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Degradation of major allergens and allergenicity reduction of soybean meal through solid-state fermentation with microorganisms

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In this study, we determined whether solid-state fermentation could degrade major allergens and reduce potential allergenicity of soybean meal (SBM). Solid-state fermentation was realized through a mixture of *Lactobacillus casei*, yeast, and *Bacillus subtilis*. High-performance liquid chromatography, size exclusion-high-performance liquid chromatography, and capillary liquid chromatography/tandem mass spectrometry coupled with electrospray ionization were used to examine the total amino acids and molecular weight distribution of the fermented soybean meal (FSBM). In addition, the potential allergenicity of FSBM was assessed by conducting *in vitro* competitive inhibition ELISA and oral sensitization and challenge of a BALB/c mice model. The results indicated that the total amino acid content increased and soy protein was degraded into polypeptides with low molecular weights that were derived from the hydrolysis of the allergen sequences N232-D383, G253-I265, E169-S215, G68-G98, A365-I375, and V153-A167. Moreover, the FSBM group exhibited a lower *in vitro* immunoglobulin E (IgE)-binding capacity than the SBM group. The BALB/c model indicated that the FSBM group manifested milder damage to the intestine, lower mMCP-1 and IgE levels, and higher IFN- γ levels as compared to the SBM group. These findings suggested that the potential allergenicity of SBM was reduced by the solid-state fermentation induced by the mixture of *Lactobacillus casei*, yeast, and *Bacillus subtilis*.

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1. Introduction

Soybean meal (SBM) is a byproduct of soybean oil extraction, and it is commonly used in the animal feed industry because of its high quality protein, wide availability, and low cost.^{1,2} Soybean, which is the raw material of SBM, is one of the eight major allergenic foods identified by the WHO/FAO. In addition, the absorption and utilization of the nutrients in SBM are hindered by the antinutritional compounds, such as trypsin inhibitors, Bowman Birk, Kunitz inhibitors, and allergenic protein, that it contains.³ Major allergic proteins, including β -conglycinin (α , α' , and β subunit) and glycinin, in SBM can trigger allergic reactions including intestine villus atrophy and other digestive distractions in newborn animals. These drawbacks limit the wide application of SBM in animal feed.⁴

At present, new technologies such as heat treatment, enzymatic hydrolysis, genetic modification, and fermentation by

microorganisms are being continuously developed to improve the nutritional value and reduce the allergenicity of SBM. Heat treatment can reduce the concentrations of antinutritional factors and allergenicity; however, excessive heat treatment affects digestibility and destroys amino acids *via* the Maillard reaction.⁵ Enzymatic hydrolysis can efficiently reduce the allergenicity of SBM, but it cannot always destroy all epitopes because of incomplete hydrolysis. In addition, it can expose internal allergens and ultimately increase the allergenicity of SBM.⁶ Moreover, it has been reported that bitter-tasting peptides are probably formed by enzymatic hydrolysis. Therefore, the utilization of hydrolysates has been limited.⁷ Genetic modification of crops has been conducted worldwide; however, the effects of this technique on the incidence of food allergies remain unclear, and it has raised controversies among consumers in many countries including the United States, Korea, and Japan.⁸

Fermentation is a traditional technique used in the food industry. Fermented soy-based products reportedly enhance the nutritional value and reduce immunoreactivity.⁹ In addition, fermented SBM (FSBM) displays excellent bioactivities including antioxidant, angiotensin converting enzyme inhibitory, and anti-thrombotic activities.¹⁰ Solid-state fermen-

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tation has been widely applied for SBM fermentation because it is more economical and profitable than other strategies.¹¹ However, only a few studies have assessed the potential allergenicity of SBM subjected to solid-state fermentation from both *in vitro* and *in vivo* perspectives. Therefore, the effects of solid-state fermentation on the allergenicity of SBM must be investigated.

In this study, we investigated the effects of solid-state fermentation on the potential allergenicity of SBM to provide theoretical basis for the development of high-quality and hypoallergenic fermented products.

2. Materials and methods

2.1. Materials

SBM was provided by Heze, Shandong, PR China. *Bacillus subtilis* (CICC No. 20641), *Lactobacillus casei* (CGMCC No. 1.539), and yeast were obtained from the China Center of Industrial Culture Collection Management (Shanghai, China), China General Microbiological Culture Collection Center (Beijing, China), and Angel Yeast Co., Ltd (Wuhan, China), respectively. The sera pool containing the specific IgE antibody against soybean from eight patients was acquired from Chongqing Manuik Technology Co., Ltd (Chongqing, China). The group of patients consisted of six males and two females who had the specific IgE levels of 5.95, 32.66, 22.4, 10.8, 6.61, 12.60, 30.60, and 12.97 kU L⁻¹. Among the eight patients, five suffered from allergic rhinitis, one had both allergic rhinitis and asthma, and the allergies of other two were unknown. Informed consent was obtained from the patients who agreed that their sera could be used for research on soybean allergenicity. Goat anti-mouse special antibodies (IgG, IgE, and IgG2a) were purchased from MBI Fermentas (Burlington, Canada). Goat anti-human special IgE was acquired from Sigma-Aldrich (St Louis, Mo., USA). Mouse IL 13 ELISA Kit, Mouse IFN gamma ELISA Kit, and Mouse MCP-1 ELISA Kit were obtained from eBioscience (San Diego, USA). All other chemicals were of analytical grade.

2.2. Inoculum preparation

For inoculum preparation, *Bacillus subtilis* was grown in the bean sprout medium containing 5% sucrose at 30 °C and 120 rpm for 20 h. *Lactobacillus casei* was cultured in the MRS broth (10 g L⁻¹ peptone, 5 g L⁻¹ yeast extract, 10 g L⁻¹ beef extract, 5 g L⁻¹ glucose, 5 g L⁻¹ sodium acetate anhydrous, 2 g L⁻¹ ammonium citrate tribasic, 2 g L⁻¹ K₂HPO₄, 0.1 g L⁻¹ MgSO₄·7H₂O, 0.05 g L⁻¹ MnSO₄·H₂O, and 1 g L⁻¹ Tween-80, at pH 6.8) at 30 °C for 20 h. The yeast extract peptone dextrose medium (20 g L⁻¹ peptone, 10 g L⁻¹ yeast extract, and 20 g L⁻¹ glucose) was prepared to cultivate yeast at 37 °C for 20 h. The microorganisms were adjusted to 10⁸ CFU mL⁻¹ with sterilized physiological saline and used as inoculum for SBM fermentation.

2.3. Solid-state fermentation

SBM was sterilized at 121 °C for 20 min and cooled to 30 °C. Then, the SBM was inoculated with 10⁸ CFU mL⁻¹ of *Bacillus subtilis* for 24 h and then with a mixture of *Lactobacillus casei* and yeast for 48 h at 30 °C under a 60% water content. The inoculated quantity was 12%. The ratio of *Bacillus subtilis*, *Lactobacillus casei*, and yeast was 2 : 1 : 1. Fermentation was terminated by maintaining the sample at -80 °C for 24 h. After this, the sample was freeze-dried and stored at -20 °C.

2.4. Protein extraction

The proteins in SBM and FSBM were extracted by the heating extraction method as previously described with slight modifications.¹² Briefly, 50 mL of extraction buffer (1.2 g L⁻¹ KH₂PO₄·2H₂O, 14.5 g L⁻¹ Na₂HPO₄·12H₂O [pH 7.4], 6 M urea, and 0.2% DTT) was added to the samples (1 g). The mixture was heated to 80 °C in a water bath for 20 min, continuously magnetically stirred, and centrifuged at 3000g for 30 min. Subsequently, the resulting supernatant was saved, dialyzed for 24 h, and stored at -20 °C.

2.5. Determination of protein in FSBM

The nutritional factors, including crude protein, acid-soluble protein, urease activity, and lysine, of FSBM were analyzed. The crude protein content was measured using an Automatic Kjeldahl Nitrogen Meter in accordance with QB/T 6432. The content of the acid-soluble protein was determined by trichloroacetic acid in accordance with QB/T 2653, and the urease activity was measured in accordance with QB/T 8622.

2.6. Quantification of the total amino acids by HPLC

The hydrolyzed amino acids were quantified by acid hydrolysis, derivatization, and high-performance liquid chromatography (HPLC) according to a previously reported strategy.¹³ Briefly, the sample (50 mg) was hydrolyzed for 24 h by adding 10 mL of 6 M HCl and phenol (1 g) at 110 °C in a vacuum closed vial. Then, the hydrolysates were dried and rinsed with ultrapure water twice. The amino acid components were determined using an automatic amino acid analyzer (S-433D, SYKAM, GER).

2.7. Determination of the molecular weight distribution

Size-exclusion HPLC (SEC-HPLC) was used to determine the molecular weight distribution of the FSBM proteins. The FSBM proteins were extracted and filtered through a cellulose acetate membrane with a pore size of 0.22 µm. The molecular weight distribution was examined using a liquid chromatograph (Shimadzu, Japan) coupled with a Shodex Protein KW-802.5 (8.0 mm × 300 mm, Tokyo, Japan) and a SPD-20A UV-vis detector. The mobile phase, *i.e.* the phosphate buffer (0.05 mol L⁻¹), was flowed at 0.5 mL min⁻¹. The extracted proteins were injected into the column at approximately 10 µL, and the eluents were detected at 220 nm.

2.8. Peptide identification by μ -HPLC-ESI-MS

Peptide identification was performed using an EASY-nLC 1000 HPLC system (Agilent Technologies, Wilmington, DE, USA) equipped with a Thermo Q-exactive Orbitrap mass spectrometer (Thermo Fisher Scientific, Waltham, MA, America). Samples were loaded on the Zorbax 300SB-C18 column (0.15 mm $\times$ 150 mm, RP-C18, Column Technology Inc, USA). Thermo EASY-nLC 1000 was equipped with a binary buffer system composed of 0.1% acetic acid (buffer A) and 84% acetonitrile in 0.1% acetic acid (buffer B). Samples were separated up to 50% by a linear gradient of buffer B in 40 min. Then, the compounds were identified using a mass spectrometer in the scan or positive ion ESI-MS mode.

2.9. *In vitro* immunoreactivity analysis

Immunoreactivities of the samples were assessed by the competitive inhibition ELISA, as previously described, with slight modifications.¹⁴

A 96-well ELISA microplate (plate A) was coated with 4 μ g per well of SBM protein in carbonate buffer and incubated overnight at 4 $^{\circ}$ C. Then, the wells were washed with PBST (0.05% Tween 20 in PBS) thrice and blocked with 250 μ L of 3% gelatin in PBS at 37 $^{\circ}$ C for 1 h. Another microplate (plate B) was also blocked and washed. Then, 60 μ L of FSBM protein mixed with an equal amount of human sera (diluted to 1:20, for IgE binding) was added to plate B and incubated for 1 h at 37 $^{\circ}$ C. After that, the mixture in plate B (100 μ L per well) was added to plate A, which was then incubated for 1 h at 37 $^{\circ}$ C. Afterwards, 100 μ L of anti-human IgE (diluted to 1:20) was added to plate A that was then incubated for 1 h at 37 $^{\circ}$ C, followed by the addition of 100 μ L of substrate solution (0.5 mg mL⁻¹ *O*-phenylenediamine, 30% H₂O₂) and further incubation at 37 $^{\circ}$ C for 15 min. Finally, the reaction was terminated with 50 μ L of sulfuric acid (2 mol L⁻¹). The absorbance was assessed at 490 nm using the Bio-Rad 1860 microplate reader (BioRad, CA, USA).

2.10. *In vivo* allergenicity assessment

2.10.1. Sensitization and challenge of mice. A BALB/c mouse model (4–5 weeks old) was used to evaluate the allergenicity of FSBM *in vivo*. Mice were purchased from the Animal Care Committee of Nanchang University and conformed to the prescribed care and use of laboratory animals (NIH Publication No. 86-23, Revised 1996). They were bred under the following conditions: temperature of 20–24 $^{\circ}$ C, relative humidity of 40%–60%, and 12 h light/dark cycles. Mice were fed with a soybean-free diet for two generations. The animal study protocol was reviewed and approved by the Animal Care Committee of Nanchang University.

The experimental scheme is depicted in Fig. 1. Mice were divided into sensitization and control groups, and sensitization was performed as previously reported with slight modifications.¹⁵ Briefly, in the sensitization group, mice were sensitized once a week with a dose of SBM protein or FSBM protein (5 mg per dose) together with cholera toxin (10 μ g per dose)

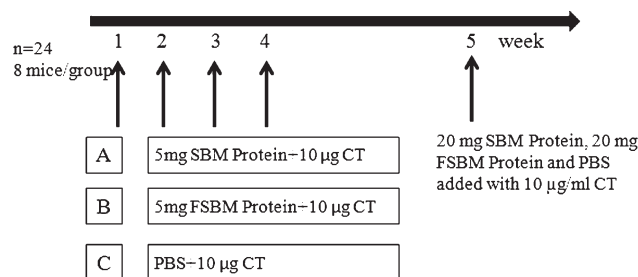


Fig. 1 Experimental scheme of *in vivo* sensitization and treatment of SBM and FSBM.

(Sigma-Aldrich, St Louis, MO, USA) in a final volume of 500 μ L of PBS. For the control group, mice were administered with 10 μ g of cholera toxin (CT) alone. After 4 weeks, mice were challenged with 20 mg of protein (Fig. 1) and then sacrificed *via* vertebral dislocation. Jejunum, blood, and spleens were harvested for the analysis of various biomarkers.

2.10.2. Morphological structure of jejunum. After mice were slaughtered, portions of the jejunum (approximately 1 cm) were obtained immediately, fixed with 10% formalin, and disposed by the standard histological techniques. The samples were cut into 5 μ m sections and then stained with hematoxylin and eosin. The morphological structures of the jejunum were observed using a light microscope (Nikon Ti Microscope, Japan).

2.10.3. Determination of the mouse mast cell protease-1 (mMCP-1) level. The serum of mice was obtained at 30 min after the challenge. The mMCP-1 level was analyzed using an ELISA kit (eBioscience, San Diego, USA) in accordance with the manufacturer's instructions.

2.10.4. Measurements of the allergen-specific immunoglobulin levels. Indirect ELISA was performed to determine the levels of the serum-specific IgE, IgG, and IgG2a, as previously described.¹⁶ Briefly, a 96-well ELISA microplate was coated with 8 μ g per well of SBM protein in carbonate buffer overnight at 4 $^{\circ}$ C. Then, the wells were washed with PBST thrice, blocked with 3% gelatin in PBS at 37 $^{\circ}$ C for 1 h, and then washed again. The sera were diluted to 1:40, 1:400, and 1:40 and then added to the wells at 37 $^{\circ}$ C for 1 h to analyze the levels of IgE, IgG, and IgG2a, respectively. Goat anti-mouse antibodies (1:4000) were used to examine the binding of IgE, IgG, and IgG2a. Phenylenediamine substrate solution (Sigma-Aldrich, St Louis, MO, USA) was added to trigger the reaction, which was terminated by H₂SO₄. The absorbance was assessed at 490 nm using a Bio-Rad 1860 microplate reader (BioRad, CA, USA).

2.10.5. Cell culture and cytokine analysis. Spleens were harvested aseptically from each mouse and transferred into an ice-cold RPMI-1640 medium (Gibco Invitrogen, Grand Island, NY). Then, spleens were ground softly with a syringe plunger, filtered through a 100 μ m nylon membrane cell strainer, shifted into a 15 mL tube, and centrifuged at 1000g for 5 min. Afterwards, the supernatants were discarded, and the resulting

pellets were resuspended in 10 mL of the RPMI-1640 medium. The process was repeated again. Red blood cell lysing buffer (3 mL) was subsequently added (Sigma-Aldrich, St Louis, MO, USA) for 5 min to remove the erythrocytes. Then, the reaction was terminated by adding 5 mL of sterile RPMI-1640 medium. Afterwards, splenocytes were centrifugated at 1000g for another 5 min, washed with 10 mL of RPMI-1640 medium twice, and suspended in the RPMI-1640 medium with 10% fetal bovine serum (HyClone, Fisher Scientific, Ottawa, ON, Canada). Viable cells were estimated by trypan blue and cultivated in 48-well plates with 200 μ L per well at a density of 2×10^6 cell per mL in the absence (control wells) or presence of samples (100 μ g per well). Concentrations of IL-13 and IFN- γ were assayed using the ELISA kits in accordance with the manufacturer's instructions (eBioscience, San Diego, USA).

2.11. Statistical analysis

All data were obtained from three independent experiments. Statistical significance was analyzed by ANOVA (SPSS version 19.0 for Windows, Chicago, IL, USA). The differences between the means were determined by Tukey's test at an α level of 0.05.

3. Results and discussion

3.1. Composition of proteins and total amino acids in SBM and FSBM

Table 1 shows the results of physicochemical indicators, including crude protein, acid-soluble protein, urease activity, and lysine, of SBM and FSBM. Compared to those in SBM, crude protein and acid-soluble protein in FSBM significantly increased by 12% and 452% (4.5-fold) after 72 h of fermentation, respectively. This result was probably due to the great proteolytic activity of the mixture of *Lactobacillus casei*, yeast, and *Bacillus subtilis*. Similarly, Teng *et al.* in 2012¹⁷ reported that after 72 h of fermentation with *B. subtilis* SB102 and *A. oryzae* AO3042, the crude protein in FSBM increased by 8.61% and 8.42% and the soluble protein increased by 32.98% and 24.15%, respectively. Moreover, Dai *et al.* in 2017¹⁸ presented that crude protein and soluble protein increased significantly from 38.25% to 44.82% and from 6.26% to 22.78% after fermentation with *Bacillus subtilis*. In addition, the lysine content significantly increased by 20%, and urease activity was hardly

Table 2 Amino acids composition of SBM and FSBM

Amino acids	SBM	FSBM
Essential amino acids		
Leucine (%)	3.26 $\pm$ 0.17	3.39 $\pm$ 0.14
Lysine (%)	2.65 $\pm$ 0.11	2.88 $\pm$ 0.15
Phenylalanine (%)	2.21 $\pm$ 0.12	3.14 $\pm$ 0.19
Valine (%)	1.96 $\pm$ 0.10	2.35 $\pm$ 0.23
Isoleucine (%)	1.79 $\pm$ 0.09	1.57 $\pm$ 0.11
Threonine (%)	1.66 $\pm$ 0.11	1.57 $\pm$ 0.16
Methionine (%)	0.55 $\pm$ 0.07	0.78 $\pm$ 0.14
Non-essential amino acids		
Glutamic (%)	8.61 $\pm$ 0.31	10.45 $\pm$ 0.63
Aspartic (%)	5.22 $\pm$ 0.22	4.70 $\pm$ 0.14
Arginine (%)	3.51 $\pm$ 0.41	1.57 $\pm$ 0.06
Serine (%)	2.34 $\pm$ 0.12	1.57 $\pm$ 0.10
Proline (%)	2.34 $\pm$ 0.15	2.35 $\pm$ 0.07
Alanine (%)	1.85 $\pm$ 0.08	1.57 $\pm$ 0.08
Histidine (%)	1.84 $\pm$ 0.11	3.14 $\pm$ 0.11
Glycine (%)	1.79 $\pm$ 0.09	1.57 $\pm$ 0.12
Tyrosine (%)	1.60 $\pm$ 0.11	2.09 $\pm$ 0.24
Cysteine (%)	0.86 $\pm$ 0.03	1.57 $\pm$ 0.18
Total (%)	44.04 $\pm$ 3.31	46.25 $\pm$ 4.16

detected. Bi *et al.* in 2015¹⁹ showed a similar result: a reduction of 67.31% for urease in FSBM with *B. subtilis* XZ1125. These results verified that the main physicochemical indicators of FSBM conformed to the standards of feedstuff-FSBM (NY/T 2218-2012).

Table 2 presents the results of total amino acids in SBM and FSBM. The total amino acids increased by 5% after fermentation. The contents of some amino acids, including isoleucine, threonine, aspartic, arginine, serine, alanine, and glycine, decreased. However, among the essential amino acids, the contents of leucine, lysine, phenylalanine, valine, and methionine in FSBM increased by 4%, 9%, 42%, 20%, and 42%, respectively. Among the non-essential amino acids, the contents of glutamic acid, histidine, tyrosine, and cysteine in FSBM increased by 21%, 71%, 31%, and 83%, respectively, as compared to those in SBM. These different results for the amino acid contents were probably due to the occurrence of microbial metabolism during fermentation. Solid-state fermentation has been used for FSBM and traditional food production, and it is believed to be better than liquid fermentation. Furthermore, many studies have reported that fermentation can improve the nutrition and utilization of soybean and reduce the anti-nutritional factors.²⁰ Therefore, in this study, the FSBM contained higher contents of proteins and total amino acids and lower contents of anti-nutritional factors; this indicated that the nutritional value of FSBM was improved, and it might become a potentially healthy food.

3.2. Molecular weight distribution

The molecular weight distribution was determined by SEC-HPLC, and the results are presented in Fig. 2 and Table 3. Molecular weight was estimated based on the calibration curve of a standard protein (Fig. 2A and B) and calculated by the formula: $Y = 8.4534 - 0.2066X$. The percentages of peptides in

Table 1 Physicochemical indicators of SBM and FSBM

Items	SBM	FSBM	Indicators
Crude protein (%)	48.12 $\pm$ 0.91	53.87 $\pm$ 1.04	≥ 45
Acid-soluble protein (%)	6.27 $\pm$ 0.74	34.58 $\pm$ 0.66	≥ 8
Urease activity (U g ⁻¹)	0.02 $\pm$ 0.00	—	≤ 0.1
Lysine (%)	2.65 $\pm$ 0.28	3.17 $\pm$ 0.32	≥ 2.5

“—” Not detected.

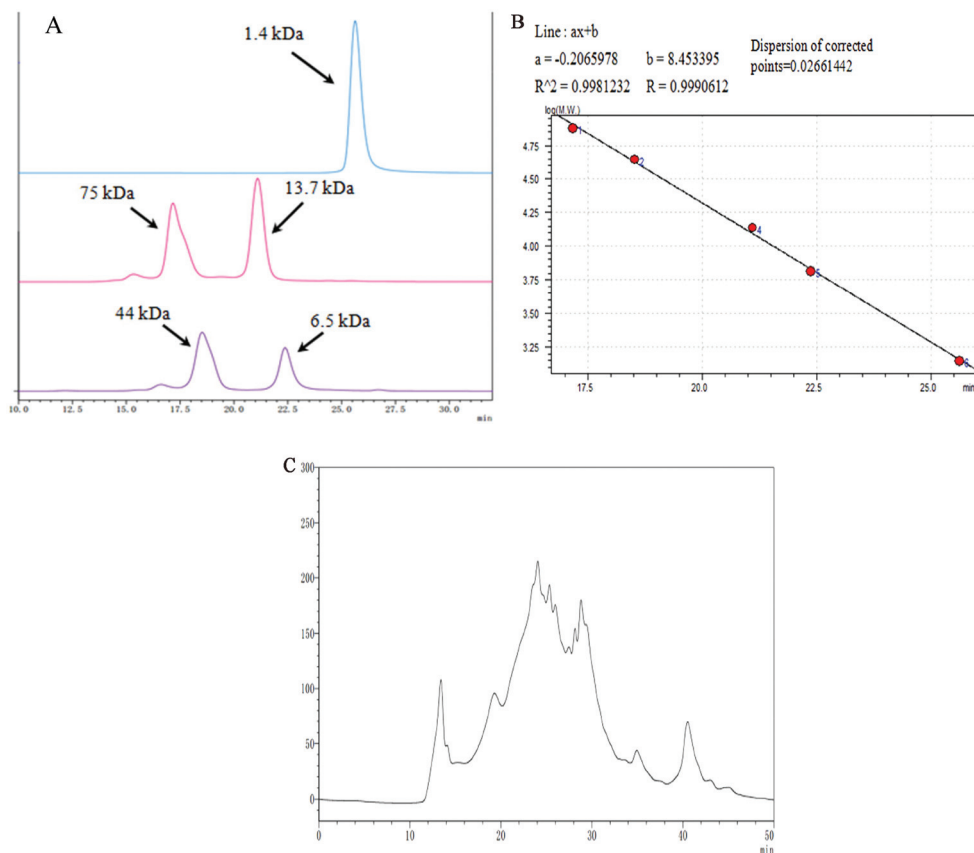


Fig. 2 Molecular weight distribution of protein. (A) SEC-HPLC profile of the molecular weight standards. (B) Standard curve of molecular weight. (C) SEC-HPLC profile of SBM fermented for 72 h.

Table 3 Molecular weight distribution of SBM fermented for 72 h

Fermentation time (h)	Molecular weight distribution (MW/Da, %)			
	>20 000	5000–20 000	2000–5000	<2000
48	29.69 ± 0.21	17.45 ± 0.61	12.41 ± 0.08	40.45 ± 0.74
72	23.17 ± 0.72	15.13 ± 0.27	14.13 ± 0.12	47.57 ± 0.26
96	21.77 ± 0.13	16.07 ± 0.76	14.63 ± 0.25	47.52 ± 1.31
120	19.13 ± 0.28	14.63 ± 0.14	15.36 ± 0.06	50.88 ± 0.93
144	19.51 ± 0.39	14.89 ± 0.23	15.28 ± 0.19	50.32 ± 0.90
168	17.82 ± 0.05	13.91 ± 0.07	15.70 ± 0.21	52.57 ± 1.61

different molecular weight ranges in the samples were then calculated by area normalization.

The chromatogram of SBM at six different fermentation times was analyzed, and the result of the SBM fermented for 72 h is presented in Fig. 2C (the others are not shown). After 72 h of fermentation, the peaks were mainly focused on the retention times from 18 min to 40 min. The content of the peptide with a molecular weight less than 20 kDa was 70.31%, and that of the peptide with molecular weight less than 2 kDa was 47.57%. Moreover, the proportion of small molecule peptides increased with the extension of the fermentation time (Table 3). These findings demonstrated that the SBM proteins were hydrolyzed during fermentation. It has been shown that

peptides with molecular weights less than 5 kDa or between 8 and 20 kDa are hypoallergenic.²¹ Therefore, the increase in the percentage of peptides with low molecular weights implied that its potential allergenicity might have been reduced during fermentation. Similarly, a previous study has shown that soy protein subjected to solid-fermentation with microorganisms is degraded into small peptides and shows weaker immuno-reactivity.²² Seo *et al.* in 2016²³ reported that the levels of β -conglycinin subunits, trypsin inhibitors, and Gly m Bd 30 K were severely decreased after fermentation with *B. subtilis* KCCM11438P such that the nutritional quality of SBM was improved. Hence, the increase in the protein content and total amino acids together with the reduced peptide size collectively

indicated that fermentation reduced the allergenicity of the SBM protein, thus making soybean more beneficial to livestock as a new type of feed ingredient.

3.3. Peptide identification

According to a search of the peptide library, the three peptides identified by μ -HPLC-ESI-MS originated from the α subunit N232-D383 of β -conglycinin,²⁴ G1 subunit G253-I265 of glycinin,²⁵ and G2 subunit E169-S215 of glycinin.²⁶ Herein, one peptide matched the fragments released from the G68-G98 subunit of glycinin G5,²⁷ and two peptides were derived from the subunits A365-I375 and V153-A167 of glycinin G5.²⁸ Sequences of the discharged peptides and the extent of hydrolysis of the allergenic protein are vital for controlling the ability of the peptides to cross the intestinal barrier and sensitization. Moreover, the conformational changes of the peptides or the masked epitopes are relevant to the accessibility of epitope.²⁸ The results revealed that partial peptides were released from the six linear epitopes of soybean allergenic protein. Pescuma *et al.* in 2015²⁹ found that the main epitopes, including V41-K60, Y102-R124, C121-L140, and L149-I162, in β -lactoglobulin were effectively degraded by *Lactobacillus delbrueckii subsp. bulgaricus* CRL 454, and then, the allergenic reactions were diminished. This phenomenon was consistent with our finding that fermentation partially degraded the linear epitopes of the allergens in SBM. IgE epitopes of food allergens are fundamental mechanisms responsible for food allergies. The epitopes include linear and conformational epitopes. The conformational epitopes are more easily destroyed by the food preparation processes, whereas the management of linear epitopes must be achieved by operating specific amino acid sequences in the protein chain.²¹ In this study, several peptides were derived from parts of linear epitopes, and the FSBM proteins were hydrolyzed into small-sized peptides during fermentation. The results showed that linear epitopes were probably destroyed.

3.4. IgE-binding capacity

IgE-binding capacity was assessed by the competitive inhibition ELISA, as shown in Fig. 3. The FSBM group exhibited the highest IC_{50} value, followed by sterilized SBM and SBM groups. The IC_{50} value signifies the concentration of the competitive antigen when its binding rate (B/B₀) is 50%. In immunology, the IC_{50} value is typically used to indicate the intensity of the antibody binding to an antigen. A higher IC_{50} value of competing antigens corresponds to a weaker binding ability of the competitive antigens to antibodies. Therefore, the FSBM group showed the lowest IgE inhibition rate among all groups, and the IgE inhibition rate of the sterilized SBM group was lower than that of the SBM group; this demonstrated that the IgE binding capacity of the SBM was decreased by both fermentation and heat treatment. To the best of our knowledge, the immunological properties of the allergenic protein are strongly related to the structural features of the allergenic protein. IgE antibodies are produced when the linear or conformational epitopes of the allergenic protein are identified by

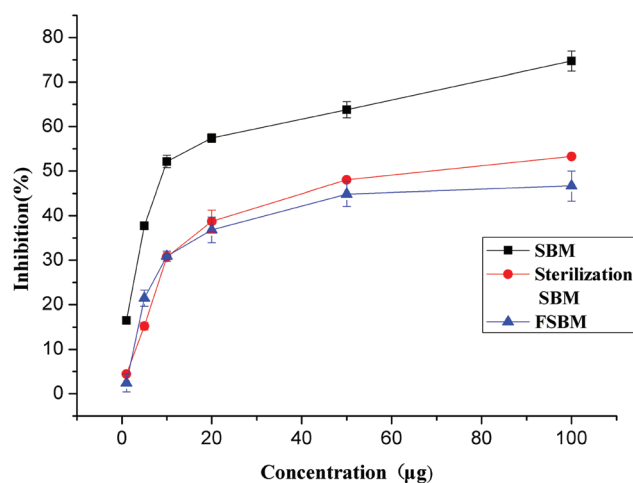


Fig. 3 IgE-binding capacities of SBM, sterilized SBM, and FSBM.

the immune system of the body. The allergenicity of the allergenic protein is presumably altered by the changes in the structure and antigenic epitopes.³⁰ Therefore, the reduction in the IgE-binding capacity observed in the fermentation group was probably due to the partial degradation of major allergens (β -conglycinin and glycinin) in soybean by microorganisms. This finding could be explained by the results of peptide identification indicating that fermentation hydrolyzed the six epitopes of soybean allergenic protein. A previous study found a similar result in which the epitopes of allergenic protein in soybean were degraded and destroyed during liquid fermentation, which led to the formation of new peptides and the reduction of allergenicity.¹³ The thermal process also reduced the IgE-binding capacity, because heat denatured the protein leading to the destruction of epitopes of IgE-binding. Soybean allergy is chiefly an IgE-mediated hypersensitivity reaction.⁸ Thus, the decreased IgE-binding capacity implied that the allergenicity of the protein in FSBM and thermal SBM were also reduced. However, although *in vitro* IgE binding capacity provides information on the immunoreactivity of the protein, clinical studies have shown that the IgE binding capacity is not completely associated with clinical symptoms.³¹ The BALB/c mouse model has been widely used for *in vivo* studies to assess the potential allergenicity of food, which supports the development of type-2 immune responses.³² Thus, it is necessary to evaluate the allergenicity of FSBM using the BALB/c mouse model.

3.5. *In vivo* assessment of potential allergenicity

3.5.1. Morphological structure of jejunum. The morphological structure of the jejunum was examined to investigate the effects of SBM on the small intestine after the mice were orally sensitized. The villus of the jejunum in the control group was dense and tact in shape, and it did not manifest any inflammation (Fig. 4A). In contrast, the jejuna in the SBM and FSBM groups were damaged (Fig. 4B and C). In the SBM group, the intestinal epithelial disorder was observed.

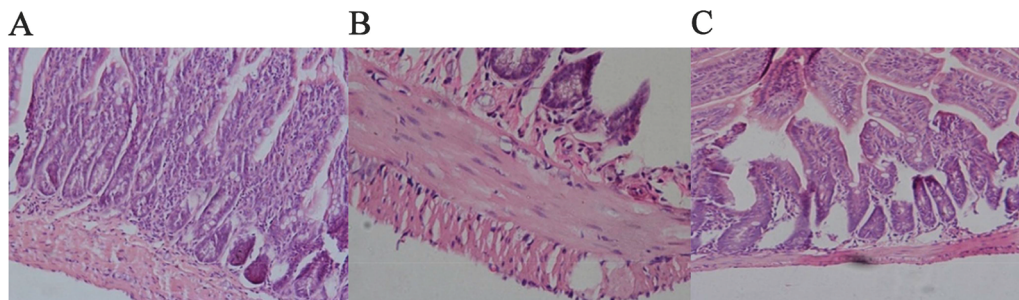


Fig. 4 Effect of different proteins on the morphological structure of jejunum in mice. Representative histological sections of the proximal jejunum were obtained from non-sensitized mice with PBS only (A), from sensitized mice fed with SBM protein (B) and FSBM protein (C) (HE, 40 $\times$).

Moreover, swelling, necrosis, and abscission occurred in the intestinal villus (Fig. 4B). Fig. 4C shows that the FSBM group displays relatively milder symptoms than the SBM group; this suggests that FSBM creates a weaker immune reaction than SBM because of differences in the composition and structure of the allergen protein that cause the sensitization of mice. Many studies have shown that morphological changes, including villous atrophy, jejunal edema, goblet cell hyperplasia, and crypt hyperplasia, in young animals are caused by transient hypersensitivity to antigenic components in food.^{15,33} Moreover, Liu *et al.* in 2008³² demonstrated that soybean glycinin and β -conglycinin caused severe gastrointestinal hypersensitivity including the degranulation of mast cells and damage of epithelium in the intestine. In our study, the allergenic protein of SBM was hydrolyzed by microorganisms. Thus, compared to SBM, FSBM created a weaker jejunum injury in mice. Although FSBM possessed allergenicity, its allergenic ability was reduced greatly by fermentation. Therefore, subsequent analyses of the levels of allergen-specific immunoglobulin, mMCP-1, and cytokines were necessary to further elucidate the mechanisms leading to the differences in the allergy symptoms between SBM and FSBM.

3.5.2. Allergen-specific immunoglobulin levels. Serum-specific IgE, IgG, and IgG2a were evaluated by indirect ELISA in the sera pool of challenged mice (Fig. 5). The IgE, IgG, and IgG2a levels of the SBM and FSBM groups were significantly higher than those of the control group. Compared to the SBM group, the FSBM group presented higher IgG and IgG2a levels, but a significantly lower ($P < 0.01$) IgE level (Fig. 5A–C). Soybean allergy is a characteristic type I allergic reaction mediated by the antigen-specific IgE antibody, and IgE plays a vital role during this period. A large number of IgE is produced by the stimulation of the allergens, and it can rapidly bind to high-affinity IgE receptors mainly on the surface of mast cells. When the allergens are ingested again and bound to IgE antibodies on mast cells, it results in the release of histamine and other inflammatory mediators (degranulation) that stimulate other cells and organs; this further leads to allergy symptoms.³⁴ Simultaneously, a large number of IgE antibodies are often observed in the sera of patients with allergic diseases.³⁵ In this study, the higher serum IgE level in the SBM group was similar to the observation in a previous study that IgE was sig-

nificantly increased in mice sensitized orally with soybean glycinin or β -conglycinin.³² However, the level of serum IgE was markedly decreased in the FSBM group; this might have been responsible for the lighter intestinal damage in the FSBM group. Furthermore, it has been reported that the allergen-specific IgG can act as a blocking antibody and inhibit IgE-mediated allergic reactions by interfering with the binding of an allergen to IgE bound on the mast cells.³⁶ Hence, the increase in the level of IgG suggested that allergic reactions in the FSBM group might be suppressed. In addition, IgG2a is a subclass of IgG, which is involved in Th1-type immune response, and interferon- γ (Th-1 cytokine) provokes the IgG subclass to IgG2a.³⁷ A polarized Th2-type response often occurs in patients with food allergies. Therefore, the enhancement of Th1-type immune responses may be beneficial for the treatment of allergic diseases. In this study, the level of IgG2a was higher in the FSBM group as compared to that in the SBM group, which was beneficial to shift towards the Th1 response in the FSBM group. Therefore, our results suggested that FSBM protein generated a milder allergenic immune reaction as compared to the SBM protein.

3.5.3. The serum mMCP-1 level. The serum mMCP-1 level was determined to reflect intestinal mast cell activation after being challenged for 30 min (Fig. 6). Compared to that of the control group, the serum mMCP-1 level was significantly elevated ($P < 0.01$) in SBM mice, whereas the serum mMCP-1 level in the FSBM group was significantly lowered ($P < 0.05$) than that in the SBM group. In addition, the level of mMCP-1 in the FSBM group was similar to that in the control group.

Mast cells are primary effector cells in hypersensitivity reactions. These cells are activated by the antigens exposed and cross-linked to allergen-specific IgE. The activation of mast cells results in the degranulation and secretion of large amounts of pro-inflammatory cytokines associated with allergy symptoms.³⁸ Serum mMCP-1 is a predominant component expressed by intraepithelial mucosal mast cells, which can reflect the degranulation of mucosal mast cells. The increase in serum mMCP-1 can promote the permeability of the epithelium, thereby inducing Th2-type inflammation responses, which ultimately lead to allergic reactions.^{39–41} Schouten *et al.* in 2009⁴¹ reported that the level of serum mMCP-1 decreased in milk allergy-mice fed with synbiotics and further reduced

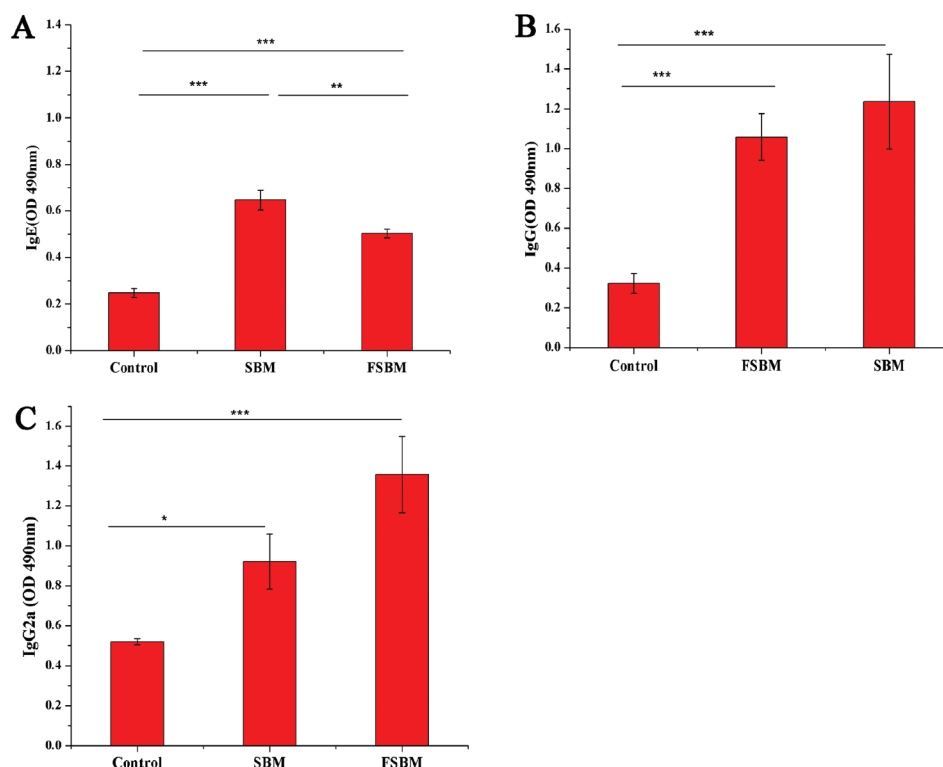


Fig. 5 Specific IgE (A), IgG (B), and IgG2a (C) levels of the sera responses in nonsensitized mice (PBS only) and mice induced with SBM and FSBM. *Significant difference ($P < 0.05$), **significant difference ($P < 0.01$), ***significant difference ($P < 0.001$).

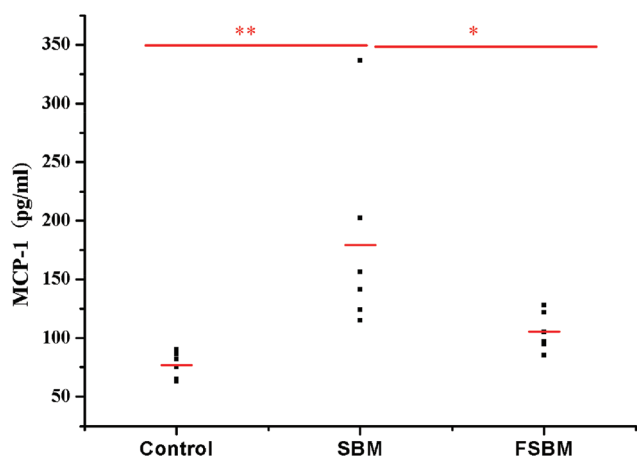


Fig. 6 mMCP-1 levels of mice among different exposure groups: control (PBS only), SBM, and FSBM. *Significant difference ($P < 0.05$), **significant difference ($P < 0.01$).

the allergy symptoms. Thus, the reduction of the serum mMCP-1 level in the FSBM group indicated that FSBM generated a weaker immunoreactivity than SBM. This finding verified the changes in the morphological structure of the jejunum and the IgE levels that were observed in this study.

3.5.4 Cytokine levels. Cytokines play important roles in regulating allergic reactions. Thus, the levels of Th1- (IFN- γ)

and Th2-related cytokines (IL-13) from the mice sensitized with FSBM and SBM were determined to evaluate the allergenic effects. As shown in Fig. 7, the levels of IL-13 and IFN- γ in the SBM and FSBM groups were significantly higher than those in the control group. Furthermore, significantly higher secretions of IL-13 and IFN- γ were detected in the FSBM group as compared to those in the SBM group.

In view of the presence of different cytokines, native T helper (Th0) cells are differentiated into Th1, Th2, and other types of T cells. Secretion of IL-12, IL-2, and IFN- γ shifts T helpers into Th1 cells, thereby promoting the release of IFN- γ . IFN- γ secreted by the Th1 cells can inhibit IgE production and induce IgG isotype switching to IgG2a. A high IgG2a level is probably beneficial to resist the Th2-type immune response. However, when IL-4 is released, activated T helper cells shift toward Th2 cells and secrete various inflammatory cytokines including IL-4, IL-5, and IL-13. These cytokines provoke Th2 cells to produce IgE and induce IgG isotype switching to IgG1, and IgG1 as well as IgE can induce systemic anaphylaxis.^{36,37} Contrary to our expectation, a higher IL-13 level was detected in the FSBM group as compared to that in the SBM group (Fig. 7A). Previous studies have shown that the IL-12 levels are higher in the allergic group as compared to those in the nonallergic group. IL-12 is generally considered a Th1-type cytokine, which has been shown to be important for the induction of Th1-dominant responses. This result indicated that IL-12 levels could not absolutely reflect the Th1 response level.^{42,43} It

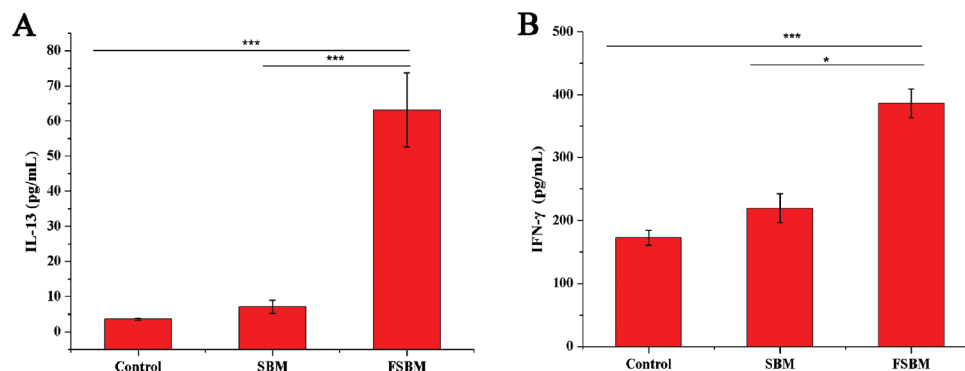


Fig. 7 IL-13 (A) and IFN- γ levels (B) secreted by the cultured spleen cells from mice treated with PBS, SBM and FSBM. *Significant difference ($P < 0.05$), *** significant difference ($P < 0.001$).

Table 4 Identification of residual peptides in FSBM

Molecular weight	Theoretical difference value	Identification of sequence	Sequence source	Score	Epitopes sequence
926.4942	-0.00025	E.ITPEKNPQ.L	β -Conglycinin α subunit I362-Q369	39.54	N232-D383
985.5201	-0.00074	L.SVIKPPTDE.Q	Glycinin G1 subunit S263-E271	37.00	G253-I265
1750.9082	-0.00449	L.SVIKPPTDEQQRPQ.E	Glycinin G1 subunit S263-Q277	51.85	
759.3818	0.00035	Q.LDQMPP.R	Glycinin G2 subunit L172-R177	32.32	E169-S215
1079.5520	-0.00072	H.LPSYSPYPR.M	Glycinin G5 subunit L83-R91	44.83	G68-G98
1210.5925	-0.00024	H.LPSYSPYPRM.I	Glycinin G5 subunit L83-M92	23.37	
782.3832	-0.00024	S.YSPYPR.M	Glycinin G5 subunit Y86-R91	27.90	
854.4043	-0.00844	R.ADFYNPK.A	Glycinin G5 subunit A365-K371	27.35	A365-I375
900.4673	-0.00543	N.TGDEPVVALS	Glycinin G5 subunit T160-I168	32.80	V153-A167

was similar to the result indicating that IL-13 levels were higher in the FSBM group than those in the SBM group in this study. Although IL-13 is generally regarded as a Th2 cytokine and it can promote the production of IgE and aggravate allergy symptoms, IL-13 levels may not always reflect the presence of allergy symptoms. A previous study has reported that IL-13 is derived from both Th2 cells and innate lymphoid cells (ILC2s); however, the precise mechanisms of allergy diseases induced by IL-13 are still unclear.⁴⁴ Oeser *et al.* in 2015⁴⁵ pointed out that T-cell-derived IL-4/IL-13 were accountable for allergic airway inflammation and airway hyperreactivity. In contrast, the number of IL-13-competent ILC2s did not correlate with the severity of lung pathology. Therefore, the mechanisms of IL-13-induced diseases should be revealed in future studies. However, a significantly higher secretion of IFN- γ was observed in the FSBM group than that in the SBM group (Fig. 7B). A previous study showed that IFN- γ in BALB/c mice could suppress Th2 cytokine secretion and inhibit Th2-mediated food hypersensitivity reactions.⁴⁶ In our present results, higher levels of IFN- γ and IgG2a and a lower level of IgE were observed in the FSBM mice; this suggested that IFN- γ promoted the production of IgG2a and suppressed the secretion of IgE, and this supported the fact that weaker intestinal damage occurred in the FSBM group as compared to that in the SBM group. Allergy symptoms induced by the allergic protein have distinct individual differences, which are attributed to the combined effect of various factors including environmental factors, genetic predisposition, and exposure conditions. Consequently, clinical

trials can be utilized to identify individual allergy symptoms (Table 4).⁴⁷

4. Conclusion

In this study, the allergenicity of SBM was evaluated after fermentation with a mixture of *Lactobacillus casei*, yeast, and *Bacillus subtilis*. The results demonstrated that the total protein and amino acid contents of FSBM were increased by the action of probiotics, and soy protein was degraded into polypeptides with low molecular weights, in which some allergenic sequences of β -conglycinin and glycinin were destroyed. In addition, the *in vitro* IgE binding capacity of the FSBM group was lower than that of the SBM group. Simultaneously, the BALB/c mice model indicated that the FSBM group showed lower levels of mMCP-1 and IgE and a higher level of IFN- γ as compared to the SBM group. Hence, a lesser degree of intestine damage was observed in the FSBM group. Therefore, our study posits that solid-state fermentation induced by the mixture of *Lactobacillus casei*, yeast, and *Bacillus subtilis* can reduce the allergenicity of SBM.

Conflicts of interest

All authors declare no conflict of interest.

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