 and histamine in the serum are critical indicators to evaluate the severity of allergic reactions. In our study, elevated levels of specific IgE, total IgE, histamine, and mMCP-1 were found in the NFRSM group compared with the NC group, whereas these allergic mediators were substantially reduced in the sera of mice in the FRSM-Mix group. Moreover, the spleen contains a mass of lymphocytes and macrophages, which will phagocytose antigen and cause spleen enlargement during allergic reaction (Xue et al., 2018), confirmed by our experimental results. These findings demonstrated that fermentation could effectively reduce the IgE-mediated allergic reaction, especially the combined fermentation, and significantly affect the sensitization of cow milk protein.

In addition to the discussion on the inflammatory mediators in serum, this study also observed the effect of oral fermented RSM on lung and jejunum structure in mice. It has been reported that when antigen induces allergic reactions, the structure and villi of the jejunum could be destroyed, and even the villi disappeared (Noti et al., 2014). Moreover, according to the report, the lungs of the sensitized mice showed infiltration of peribronchial inflammatory cells, with disruption of normal lung structure (Singh et al., 2006). Compared with the NFRSM group, oral fermented RSM improved the damage of various degrees of the jejunum and lung structure in mice, especially in the FRSM-Mix group. This indicates that compound fermentation can effectively reduce the sensitization of RSM. The jejunum villus length, crypt depth and villi-to-crypt ratio in mice are the most susceptible parameters of intestinal inflammation (Ana et al., 2015). Indeed, in our study, we found that the jejunum villus length and the villi-to-crypt ratio in the FRSM-Mix group was similar to that of the NC group with no significant difference. These results also get support by reducing the concentration of inflammatory mediators, as discussed before. In general, oral challenges with compound fermented RSM can effectively prevent the development of intestinal inflammation.

To further assess the sensitization of the cow milk protein after fermentation, we analyzed the cytokines and transcription factors expression levels of spleens and jejunum in the BALB/c. It has been confirmed that the development and activation of Th2 cells and B cells are the major factors in IgE production (Fallon et al., 2006). It was also reported that the cytokine IL-4 produced by Th2 cells plays a crucial role in IgE-mediated CMPA by inducing the IgE class switching and its production (Dourado et al., 2010; Kumar et al., 2012). Moreover, IFN-$\gamma$ produced by Th1 cells may reduce IgE production to relieve allergic response by inhibiting the production of IL-4 (Wróblewska et al., 2020; Stott et al., 2013). Thus, the balance of Th1/Th2 can effectively reflect the degree of hypersensitivity, and allergies generally tend to be excessive Th2-type immune responses (Stott et al., 2013). Compared with the NFRSM group, the production of Th2-type cytokines (IL-4 and IL-5) in the FRSM-Mix group was significantly reduced, whereas the production of Th1-type cytokines (IFN-$\gamma$) was significantly enhanced in this study. These findings were consistent with our expectations and previous conclusions. This result indicates that the protein after fermentation may suppress allergies by upregulating the expression of Th1 cytokines and downregulated Th2 cytokines.

Moreover, the mRNA relative expression of Th1 (T-bet) and Th2 (GATA-3) results was consistent with its cytokine changes. It is well known that T-cells may secrete cytokines (TGF-$\beta$, IL-10) when given small doses of antigens (Faria and Weiner, 2005), and these cytokines are anti-inflammatory and regulatory cytokines during the induction of tolerance (Sierra et al., 2010; Akdis and Akdis, 2014). Previous studies have shown that increased secretion of IL-10 and TGF-$\beta$ in the splenocyte supernatant may indicate the beginning of regulatory cells immune regulation (Wróblewska et al., 2020; Yu et al., 2016). In our study, we observed that the production of TGF-$\beta$ and IL-10 in the FRSM-Mix group were significantly higher than those of the NC and NFRSM group. Meanwhile, in recent years, more and more studies have shown that the imbalance of Treg /Th17 is also one of the key factors resulting in allergic diseases (Okamoto et al., 2012; Xue et al., 2019). Thus, the transcription levels of the different transcription factors determine the balance of Treg/Th17, ROR$\gamma$t for Th17 cells and Foxp3 for Treg cells. The expression levels of FoxP3 play a crucial role in maintaining intestinal homeostasis against allergic responses and production of antigen-specific Treg (Tsuji and Kosaka, 2008), and dyshomeostasis caused by Treg cell deficiency in the intestinal tract can lead to increased Th2 response in allergic diseases (Yu et al., 2016). Our study observed upregulated FoxP3 and downregulated ROR$\gamma$t expression in the jejunum of the FRSM-Mix group. This observation suggested that an increase in Treg cells may modulate allergic responses by regulating the balance of Treg/Th17. This finding is consistent with previous research results by Xue et al. (2019).

Several studies have demonstrated that different lactic acid bacteria have different proteolytic systems (Savijoki et al., 2006). Due to the differences in their proteolytic systems, it is expected that the capability of lactic acid bacteria strains to reduce the antigenicity of milk proteins would not be the same. Notably, *L. plantarum* was promoted its growth in the hydrolytic product of the *L. helveticus* and improved the hydrolysis of the protein (Bergamini et al., 2013). Wróblewska et al. (2020) also found that co-fermentation could better regulate Th1/Th2 balance and reduce allergy marker levels, thus reducing sensitization in dairy products (Wróblewska et al., 2020). Our previous results also proved that co-fermentation could split more allergic epitopes to reduce sensitization of cow milk (Zhao et al., 2021). Therefore, the mixture of strains had better effects than a single strain.

## CONCLUSIONS

This study first investigated the fermented cow milk protein from the perspectives of structural character-

istics and potential allergenicity to illustrate the relationship between conformational changes and hypoallergenicity. Our results showed that the fermentation significantly altered the conformational structures of the cow milk protein, especially the tertiary structure. In addition, results also showed that the levels of Th2-related cytokines (IL-4, IL-5) and transcription factors (T-bet) decreased, and that of a Th1-related cytokine (IFN-γ) and transcription factors (GATA-3) increased slightly in the FRSM-Mix group. Simultaneously, upregulated FoxP3 and downregulated RORγt expression in the FRSM-Mix group were observed. This observation suggested that fermentation could effectively reduce the sensitization of cow milk protein by regulating the imbalance of Th1/Th2 and Treg/Th17 in Balb/c mice. Fermentation may be an effective processing method to reduce sensitization and provide a theoretical basis for developing hypoallergenic dairy products and could also be clinically beneficial.

## ACKNOWLEDGMENTS

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