

Allergenicity reduction and rheology property of *Lactobacillus*-fermented soymilk

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Abstract

BACKGROUND: This study aimed to investigate the reduction in the potential allergenicity of soymilk, and its rheological properties, after fermentation with *Lactobacillus*. Soymilk (SM) was fermented with *Lactobacillus brevis* and *Lactobacillus* sp. The molecular weight of fermented soymilk (FSM) was characterized using sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), and the potential allergenicity of FSM was analyzed using enzyme-linked immunosorbent assay *in vitro* and the BALB/c mouse model to detect changes in histamine, mouse mast cell protease-1 (mMCP-1), allergen-specific IgG/IgE, and cytokine levels and histomorphology of jejunum *in vivo*.

RESULTS: The SDS–PAGE and enzyme linked immunosorbent assay (ELISA) showed that allergens of soybean (β -conglycinin and acidic subunit of glycinin) were almost degraded, and their immunoreactivity was decreased. In the BALB/c mouse model, the FSM group did not show anaphylactic shock symptoms compared with the SM group. Moreover, a tendency toward decreased serum allergen-specific IgG/IgE levels, plasma histamine levels, and mMCP-1 concentrations was observed in the FSM group. Furthermore, Th2-related cytokines were decreased, while IFN- γ production increased in spleen cell cultures. The intestinal villus was slightly damaged after the challenge. All these findings indicated that the Th1/Th2 balance in the FSM group shifted toward a Th1 response, ultimately reducing the potential allergenicity of FSM. Rheological assessment suggested that FSM has good viscous and pseudoplastic properties.

CONCLUSION: Fermentation might be a promising method for producing tasty, hypoallergenic soybean products.

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Keywords: soymilk; *lactobacillus*; fermentation; potential allergenicity; rheology

INTRODUCTION

Soy foods produced from soybean seeds, such as soymilk (SM) and tofu, have been traditional foods in East Asia for a long time. The consumption of SM, which is traditionally processed through soaking, grinding, filtering, and heating,^{1,2} has greatly increased as it provides an excellent source of proteins.³ However, as a raw material for food, soybean seeds are not widely used in Western countries. Some consumers dislike the unpleasant odor and 'grass/bean' flavor, and some prefer a bland or neutral flavor from bean products.⁴ Soybean is also a major allergenic food for humans and animals. It is included in the list of the 'Big Eight' allergenic foods.⁵ Allergic individuals who consume soybean-containing foods may experience several immunological symptoms, including urticaria, rhinitis, pruritus, asthma, anaphylactic shock, and even death.⁶ An epidemiological survey showed that approximately 0.5% of the general population, 0.4%–3.1% of the referred population, and 12.9% of allergic children are allergic to soybean products.⁷

So far, the only way to prevent food allergies is complete avoidance of the offending food. Recently, the need to control soybean allergy by processing treatments rather than avoidance has promoted the development of new technologies,⁸ including physical,⁹ chemical,¹⁰ and biological treatments.¹¹ Fermentation is

a traditional processing technology in the food industry. Previous studies have shown that fermentation can improve the bioavailability of nutrients and reduce pathogenic bacteria in the gastrointestinal tract. It is also assumed that the allergenicity of the product may be reduced by fermentation. Shiu *et al.*¹² found that the content and profile of free amino acids in *B. subtilis* E20 fermented soybean meal are respectively higher and better compared with those in soybean meal and that the fermented soybean meal is a good substitute for fish meal in feed. The content of *Enterobacteriaceae* in the intestine of pigs that were fed with fermented feed

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was significantly lower than in those fed with dry feed.¹³ Furthermore, Shi *et al.*¹⁴ found that fermentation with *Lactobacillus casei* can effectively decrease the antigenicity and allergenicity of milk proteins.

Recently, many studies have reported that SM fermented with lactic acid bacteria has high nutritional value and is beneficial to consumers due to its hypolipidemic, anti-atherogenic, and anticancer properties.¹⁵ Despite the fact that fermented soymilk (FSM) has several benefits for consumers, it has not yet been widely accepted due to its beany flavor, rigid, and rough texture, and potential allergenicity. Daisuke¹⁵ found that the addition of L-valine to SM can control the metabolic flux of *Streptococcus thermophilus* and reduce the amount of diacetyl, which was responsible for the unpleasant smell in FSM. In another report, the unpleasant smell in FSM was reduced after soaking soybeans in NaHCO₃.¹⁶ In terms of the texture of FSM, Peng *et al.*¹ found that blanching at temperature above 70 °C can result in a soft and weak SM gel.

As far as we are aware, only a few studies have focused on reducing the allergenicity of SM by using *Lactobacillus*. In addition, various *Lactobacillus* strains and fermentation conditions can influence the reduction of the allergenicity of FSMs. In our previous study, we found that the immunoreactivity of SM was related to the molecular weight (MW) of the SM protein. Song *et al.*¹⁷ also suggested that hydrolyzed and fermented soy ingredients show negligible immunoreactivity if the MW of the SM protein is less than 20 kDa. We proposed that fermentation conditions should be based on the analysis of percentage of total protein content with a MW below 20 kDa in hypoallergenic FSM.

This research aimed to evaluate the allergenicity of FSM *in vitro* (inhibition enzyme-linked immunosorbent assay – ELISA) and *in vivo* (BALB/c animal model) via liquid state fermentation of SM using *Lactobacillus brevis* (China Center of Industrial Culture Collection (CICC) 23 474) and *Lactobacillus* sp. (CICC 23470) (specific to growth in soymilk). Meanwhile, the rheological properties (rheological parameters and flow behaviors) were also analyzed.

MATERIALS AND METHODS

Materials

Soybean (Dong Nong 48) was provided by the Key Laboratory of Soybean Biology at the Ministry of Education, Northeast Agricultural University, China. *Lactobacillus brevis* CICC 23474 and *Lactobacillus* sp. CICC 23470 were purchased from CICC. Polyclonal rabbit anti-soybean sera (IgG) were procured from Sigma-Aldrich (St Louis, MO, USA). Horseradish peroxidase (HRP)-labeled mouse IgG, IgG1, and IgE antibodies were also procured from Sigma-Aldrich (St Louis, MO, USA). The sera containing specific IgE antibodies against soybeans were collected from eight patients admitted to the First Affiliated Hospital of Guangxi Medical University (Nanning, China). The serum sample collection was approved by the internal ethical committee of the hospital.

Preparation and fermentation of SM

Soybeans were soaked in deionized (DI) water at room temperature overnight. Then, they were ground with an Oster blender (1:6 w/v DI water) for 5 min and filtrated through 400 mesh filters. The filtrate was termed raw SM, and the raw SM was boiled for 5 min to form SM. Fermentation conditions were as follows: inoculation amount, 1:24 (v/v); ratio of *Lactobacillus brevis* CICC 23474 to *Lactobacillus* sp. CICC 23470, 3:2; fermentation temperature, 33 °C; and fermentation time, 24 h.

The pH values of all defatted SM and fermented samples (1:15, w/v, N-hexane, 4 °C, 4 h) were adjusted to 8.0 with 1 M NaOH. The samples were then mixed with a twofold volume of Tris–HCl (0.05 M, pH 8.0), and the resulting slurry was stirred for 4 h. The supernatant containing the extracted soy proteins was obtained by centrifugation (9000×g, 30 min) at 4 °C. This procedure was repeated twice. The proteins were lyophilized for further analysis.

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE)

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis was performed using a Mini-PROTEAN system (Bio-Rad Laboratories, Hercules, CA, USA) with 12% separating gel and 4% stacking gel. The peptides with low MW were analyzed using tricine-SDS-PAGE with 4%, 10%, and 16.5% acrylamide. The gels were immersed in a fixative solution containing ethanol, acetic acid, and water (4:1:5, v/v/v) for 30 min.¹⁸

The gel was stained with Coomassie Brilliant Blue (R-250) and destained with destain buffer (acetic acid: methanol: water = 3:2:31, v/v). Subsequently, the gel was scanned using a SQ-GS800 scanning densitometer (Bio-Rad Laboratories, USA). The band intensity was calculated using Quantity One (Bio-Rad Laboratories, USA).

ELISA

The IgG- and IgE-binding capacity of SM and FSM proteins were assessed through competitive inhibition ELISA, as previously reported, with few modifications.¹⁹ In brief, the native SM protein was coated (2 and 10 µg/mL for IgG and IgE tests, respectively, 100 µL per well) in carbonate buffer overnight at 4 °C. For the competitive proteins, 60 µL/well of samples (1, 2, 5, 10, and 20 µg/mL for IgG-binding; 20, 40, 80, 160, and 320 µg/mL for IgE-binding) was mixed with 60 µL/well of rabbit anti-soybean sera (diluted 1:5000, for IgG binding test) or soybean-allergic patients' serum pool (diluted 1:10, for IgE binding test). For the IgE binding test, HRP-conjugated streptavidin (diluted 1:60) was also added. The reaction was developed in 100 µL of substrate solution of o-phenylenediamine. Afterward, the reaction was terminated with the addition of 2 mol L⁻¹ H₂SO₄. Absorbance was read with a microplate reader (Bio-Rad Laboratories) at 490 nm.

Animals

All animal-use protocols were previously reported by Meng *et al.*²⁰ Twenty-four female BALB/c mice, which were bred for two generations with a soybean-free diet, were used in this study. Eight animals were housed in a stainless-steel cage, and room temperature was controlled at 25 ± 2 °C, humidity was 40–60% with a 12 h/12 h light / dark cycle. Animals used in this study were approved by the Animal Care Committee of Nanchang University under the guidelines for the care and use of laboratory animals (NIH Publication No. 86–23, Revised 1996).

Oral sensitization and challenge of mice

The sensitization and challenge of mice were based on the studies by Yang *et al.*²¹ and Meng *et al.* with slight modification. Briefly, 10 mg of proteins was homogenized in 100 µL of phosphate buffered saline (PBS) and mixed with 10 µg of cholera toxin (Sigma-Aldrich, St Louis, MO, USA) as an adjuvant (n = 8 per group). On day 35, the mice were orally challenged with 50 mg of proteins (100 µL). Then, 40 min after the final challenge, allergic or anaphylactic symptoms were monitored using a validated anaphylactic

Table 1. Anaphylactic symptom scoring

Score	Symptoms
0	No symptoms
1	Scratching nose and mouth
2	Swelling around the eyes and mouth; pillar erection; reduced activity; high breathing rate
3	Shortness of breath; blue rash around the mouth and tail; higher breathing rate
4	No activity after stimulation, shivering and muscle contractions
5	Death by shock

scoring table (Table 1). Blood samples and mouse spleens were then collected to measure the following biomarkers: immunoglobulins, cytokines. More specific instructions are shown in Fig. 1a.

Soybean-specific immunoglobulins

The soybean-specific serum levels of IgE, IgG, IgG1, and IgG2a were measured using ELISA. Briefly, SM proteins (8 µg/mL) were incubated in carbonate buffer. The sera of mice were then diluted to analyze of specific IgE (1:10), IgG (1:100), specific IgG1 (1:40) and IgG2a (1:40). Then, HRP-labeled goat anti-mouse IgE, IgG, IgG1, or IgG2a (1:5000) antibodies were added to the plate, and the reaction was developed.

Measurement of morphological structure of duodenum

The mice were slaughtered, 5 cm segments of the duodenum were removed immediately. After fixed with paraformaldehyde (40 g/kg), stained with hematoxylin and eosin, then the duodenum was subject to a standard histological protocol.

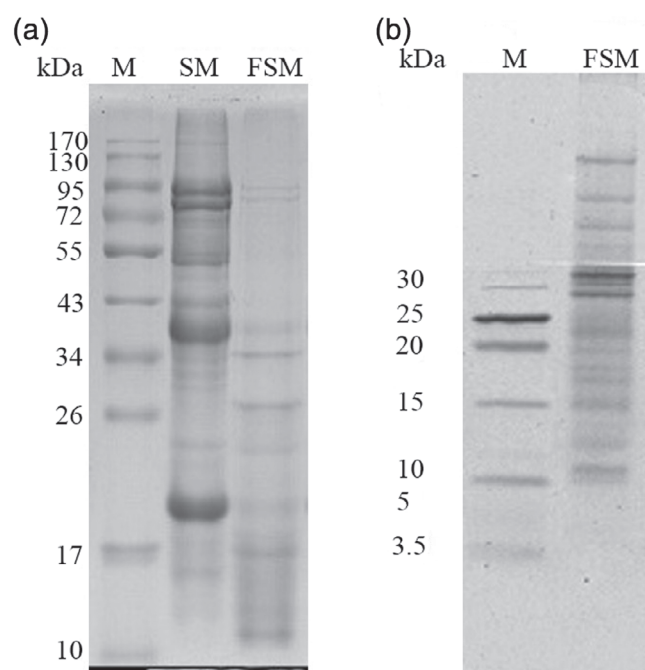


Figure 2. (a) SDS–PAGE profiles of soymilk, b Tricine–SDS–PAGE profiles of fermented soymilk, M: molecular mass markers; SM: soymilk protein; FSM: fermented soymilk protein.

Mouse mast cell protease-1 (mMCP-1) levels

The content of mMCP-1 was measured using an ELISA kit (eBioscience, San Diego, USA). Mouse serum was collected after 30 min of challenge, and the operations were carried out according to the manufacturer's instructions.

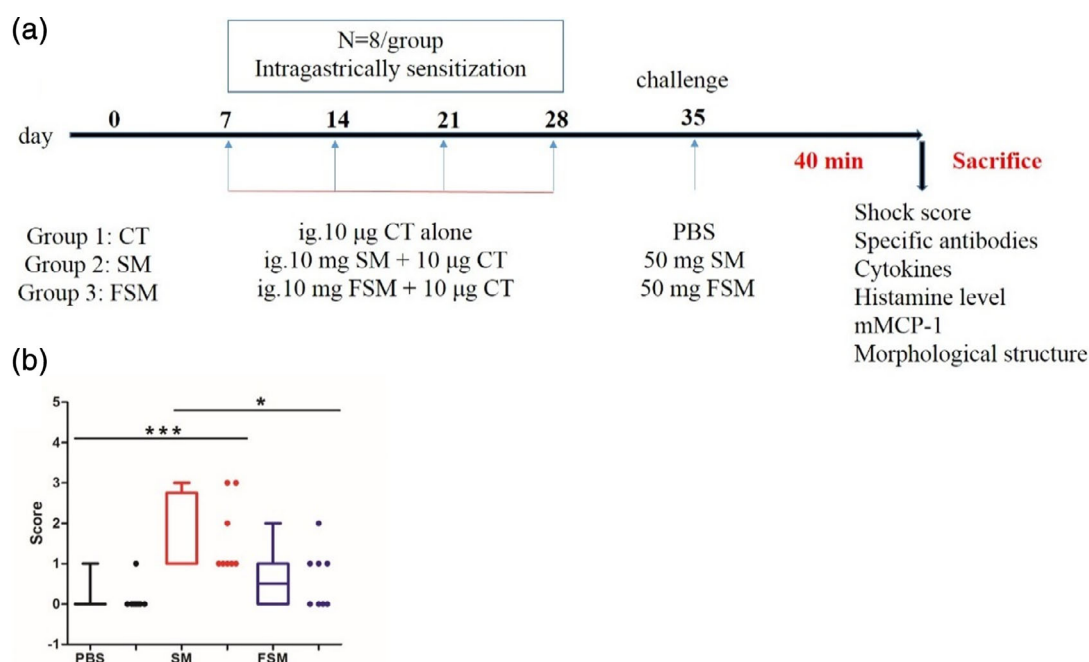


Figure 1. Oral immunization of BALB/c mice with SM or FSM. (a) Experimental scheme. BALB/c mice were sensitized weekly by intragastric gavage with SM or FSM using CT as adjuvant. Control group received CT only. (b) Score of hypersensitivity symptoms. The symptoms of hypersensitivity were scored on a scale from 0 (no symptoms) to 5 (death).

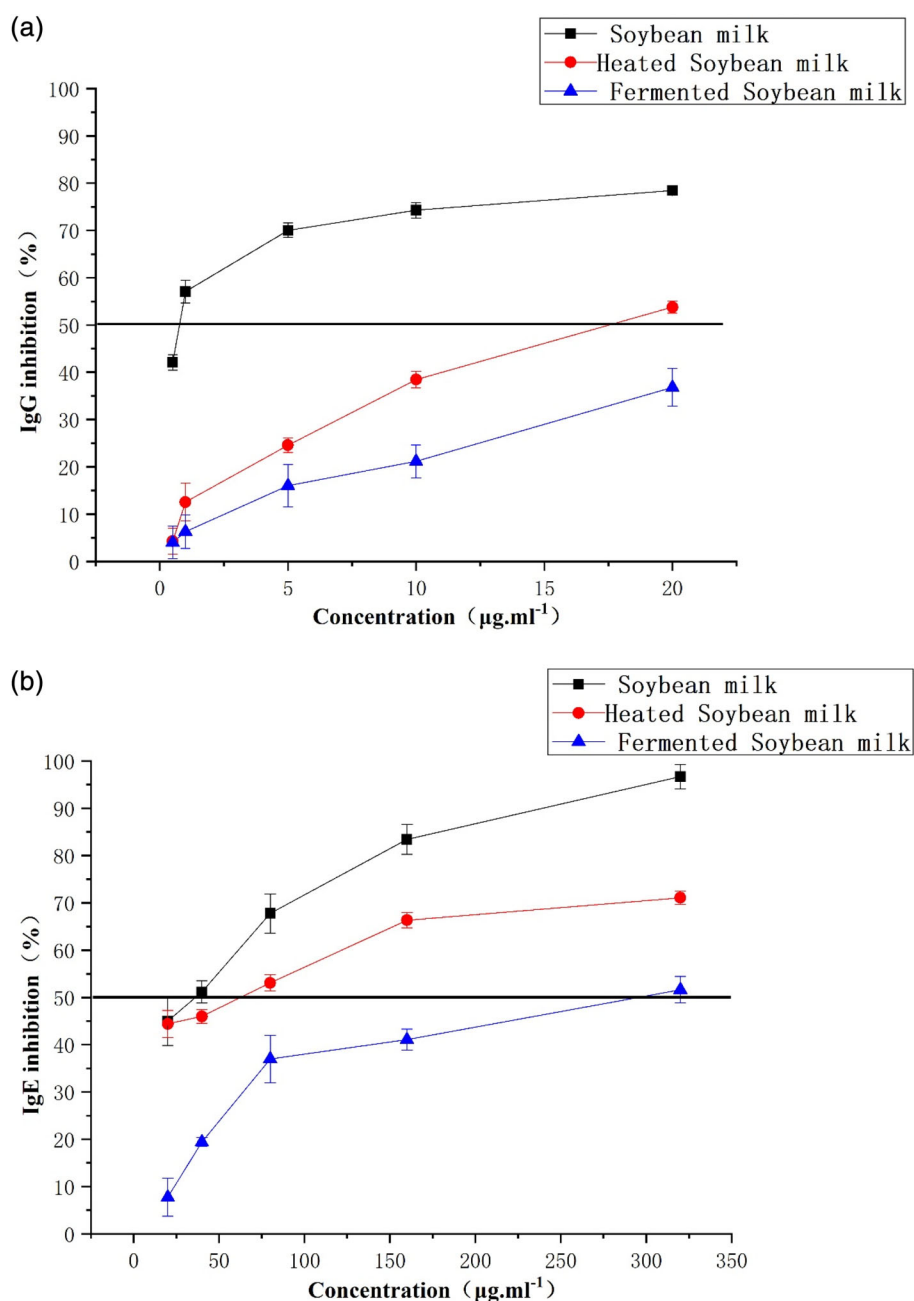


Figure 3. IgG a and IgE b binding ability of SM, heated SM, and FSM.

Cytokine levels

Splenic cell culture was based on the studies by Yang *et al.*²² Briefly, each well was inoculated with 2×10^6 cells (24-well plate), and then PBS, SM, and FSM (50 mg/mL) were added to be incubated with splenic cells for 72 h (37 °C and (CO₂:air = 1:19, v/v)). Subsequently, the supernatants were collected after centrifugation. The content of Th 1 (IFN- γ) and Th 2 (IL-4, IL-5, IL-10, IL-13) cytokines were measured through ELISA kits (eBioscience, San Diego, USA).

Plasma histamine levels

Plasma histamine levels were assessed with the ELISA kit (Demeditec Diagnostics GmbH, Germany) after mouse plasma was collected. All the operations were in accordance with the manufacturer's protocol.

Rheological measurements

The rheological properties of SM were measured using a controlled-stress rheometer (DHR-2, TA, USA), as previously reported, with a few modifications.²³

Modulus (G' , G'') was obtained using an oscillatory frequency sweep test at a 100 mm gap oscillatory frequency varying from 0.01 Hz to 10 Hz at a constant strain of 0.1% to ascertain viscoelastic properties. Shear rate sweep was used to assess the flow behavior of FSM (upward curve: shear rate from 0/s to 100/s, downward curve: shear rate from 100/s to 0/s). The power law or Ostwald-de Waele model was used for the flow behavior data and is represented by the equation:

$$\eta_a = \tau/\dot{\gamma} = k\dot{\gamma}^{n-1}$$

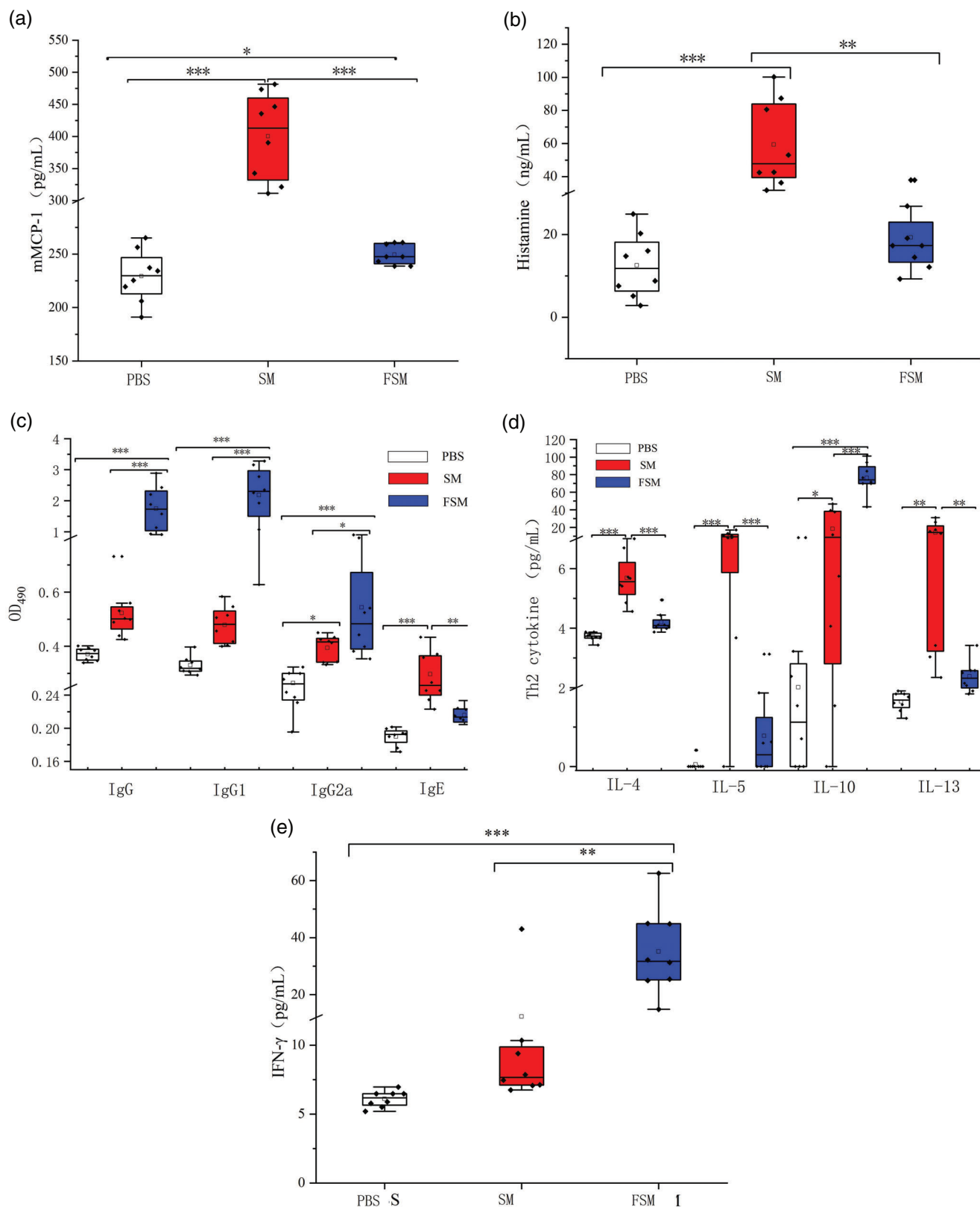


Figure 4. mMCP-1a and plasma histamine b levels of mice among different exposure groups c Specific IgG, IgG1, IgG2a and IgE levels of the sera responses in nonsensitized mice d IL-4, IL-5, IL-10, IL-13, e IFN- γ secreted by the cultured spleen cells from mice treated with PBS, SM and FSM. *Significant difference at $P < 0.05$, ** significant difference at $P < 0.005$, *** significant difference at $P < 0.001$.

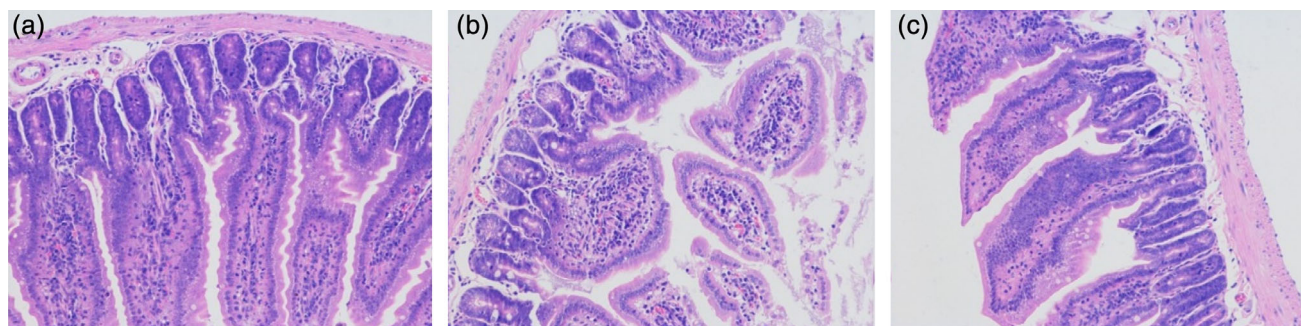


Figure 5. Morphological structure of jejunum in mice treated with PBS a, SM b and FSMc group (HE, 200×).

where η_a is the apparent viscosity (Pa s), τ is the shear stress (Pa), γ is the shear rate (s^{-1}), k is the consistency index (Pa sn), and n is the flow behavior index.

Statistical analysis

Data were presented as means $\pm$ standard deviations (SD), and were processed by Statistical Package for the Social Sciences 22.0 software (SPSS, Inc., Chicago, IL, USA). One-way ANOVA and Dunnett's multiple comparison test were used to assess the differences between groups. Differences were considered statistically significant at $P < 0.05$.

RESULTS AND DISCUSSION

SDS-PAGE profile of FSM

The SDS-PAGE and tricine-SDS-PAGE patterns of FSM protein are shown in Figs 2a and b, respectively. The SDS-PAGE patterns indicated that almost all high-MW proteins in soybean including β -conglycinin and acidic subunits of glycinin, considered to be major allergenic proteins, were hydrolyzed to small peptides (Fig. 2a). In tricine-SDS-PAGE, the patterns also showed that FSM protein was almost hydrolyzed by protease into small peptides with MW ranging from 3.4 kDa to 30 kDa, accounting for 56.5% of the total protein. Meanwhile, the peptides with MW less than 20 kDa accounted for 45.6%. A new band with 32 kDa MW in the lanes can be identified as a fragment of high-MW protein. Moreover, there was a slight change in the basic subunits of glycinin, and this phenomenon was also observed in the study by Meinschmidt *et al.*²⁴ Govindaraju and Srinivas²⁵ concluded that acidic subunits are more likely to undergo enzymatic hydrolysis than alkaline subunits. The reduction of cleavage may be due to the more hydrophobic amino acids in alkaline subunits, which formed resistant hydrophobic core and make it difficult to hydrolyze. As with the results of enzymatic hydrolysis, the fermentation exerted a smaller effect on the basic subunits of glycinin than its acidic subunits.

Immunoreactivities of FSM

The effects of heating combined with fermentation on the immunoreactivities of SM were investigated. Native SM exhibited the highest IgG-binding inhibition curve, and heat treatment lowered the curve. By contrast, FSM showed the lowest IgG-binding inhibition curve. The IC_{50} values of native and heated SM were 0.76 and 18.26 $\mu g/mL$, respectively (Fig. 3a). The IC_{50} value of FSM was higher than that of heated SM. These findings were also observed in IgE-binding, and the IC_{50} values of native, heated, and FSM were 36.15, 62.67, and 294.93 $\mu g/mL$, respectively. The results

suggested that the IgG- and IgE-binding capacity of SM could be reduced to some extent by thermal denaturation. However, thermal denaturation is not the best processing method to reduce the immunoreactivities of SM because the denaturation temperature of soybean protein is high.²⁶

The immunological characteristics of allergens are closely related to the structure of proteins. Alterations in allergenicity are based on changes in protein structure, especially in allergenic epitopes. After being heated, the structure of SM protein was unfolded, and some critical epitopes were destroyed, leading to a reduction in SM immunoreactivities. Moreover, the denatured proteins could be hydrolyzed or modified by the protease produced from the metabolism of lactic acid bacteria, and several epitopes were destroyed during fermentation. Fermented soymilk therefore showed the lowest immunoreactivities among the samples, and it can be speculated that fermentation could reduce the potential allergenicity of SM.

Anaphylactic shock score

After challenging with corresponding proteins (SM, FSM) or PBS, the symptoms of anaphylactic shock were observed in Fig. 1b. Eight mice in SM group showed anaphylactic shock symptoms (median score 2, $P < 0.001$ compared with PBS mice), which indicated that the allergic response was induced when the mice were sensitized to SM. Moreover, FSM group showed a relatively mild anaphylactic shock symptom, and there was no significant difference between the FSM and PBS groups.

Release of bioactive medium

There was a significantly increased trend in plasma histamine levels in the SM group when compared with the PBS group at 30 min after the challenge. This suggested that SM activated the effector cells. Interestingly, the content of plasma histamine was dramatically decreased in the FSM group, which was reduced by 78.83% compared with the SM group, while there was no significant difference between the PBS and the FSM group (Fig. 4a). Furthermore, serum mMCP-1 was measured as another marker, which could indicate allergen-induced activation of mast cells (Fig. 4b). A similar trend was observed between histamine and mMCP-1 levels. The SM group exhibited higher serum mMCP-1 levels than the PBS group ($P < 0.001$). The serum mMCP-1 levels were lower in mice orally challenged with FSM than those with SM (42.71%, $P < 0.05$). The results suggested that FSM exerts an inhibitory effect on the development of allergic clinical manifestations, which was consistent with the observed anaphylactic shock symptoms.

In food allergy, the specific IgE binds to the high-affinity IgE receptor (Fc ϵ RI) and cross-links with allergens in mast cells, thereby

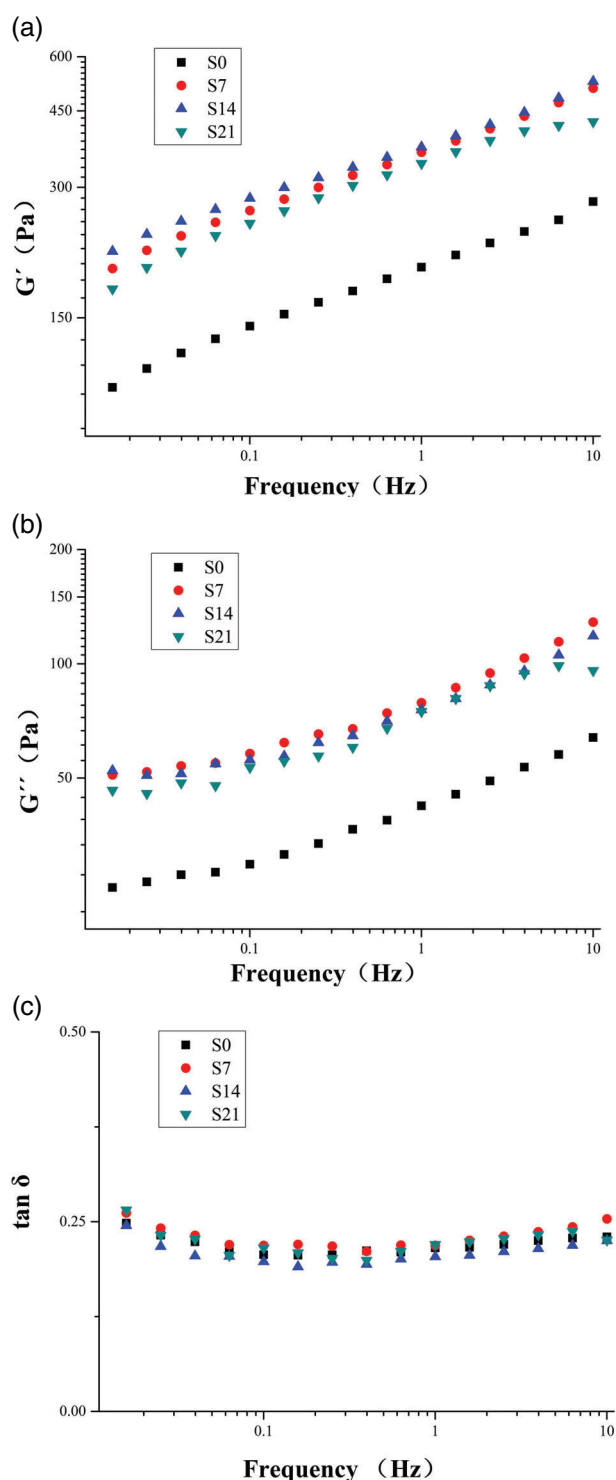


Figure 6. Frequency sweeps of fermented soymilk at 0, 7, 14, 21 days of storage at 4 °C.) G' = storage, G'' = loss modulus and $\tan \delta = G'/G''$.

triggering the degranulation of effector cells. Subsequently, histamine, mMCP-1, and various proteases are released, which causes allergic symptoms.²⁷ When the sensitized mice were challenged with FSM, the serum mMCP-1 concentrations decreased compared with those of the SM group, indicating that FSM might alter the activation and function of mast cells. Moreover, as another marker of effector cells, basophils play a key and non-redundant role in

the immune system, representing less than 1% of peripheral blood leukocytes.²⁸ Histamine is another marker of allergen-induced cell activation, and the level of histamine can indicate the activation of mast cells and basophils. The FSM group had a lower content of histamine than SM group, which suggested that histamine released from the activated mast cells or basophils was consistent with the weaker systemic allergic symptoms.

Allergen-specific immunoglobulin levels

The levels of serum-specific IgE, IgG, IgG1, and IgG2a against SM were measured (Fig. 4c). The SM group generated a significantly higher IgG ($P < 0.001$) and IgE ($P < 0.01$) response compared with the PBS group. In the FSM group, a significant increase in IgG and IgG1 levels ($P < 0.001$) was observed, while the level of IgE was decreased. For IgG2a, a slight increase was found in the SM group, and the content of IgG2a significant increase in the FSM group ($P < 0.05$).

Previous studies have shown that both IgE and IgG are involved in an allergic reaction in murines. BALB/c mice sensitized with soybean proteins by gavage could produce high levels of IgE and IgG antibodies.²⁹ Similarly, a significant increase in specific IgE and IgG levels was observed in mice that were orally sensitized with soybean proteins. After SM was fermented, the IgE level was decreased. The analysis of the subclasses of IgG antibodies showed that the increase in IgG level was mainly due to the increase in IgG1. Our result is consistent with Chin *et al.*'s results: when the mice were subjected to FSM, IgG1 and IgG2a levels were increased.³⁰ In addition to the induction of specific IgE and IgG, the IgG1 isotype is also important to evaluate the allergenicity of animal models. Combine the results of specific immunoglobulin, it implied that fermentation led to a change of immune response to SM.

Cytokine production in splenic cells

To determine whether FSM can alter the production of cytokines in the immune response, the content of Th1-related (IFN- γ) and Th2-related cytokines (IL-4, IL-5, IL-10, and IL-13) was analyzed. As shown in Fig. 4d, the levels of IL-4, IL-5, and IL-13 secreted by Th2 cells were significantly increased in the SM group (IL-4, IL-5, $P < 0.001$; IL-13, $P < 0.01$) and significantly decreased in the FSM group (IL-4, IL-5, $P < 0.001$; IL-13, $P < 0.01$) compared with the control (PBS) group. However, IL-10 showed an opposite trend. The levels of IL-10 in the FSM group were significantly increased. On the other hand, the level of IFN- γ in the FSM group (Fig. 4e) was significantly higher than that of SM group, and there was significant difference compared with the control group ($P < 0.001$).

IL-10 exerted a regulatory effect on inflammation, which might be attributed to the reduction of specific IgE. This observation is consistent with the increased levels of IL-10 in the FSM group. This finding led us to speculate that IL-10 is not derived from Th2 cells but from Th1 cells or regulatory T cells. O'Garra³¹ also reported this hypothesis.

As far as we are aware, IL-4, IL-5, and IL-13 are Th2-related cytokines and induce the growth and differentiation of B cells. Given that soybean allergy is mainly mediated by IgE hypersensitivity reactions of Th2-related cytokines, these cytokines are the key factors in the sensitization and effector stages of the allergic inflammatory reaction.³² In the sensitized mice, FSM inhibited the production of IL-4 but induced high levels of IL-10 and IFN- γ , which was consistent with the study by Velez *et al.*³³ IFN- γ can promote the maturation of B-lymphocytes and production

Table 2. Flow behavior of fermented soymilk

Samples	up			down		
	κ (Pa s ⁿ)	n	R	κ (Pa s ⁿ)	n	R
FSM 0 day 6.87 ± 0.017 ^b	0.359 ± 0.009 ^d		0.9941	7.89 ± 0.02 ^{c,d}	0.326 ± 0.008 ^c	0.9977
FSM 7 day 9.28 ± 0.072 ^a	0.343 ± 0.009 ^a		0.9872	12.19 ± 0.06 ^d	0.327 ± 0.006 ^c	0.9975
FSM 14 day 5.85 ± 0.049 ^c	0.414 ± 0.021 ^b		0.9234	9.79 ± 0.206 ^b	0.376 ± 0.003 ^a	0.9976
FSM 21 day 5.62 ± 0.101 ^d	0.451 ± 0.003 ^c		0.9433	8.50 ± 0.187 ^a	0.369 ± 0.005 ^a	0.9974

Means in the same row with different small letter superscripts are significantly different.

of IgG2a. As FSM can reduce IgE synthesis and increase IgG synthesis, it can shift the Th1/Th2 balance toward Th1 by regulating B cells to produce IgG instead of IgE. Dong *et al.*³⁴ also found that the allergen-specific IgG2a and IFN- γ increased and IgE levels decreased after the immunomodulating properties of the antigen were modified.

Morphological structure of jejunum

As shown in Fig. 5, the intestines of the SM and FSM groups were damaged, and focal necrosis and exfoliation of villus were observed in the jejunum compared with the PBS group. However, the mice in the FSM group exhibited relatively mild symptoms, but there was a certain degree of atrophy of intestinal villus compared with that in the PBS group.

In parallel with the increased concentration of histamine and mMCP-1, however, the epithelium was less damaged by FSM, suggesting that FSM led to a weaker immune reaction than SM. This phenomenon may be explained by the downregulation of mast cell activation, thus reducing epithelial permeability, which decreases the Th2-type inflammatory responses that might result in an intermediate- or late-phase response in food allergy. Yang *et al.*²¹ and Watanabe *et al.*³⁵ observed similar morphological changes in animals with food allergy.

Rheological measurements

Storage modulus (G'), loss modulus (G''), and loss tangent ($\tan \delta = G''/G'$) for the samples during cold storage are represented in Fig. 6. G' and G'' are measured to characterize the degree of solid-like and liquid-like characteristics of a gel, respectively.³⁶ The FSM showed weak gel-like and dominant elastic properties (G' was higher than G'' with $\tan \delta$ less than 1, Fig. 6c).³⁷ The differences in storage modulus reflected the gelation characteristics of various FSMs. The values of G' during the storage time were higher than its initial value (day 0), and the highest value was observed at 14 days, which then decreased, indicating that gel strength changed along with storage time. Donkor *et al.*²³ also measured the value of G' in set-type soy yogurt, and their results were consistent with our study. The increased values during storage time may be due to the change in pH. As the pH decreases during storage time, the negative charge of the denatured protein is neutralized, which reduces the electrostatic repulsion between protein complexes; then, the structure of the gel becomes harder.³⁸ Furthermore, the soy protein subunit composition also contributed to the changes in G' . Nik *et al.*³⁹ found that G' is related to the soy protein subunit composition and that its value increases with the absence of subunits. For this situation, we can speculate that the soy protein subunits (almost all β -conglycinin and acidic subunits of glycinin, Fig. 2a), which are regarded as major allergens, were hydrolyzed to small peptides along the increase of the G' values (Fig. 6).

For the flow behavior, the values of parameters n , k , γ , and η^a are presented in Table 2. The experimental data fitted well with the power law with R^2 values ranging from 0.92 to 0.99 for the ascending curves and 0.99 for the descending curves. High k values and low n values ($n < 1$) indicated that the products were viscous and had pseudoplastic properties (Table 2). A similar rheological behavior was reported by Donkor *et al.*²³ in set-type soy yogurt. Similar to the G' values, the values of k shared the same trend in the ascending and descending curves. The highest values of k were observed at 7 days, which then decreased. A high consistency index value may be attributed to the metabolic activity of probiotic organisms, which contributed to greater pseudoplastic properties of the newly formed gels. Furthermore, changes were observed as the number of probiotic organisms increased in the early days of storage. This finding suggested that the allergenicity of FSM might decrease during the storage time. Unfortunately, we failed to measure the allergenicity of FSM at the end of storage time. Our findings contradicted those of Bueno and Garcia-Cruz⁴⁰ and Charles,⁴¹ who reported that the microorganism contribution in fermented broths was insignificant.

CONCLUSION

In the present study, the allergenicity of FSM both *in vitro* and *in vivo* was assessed. Fermented soymilk exhibited low immunoreactivities *in vitro*. In the animal model, FSM also presented a low potential allergenicity *in vivo*, suppressing serum-specific IgE antibody and plasma histamine levels and mast cell activation and slightly damaging the morphological structure of the jejunum. Moreover, FSM shifted the Th2/Th1 balance toward a Th1-type response relative to the response against native SM. These findings suggested that FSM has the potential to be a safe food for individuals at risk of soybean allergy. However, the clinical relevance of these findings still needs to be confirmed by further research – for example, controlled human challenge studies. The rheological properties of FSM were also measured. FSM showed a clear gel-like behavior and had viscous and pseudoplastic properties and there was no difference between FSM and set-type soy yogurt.

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