Mixed fermentation of soybean meal by protease and probiotics and its effects on the growth performance and immune response in broilers

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ABSTRACT
Soybean meal (SBM) contains several anti-nutritional factors, which limit the use of soy protein. Exogenous enzyme supplementation and microbial fermentation can reduce anti-nutritional factors and increase the bioavailability of nutrients in SBM. In this study, we investigated the optimal parameters for the solid-state fermentation (SSF) of SBM by protease in combination with probiotics and evaluated its effect on the growth performance and immune response in broilers. Protease in combination with Bacillus subtilis significantly increased the degradation of soybean protein and soybean allergens in a dose-dependent manner during the SSF of soybean meal. Broilers fed 10% fermented SBM (FSBM), which was produced with protease and B. subtilis, consumed more daily feed on average during the entire feeding period than the SBM group did (P < 0.05). Dietary supplementation with 10% FSBM attenuated IL-4 mRNA expression in the spleen and bursa of Fabricius of broilers (P < 0.05). Furthermore, 10%-FSBM-fed broilers had significantly lower serum anti-soybean IgG levels than SBM-fed broilers (P < 0.05). These results suggest that the supplementation of B. subtilis and protease during the SSF of SBM reduces anti-nutritional factors. Moreover, dietary supplementation of 10% FSBM could inhibit the allergic immune response in broilers.

Introduction
Soybean meal (SBM) contains several anti-nutritional factors, which limit the use of soy protein. Exogenous enzyme supplementation and microbial fermentation can reduce anti-nutritional factors and increase the bioavailability of nutrients in SBM. In this study, we investigated the optimal parameters for the solid-state fermentation (SSF) of SBM by protease in combination with probiotics and evaluated its effect on the growth performance and immune response in broilers. Protease in combination with Bacillus subtilis significantly increased the degradation of soybean protein and soybean allergens in a dose-dependent manner during the SSF of soybean meal. Broilers fed 10% fermented SBM (FSBM), which was produced with protease and B. subtilis, consumed more daily feed on average during the entire feeding period than the SBM group did (P < 0.05). Dietary supplementation with 10% FSBM attenuated IL-4 mRNA expression in the spleen and bursa of Fabricius of broilers (P < 0.05). Furthermore, 10%-FSBM-fed broilers had significantly lower serum anti-soybean IgG levels than SBM-fed broilers (P < 0.05). These results suggest that the supplementation of B. subtilis and protease during the SSF of SBM reduces anti-nutritional factors. Moreover, dietary supplementation of 10% FSBM could inhibit the allergic immune response in broilers.

Feeding of FSBM produced by Aspergillus species significantly increases the activity of intestinal digestive enzymes and improves growth performance during the growing period of broilers (Mathivanan et al. 2006; Feng et al. 2007a, 2007b). In addition to fungal fermentation, solid-state fermentation (SSF) of SBM by Lactobacillus species and Clostridium butyricum may reduce the soy carbohydrate and soy protein contents of FSBM (Su et al. 2018). Bacillus species can produce lactic acid and remove trypsin inhibitor during fermentation. Thus, they increase protein hydrolysis and liberate free amino acids (Mukherjee et al. 2016). Feeding of FSBM produced by B. subtilis during the early phase can improve growth performance in broilers (Kim et al. 2016). Exogenous enzyme supplementation is also widely used to enhance the degradation of soybean protein and inactive anti-nutritional factors during the SSF of SBM (Ma and Wang 2010; Amadou et al. 2011). Dietary enzyme supplementation improves the nutritional status and growth performance of broilers (Abudabos 2012; Abudabos and Yehia 2013; Abd El-Hack et al. 2018). However, to our knowledge, no study has reported the fermentation conditions of exogenous proteases and probiotics for the SSF of SBM. Furthermore, the effect of FSBM produced by

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protease and probiotics on the growth performance and immune response in broilers remains unverified.

Therefore, this study investigated the optimal parameters for the mixed SSF of SBM by protease and probiotics and evaluated the effect of FSBM on broilers. The results provide valuable information regarding the fermentation conditions of SBM by enzymes and probiotics. These conditions are suitable for use on an industrial scale to mass-produce functional fermented products.

Materials and methods

Microorganisms and culture conditions

All chemical reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless specified otherwise. *B. subtilis* (ATCC 6051) and *A. oryzae* (ATCC 11493) were purchased from the Food Industry Research and Development Institute (Hsinchu, Taiwan). After thawing, *B. subtilis* was inoculated into an Erlenmeyer flask containing brain heart infusion broth at 30°C for 18 h with shaking. *A. oryzae* was inoculated into an Erlenmeyer flask containing potato dextrose agar and incubated at 25°C for 5 d with shaking.

SSF

SBM was ground to fine powder and mixed with water to the required relative moisture content in a space bag and autoclaved at 121°C for 30 min. The cooled substrates were mixed with different activities of protease (measured in casein digestion units, CDU). To study the effects of the initial moisture content and protease activity, fermentations were performed with different initial moisture contents (40%, 50%, and 100%, w/w) and activities of protease (10^4, 10^5, and 10^6 CDU/kg) in a chamber at 25°C. To study the effects of the initial moisture content, protease activity, and *A. oryzae*, fermentations were performed with different initial moisture contents (40%, 50%, and 100%, w/w), different activities of protease (10^4, 10^5, and 10^6 CDU/kg), and 10% inoculums (v/w) of *B. subtilis* in a chamber at 30°C. To study the effects of the initial moisture content, protease activity, and *A. oryzae*, fermentations were performed with different initial moisture contents (40%, 50%, and 100%, w/w), different activities of protease (10^4, 10^5, and 10^6 CDU/kg), and 10% inoculums (v/w) of *A. oryzae* in a chamber at 25°C. Different fermentation durations (6–24 h) were employed to study their effects on the SSF of SBM. All the experiments were performed three times. After fermentation, the FSBM samples were dried at 50°C for 24 h and homogenized through mechanical agitation. The fermented powder was then stored at 4°C prior to analysis.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and western blot

The crude protein extracts from FSBM were separated through sodium dodecyl sulfate-polyacrylamide gel electrophoresis. After electrophoresis, the gel was stained with Coomassie Brilliant Blue R-250 to visualize the total protein content. Protein lysate was separated through sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transblotted onto a polyvinylidene fluoride membrane (Perkin Elmer, Norwalk, CT, USA). The β-conglycinin primary antibody was purchased from Abmart (Berkeley Heights, NJ, USA). The secondary antibody coupled to horseradish peroxidase was used in the diaminobenzidine staining procedure. The Western blotting procedure was performed according to the manufacturer’s instruction.

Fast protein liquid chromatography (FPLC)

The FSBM was dissolved in distilled water and centrifuged for 10 min at 6000 × g. The supernatant was then tested for soy protein degradation. Chromatographic analyses were performed using the AKTAprime plus system (GE Healthcare, Marlborough, MA, USA). Samples were separated on a TOYOPEARL HW-50F column (Sigma-Aldrich, St. Louis, MO, USA) calibrated with molecular mass standards (GE Healthcare, Marlborough, MA, USA). The flow rate was 500 μL/min, and 50 mM phosphate buffer was used as a mobile-phase solution. The soy protein content was determined at a wavelength of 280 nm. The recorder was set to 60 min.

Animal study

Fifty-four female Ross 308 broilers (1 d old) from a local commercial hatchery were weighed and randomly allotted to one of three treatments, with three replicate cages of six birds per cage. Broilers were reared in stainless-steel and temperature-controlled cages (190 cm × 50 cm × 35 cm) for 3 weeks. Basal diets were formulated based on the National Research Council recommendations, as listed in Table 1. SBM used in the present study is high-protein SBM (47% CP). Thus, no additional lysine was added in the basal diet. The three groups were categorized according to their feeding regimens: (1) basal diet (CTRL), (2) 5% FSBM produced by protease and *B. subtilis*, or (3) 10% FSBM produced by protease and *B. subtilis*. Feed and water were offered *ad libitum*. The temperature was set at 32°C on the first day and gradually reduced to 24°C by the end of the experiment. The lighting schedule included

| Table 1. Nutrient composition of the basal diet. |
|-----------------------------------------------|
| Ingredient % | CTRL | 5% FSBM | 10% FSBM |
|----------------|------|---------|---------|
| Corn, yellow | 51.6 | 51.8 | 52 |
| Soybean meal (47% CP) | 38.5 | 33.2 | 28 |
| Soybean oil | 5.9 | 6 | 6 |
| CaCO3 (38%) | 2 | 2 | 2 |
| CaHPO4 | 1 | 1 | 1 |
| Salt | 0.4 | 0.4 | 0.4 |
| Choline (50%) | 0.02 | 0.02 | 0.02 |
| Vitamin, premix | 0.1 | 0.1 | 0.1 |
| Mineral, premix | 0.1 | 0.1 | 0.1 |
| Methionine (99.5%) | 0.3 | 0.3 | 0.3 |
| Fermented soybean meal | 0 | 5 | 10 |
| Calculated composition | | | |
| Crude protein % | 23.05 | 23.05 | 23.10 |
| Ca % | 1.134 | 1.131 | 1.128 |
| P % | 0.579 | 0.575 | 0.572 |
| Lysine % | 1.224 | 1.222 | 1.224 |
| Methionine + Cystine % | 1 | 1 | 1 |
| ME, kcal/kg | 3217.81 | 3219.60 | 3215.68 |

aSupplied per kilogram of diet: retinol, 6000 IU; cholecalciferol, 900 IU; tocopherol, 30 IU; menadione, 3 mg; riboflavin, 6 mg; pantothenic acid, 18 mg; niacin, 60 mg; and cobalamin, 30 μg.

bSupplied per kilogram of diet: Cu, 20 mg; Zn, 100 mg; Fe, 140 mg; Mn, 4 mg; Se, 0.1 mg; and I, 0.2 mg.
Table 2. Primer sequences for quantitative reverse-transcription PCR.

| Genes  | Primer sequences (5′-3′)                                           | Accession no. | Annealing temperature (°C) |
|--------|---------------------------------------------------------------------|---------------|----------------------------|
| IL-4   | F: TGTGCCACGCTGCTGTTACA R: CTTGTGGCAGTGCTGGCTCTCC                  | AJ621249.1    | 60.9                       |
| IL-10  | F: AGCAGATCAAGGAGAGGTTCC R: ATCAGCAGGTACTCCCTTCC                  | AJ621614      | 60.9                       |
| β-actin| F: CCACCGGAATGCCTCTAAAC R: GAAGGTCGACGGCAGCTTCT                   | X00182        | 60.0                       |

Figure 1. Effect of protease and probiotics on the soy protein content of FSBM. (A) Effects of different protease activity (10^4, 10^5, and 10^6 CDU/kg), initial moisture (40%, 50%, and 100%), and fermentation durations (6, 12, and 24 h) on the soy protein content of FSBM. (B) Effects of different protease activity (10^4, 10^5, and 10^6 CDU/kg), initial moisture (40%, 50%, and 100%), and fermentation durations (6, 12, and 24 h) on the soy protein content of FSBM produced by A. oryzae. (C) Effects of different protease activity (10^4, 10^5, and 10^6 CDU/kg), initial moisture (40%, 50%, and 100%), and fermentation durations (6, 12, and 24 h) on the soy protein content of FSBM produced by B. subtilis. M represents the standard protein marker. Three experiments were conducted, and one representative result is displayed.
22 h of light and 2 h of dark throughout the experiment. The individual body weight, average daily feed intake, average daily gain, and feed conversion ratio (FCR) were recorded every week. Two chicks per replicate cage were sacrificed through cervical dislocation at 21 d of age, and the blood, spleen, and bursa of Fabricius were collected for analysis. Plasma samples (5 mL) were collected through cardiac puncture, centrifuged at 3000 × g for 10 min, and then stored at −20°C. The spleen and bursa of Fabricius of the birds were excised and subsequently frozen at −20°C for further analysis.

**Serum immunoglobulin G and immunoglobulin A analysis**

Blood samples were collected in nonheparinized tubes through cardiac puncture from two random chicks per replicate cage at 21 days old. Blood samples were centrifuged for 15 min at 3000 × g at 4°C. The serum was then stored at −4°C until the immunoglobulin G (IgG) and immunoglobulin A (IgA) concentrations were determined. The serum immunoglobulin levels were measured using an enzyme-linked immunosorbent assay kit (Bethyl Laboratories, Inc., Montgomery, TX, USA).

**Quantitative reverse-transcription polymerase chain reaction**

Six birds from each group were randomly selected, sacrificed, and examined for gene expression. Total RNA was isolated from the spleen and bursa of Fabricius and homogenized in TRIzol Reagent (Invitrogen, Carlsbad, CA, USA) using a homogenizer (SpeedMill PLUS, Analytik Jena, Jena, Germany). Total RNA was then purified and reverse transcribed by a Transcriptor...
Reverse Transcriptase kit (Roche Applied Science, Indianapolis, IN, USA). Quantitative reverse-transcription polymerase chain reaction (PCR) was performed using a MiniOpticon real-time PCR detection system (Bio-Rad, Hercules, CA, USA) and KAPA SYBR FAST qPCR Kit (Kapa Biosystems, Boston, MA, USA). PCR was performed using 40 cycles at 95°C for 30 s, 58–60°C for 60 s, and 72°C for 30 s, and β-actin mRNA was determined as the internal control gene. The sequence of primers for quantitative reverse-transcription PCR is listed in Table 2. The mRNA expression of each gene was normalized to the β-actin mRNA expression in the same sample. The threshold cycle (Ct) values were obtained, and the relative gene expression was calculated using the formula \((1/2)^{\Delta \text{Ct target genes} - \Delta \text{Ct β-actin}}\).

**Ethics statement**

All experiments were performed in accordance with the approved guidelines. The animal protocol was approved by the National Ilan University Institutional Animal Care and Use Committee.

**Statistical analysis**

Replicate means are the experimental units in the statistical analysis. The data were analyzed in a completely randomized design using the general linear model (GLM) procedure of the SAS software (SAS Institute, Cary, NC, USA). Means were compared using Tukey test when the probability values were significant \((p < 0.05)\).

**Results**

**Protease in combination with B. subtilis promotes soy protein degradation**

The protease activity plays a critical role in soy protein degradation. We first investigated the effects of the initial moisture content, incubation duration, and protease activity on soy protein degradation. The degradation efficiency of 75-kDa soy protein was linearly correlated with increased protease activity (Figure 1A). The content of 20-kDa soy protein was associated with increasing protease activity (Figure 1A). Furthermore, \(10^4\) CDU/kg protease in combination with A. oryzae treatment promoted the degradation of 75-kDa soy protein at 100% initial moisture during 12- and 24-h fermentation periods (Figure 1B). As expected, increased protease activity \((10^2\) and \(10^6\) CDU/kg) enhanced the degradation of 75-kDa soy protein during the SSF of SBM by A. oryzae (Figure 1B). Moreover, \(10^4\) CDU/kg protease in combination with A. oryzae treatment further degraded 50-kDa soy protein at 100% initial moisture during a 24-h fermentation period (Figure 1B). Similar to A. oryzae, B. subtilis treatment combined with \(10^4\) CDU/kg protease promoted the degradation of 75-kDa soy protein at 100% initial moisture during a 24-h fermentation period (Figure 1C). In contrast to the A. oryzae treatment, B. subtilis treatment with \(10^6\) CDU/kg protease completely degraded the 75-kDa soy protein (Figure 1B and 1C). Similarly, \(10^6\) CDU/kg protease in combination with B. subtilis treatment further degraded the 50-kDa soy protein at 100% initial moisture during a 24-h fermentation period (Figure 1C).

We then tested whether protease and probiotics could further reduce the β-conglycinin content of SBM at different initial moisture contents and incubation durations. The content of the α subunit of β-conglycinin (75 kDa) was significantly reduced in the presence of increased protease activity \((10^6\) CDU/kg; Figure 2A). In combination with A. oryzae treatment, \(10^4\) and \(10^5\) CDU/kg protease promoted the degradation of the α subunit of β-conglycinin at 100% initial moisture during 12- and 24-h fermentation periods (Figure 2B), and \(10^6\) CDU/kg protease completely degraded the α subunit of β-conglycinin under all fermentation conditions (Figure 2B). Similar results were also observed for protease in combination with B. subtilis treatment (Figure 2C).

We further examined the differences in the degradation efficiency of the α and β subunits of β-conglycinin of A. oryzae and B. subtilis in the presence of \(10^6\) CDU/kg protease at 100% initial moisture during a 24-h fermentation period. Both protease alone and protease in combination with A. oryzae treatment sufficiently degraded the α subunit of β-conglycinin compared with untreated SBM (Figure 3A). In addition to the degradation of the α subunit of β-conglycinin, protease in combination with B. subtilis treatment further degraded the β base of the β-conglycinin subunits (Figure 3A). Moreover, FPLC demonstrated that large-molecular-weight soy protein was reduced in the presence of protease and probiotics compared with untreated SBM (Figure 4A and 4B). The levels of low-molecular-weight (<6.5 kDa) soy protein were significantly higher when protease was used in combination with B. subtilis rather than A. oryzae (Figure 4B, black arrow). These findings indicate that optimal SSF for soy protein degradation utilizes protease in combination with B. subtilis treatment at 100% initial moisture for 24 h.
Figure 4. FPLC patterns of soy protein degradation by protease and probiotics. The SBM was fermented with $10^5$ CDU/kg protease and probiotics (A. oryzae or B. subtilis) at an initial moisture content of 40% for 24 h. (A) FPLC measurement of standard patterns of soy protein. (B) FPLC measurement of soy protein degradation in FSBM produced by protease and probiotics. The two black lines is used as an indicator of standard proteins (66 and 6.5 kDa) based on its retention time. Three experiments were conducted, and one representative result is displayed.
for three weeks. However, no differences were statistically confirmed in the body weight between the different diets during the entire feeding period (Table 3). Broilers fed 5% FSBM had a lower feed intake than broilers fed other treatments from Day 1 to 7 (Table 3, \( P < 0.05 \)). The group with high-concentration FSBM supplementation exhibited higher feed intake throughout the feeding period than the groups with other treatments (Table 3, \( P < 0.05 \)). No statistical differences were confirmed in the FCR between different FSBM supplementations during the entire feeding period (Table 3). To examine the effect of different concentrations of FSBM on the inflammatory gene expression in the spleen and bursa of Fabricius, broilers were fed with 5% and 10% FSBM. Supplementation of 5% FSBM in the diet for 21 d had no significant effect on \( \text{IL-4} \) mRNA expression in the spleen compared with the control group (Figure 5A), whereas \( \text{IL-4} \) mRNA expression in the spleen was significantly reduced after 10% FSBM supplementation in broilers (Figure 5A, \( P < 0.05 \)). Similarly, 5% FSBM did not inhibit \( \text{IL-10} \) mRNA expression in the spleen of broilers at 21 d of age (Figure 5B). However, \( \text{IL-10} \) mRNA expression was significantly reduced in the spleens of broilers after treatment with 10% FSBM (Figure 5B, \( P < 0.05 \)). In the bursa of Fabricius of broilers, \( \text{IL-4} \) mRNA expression was not affected by 5% FSBM treatments over 21 d (Figure 5C), whereas 10% FSBM treatments efficiently inhibited \( \text{IL-4} \) mRNA expression in broilers (Figure 5C, \( P < 0.05 \)). However, no differences in \( \text{IL-10} \) mRNA were statistically confirmed for different FSBM supplementations in the bursa of Fabricius of broilers (Figure 5D). Moreover, supplementation of

Figure 5. Effect of FSBM produced by \( B. \ subtilis \) on the immune-response-associated gene expression in the spleens and bursa of Fabricius of broilers. Effects of control (CTRL), 5% FSBM produced by \( B. \ subtilis \) (5% FSBM), and 10% FSBM produced by \( B. \ subtilis \) (10% FSBM) on \( \text{IL-4} \) (A) and \( \text{IL-10} \) (B) mRNA expression in the spleens of broilers at 21 d. Effects of control (CTRL), 5% FSBM produced by \( B. \ subtilis \) (5% FSBM), and 10% FSBM produced by \( B. \ subtilis \) (10% FSBM) on \( \text{IL-4} \) (C) and \( \text{IL-10} \) (D) mRNA expression in the bursa of Fabricius of broilers at 21 d. The values are expressed as mean ± standard deviation (\( n = 3 \)). The means with different letter superscripts are significantly different (\( P < 0.05 \)).

Table 3. Effect of FSBM on the growth performance of broilers.

| Group     | CTRL       | 5% FSBM    | 10% FSBM   | \( p \) value |
|-----------|------------|------------|------------|----------------|
|           | Mean      | SD         | Mean       | SD             | Mean       | SD         |           |               |
| Body weight (g) |           |            |            |                |            |            |           |               |
| 1 d        | 38.94     | 0.19       | 38.89      | 0.10           | 38.78      | 0.10       | 0.3990   |
| 7 d        | 130.00    | 5.77       | 121.11     | 6.94           | 130.00     | 5.77       | 0.2076   |
| 14 d       | 326.67    | 20.82      | 288.89     | 33.72          | 324.44     | 18.36      | 0.2012   |
| 21 d       | 601.11    | 32.62      | 602.22     | 27.76          | 617.78     | 29.63      | 0.7594   |
| Average daily gain (g/d) |           |            |            |                |            |            |           |               |
| 1–7 d      | 13.01     | 0.81       | 11.75      | 1.00           | 13.03      | 0.82       | 0.2061   |
| 8–14 d     | 28.10     | 2.18       | 23.97      | 5.14           | 27.78      | 1.92       | 0.3517   |
| 15–21 d    | 41.00     | 2.18       | 42.17      | 7.16           | 42.70      | 2.79       | 0.90004  |
| 1–21 d     | 27.03     | 0.95       | 26.30      | 1.32           | 27.84      | 1.41       | 0.3770   |
| Average daily feed intake (g/d) |           |            |            |                |            |            |           |               |
| 1–7 d      | 16.83\(^a\)  | 1.45       | 14.29\(^b\) | 0.10          | 15.67\(^*\) | 0.27       | 0.0289   |
| 8–14 d     | 40.32     | 2.44       | 33.49      | 7.29           | 38.89      | 3.17       | 0.2619   |
| 15–21 d    | 59.52     | 8.30       | 53.71      | 5.61           | 60.00      | 1.26       | 0.6364   |
| 1–21 d     | 34.89\(^a\) | 1.51       | 34.50\(^b\) | 1.19           | 38.25\(^*\) | 1.30       | 0.0262   |
| FCR (daily feed intake/daily gain) |           |            |            |                |            |            |           |               |
| 1–7 d      | 1.29      | 0.04       | 1.22       | 0.11           | 1.22       | 0.09       | 0.5443   |
| 8–14 d     | 1.44      | 0.06       | 1.40       | 0.02           | 1.40       | 0.04       | 0.4705   |
| 15–21 d    | 1.49      | 0.21       | 1.30       | 0.11           | 1.41       | 0.07       | 0.3302   |
| 1–21 d     | 1.41      | 0.06       | 1.31       | 0.03           | 1.41       | 0.06       | 0.1297   |

\(^{a,b}\)Means within a row with no common superscript are significantly different (\( P < 0.05 \)).
5% FSBM in the diet for 21 d had no significant effect on anti-soybean allergen IgG levels compared with the control group (Figure 6A), whereas anti-soybean allergen IgG levels were significantly reduced after treatment with 10% FSBM (Figure 6A, P < 0.05). By contrast, no significant differences were observed in anti-soybean allergen IgA levels for different FSBM supplementation at 21 d of age (Figure 6B). These results indicate that supplementation of FSBM in the diet has no effect on the growth performance of broilers. However, FSBM produced by protease and B. subtilis could alleviate the immune response caused by soybean allergens in broilers.

Discussion

We demonstrated that in optimal SSF conditions for soy protein degradation, protease is used in combination with B. subtilis treatment at 100% initial moisture during a 24-h fermentation period. FSBM produced by protease and B. subtilis did not affect the growth performance of broilers. Dietary supplementation with FSBM significantly reduced the allergic immune-response-associated gene expression and serum anti-soybean allergen IgG levels in broilers.

Several reports have demonstrated that soybean oligosaccharides and crude proteins can be degraded during microbial fermentation by producing a variety of enzymes (Hong et al. 2004; Adeyemo and Onilude 2014; Su et al. 2018). Exogenous enzyme supplementation also increases the degradation of soybean protein and inactive anti-nutritional factors in the fermentation of SBM (Ma and Wang 2010; Amadou et al. 2011). Protease supplementation efficiently improved the nutritional value of SBM during SSF through the degradation of the large-molecular-weight soybean protein (Wang et al. 2014). We previously demonstrated that oligosaccharides in SBM were efficiently reduced during SSF by Lactobacillus species and C. butyricum (Su et al. 2018). Here, we discovered that protease supplementation increased soy protein degradation in a dose-dependent manner. Previous studies have indicated that A. oryzae and B. subtilis can degrade anti-nutritional factors and increase small-size peptides in the fermentation of SBM by producing multiple hydrolysis enzymes (Hong et al. 2004; Teng et al. 2012). In this study, a significantly larger improvement in soy protein and soybean allergen degradation during SSF was observed when probiotic supplementation was used in combination with protease than when protease treatment was used alone. Furthermore, a previous study demonstrated that trypsin inhibitor and large-size protein in FSBM were lower for treatment with B. subtilis than for treatment with A. oryzae (Teng et al. 2012). In this study, the anti-nutritional factors and small-size protein content in FSBM exhibited a larger improvement when protease was used in combination with B. subtilis treatment rather than A. oryzae treatment. Whether B. subtilis has a higher capacity to produce enzymes than does A. oryzae remains to be investigated.

Newborn chicks with immature digestive systems are inefficient in nutrient utilization from SBM because of insufficient endogenous enzymes (Batal and Parsons 2002; Chot et al. 2010). Feeding of SBM fermented with Aspergillus species was reported to elevate the activities of intestinal digestive enzymes and intestinal morphology in broilers (Feng et al. 2007b). Furthermore, feeding broilers with FSBM produced by Aspergillus species could improve growth performance (Hirabayashi et al. 1998; Mathivanan et al. 2006; Feng et al. 2007b). Similarly, feeding of FSBM produced by B. subtilis during the early phase benefits the growth performance of broilers (Kim et al. 2016). Although changes in growth performance did not reach statistical significance in the present study, FSBM produced by protease and B. subtilis still caused a similar trend in improving the FCR in broilers between Day 1 and Day 21. Microbial fermentation has been demonstrated to degrade anti-nutritional factors and increase small-size peptides (Hong et al. 2004; Teng et al.
2012) and therefore increase the availability of nutrients in broilers. Thus, the improved growth performance in FSBM-fed broilers may be caused by increased levels of amino acids and reduced anti-nutritional factors resulting from fermentation. However, the nutritional value of FSBM produced by protease and B. subtilis should be reanalyzed in a future study.

SBM containing soybean allergens (glycinin and β-conglycinin) has been reported to induce local mucosal T-cell and B-cell infiltration (Dreau et al. 1995), which leads to impaired growth performance in broilers (Palacios et al. 2004). These soybean allergens can be broken down by fermentation processes using bacteria or exogenous enzymes (Yamanishi et al. 1995; Feng et al. 2007b; Wang et al. 2011). In this study, protease in combination with B. subtilis treatment degraded the α (75 kDa) and β (50 kDa) subunits of β-conglycinin during the SSF of SBM. This result is consistent with a previous observation that the α and β subunits of β-conglycinin could be degraded by B. subtilis during the SSF of SBM (Zhang et al. 2018). Broilers fed FSBM produced by Aspergillus species had a higher level of total serum IgA and IgM than those fed the basal diet (Feng et al. 2007b). In this study, the levels of anti-soybean allergen IgG in broilers were significantly reduced by FSBM produced by protease and B. subtilis in a dose-dependent manner. Although it did not reach statistical significance, FSBM produced by protease and B. subtilis with low levels of soybean allergens also caused a similar decreasing trend in the serum levels of anti-soybean allergen IgA. A previous study reported that intestinal mucosal immune responses to soybean allergens were elevated by high levels of inflammatory cytokines, such as IL-4 and IL-10 (Sun et al. 2008). Glycinin and β-conglycinin in SBM could promote intestinal epithelial cells to express inflammatory cytokines (Xu et al. 2010). Reduced IL-4 and IL-6 mRNA expression was observed in the small intestine of pigs fed FSBM (Zhang et al. 2018). In this study, IL-4 mRNA expression in the spleen and bursa of Fabricius of broilers consistently decreased with increasing FSBM supplementation. The results of serum immunoglobulin levels and inflammatory gene expression implied that reduced soybean allergen contents in FSBM could improve allergic immune response in broilers.

In conclusion, supplementation of B. subtilis and protease during the SSF of SBM increases the protein degradation and reduces the soybean allergen content. FSBM produced by B. subtilis and protease with high levels of small-size protein and low levels of soybean allergen could improve immune response in broilers.

Disclosure statement

No potential conflict of interest was reported by the authors.

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