R. Bras. Zootec., 48:e20170298, 2019
https://doi.org/10.1590/rbz4820170298

Non-ruminants
Full-length research article

Revista Brasileira de Zootecnia

Brazilian Journal of Animal Science
ISSN 1806-9290
www.rbz.org.br

Optimization of solid-state fermentation conditions of *Bacillus licheniformis* and its effects on *Clostridium perfringens*-induced necrotic enteritis in broilers

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Received: November 20, 2017
Accepted: January 12, 2018

How to cite: Lin, E. R.; Cheng, Y. H.; Hsiao, F. S. H.; Proskura, W. S.; Dybus, A. and Yu, Y. H. 2019. Optimization of solid-state fermentation conditions of *Bacillus licheniformis* and its effects on *Clostridium perfringens*-induced necrotic enteritis in broilers. Revista Brasileira de Zootecnia 48:e20170298. https://doi.org/10.1590/rbz4820170298

ABSTRACT - In the present study, we examined the growth parameters of *Bacillus licheniformis* in solid-state fermentation (SSF) and evaluated the effects of *Bacillus licheniformis*-fermented products on *Clostridium perfringens*-challenged broilers. During four and six days of SSF, the highest viable biomass was observed at 5% glucose, 10% soybean meal, 3% yeast, and 50% initial moisture content. The *Bacillus licheniformis* SSF products were heat- and acid-resistant. Furthermore, the fermented products were able to inhibit the growth of *Clostridium perfringens* and *Staphylococcus aureus in vitro*. In feeding experiments, in a similar manner to the antibiotic treatment group, dietary supplementation of *Bacillus licheniformis*-fermented products significantly improved intestinal morphology and necrotic lesions under *Clostridium perfringens* challenge, accompanied by increased *IFN-γ* mRNA expression in the spleen and bursa of Fabricius. These results together suggest that *Bacillus licheniformis*-fermented products have potential for development as feed additives and use as possible substitutes for antibiotics to treat *Clostridium perfringens* in the poultry industry.

Keywords: broiler, disease, fermentation, probiotics

Introduction

Necrotic enteritis (NE) is an extremely common and important avian enteric disease caused mainly by *Clostridium perfringens* (Van Immerseel et al., 2004; Timbermont et al., 2011; Abudabos et al., 2018). It leads to enormous economic losses in the poultry industry worldwide (Van der Sluis, 2000; Timbermont et al., 2011). *Clostridium perfringens* is a Gram-positive anaerobic spore-forming bacterium found in the gastrointestinal tract of broilers (Van Immerseel et al., 2004; Dahiya et al., 2005). The disease prevalently occurs in broilers aged from two to six weeks and causes sudden death with mortality rates up to 50% (Kaldhusdal and Løvland, 2000; Lee et al., 2011).

Antibiotics have been commonly used worldwide as growth promoters and for prophylactic treatment of *Clostridium perfringens*-induced NE in poultry (Van Immerseel et al., 2004; Caly et al., 2015). However, the European Union has banned the use of antibiotics, leading to an increase in NE outbreaks in broilers in European countries (Van der Sluis, 2000; Van Immerseel et al., 2004). Therefore, alternative strategies to prevent NE in broilers are needed in the poultry industry. Over the past few years, it has been demonstrated that dietary supplementation of probiotics can inhibit the growth of gastrointestinal pathogens and subsequent diseases by producing antimicrobial substances (Patterson and Burkholder, 2003; Lutful Kabir, 2009; Cheng et al., 2018). Among *Bacillus* species, *Bacillus licheniformis* has been

identified in the gastrointestinal tract of broilers with activity against *Clostridium perfringens in vitro* (Barbosa et al., 2005). Furthermore, it has been reported that dietary supplementation of *Bacillus licheniformis* improves growth performance of broiler chickens (Liu et al., 2012; Al-Sagan and Abudabos, 2017). *Clostridium perfringens*-induced NE is alleviated in *Bacillus licheniformis*-fed broiler chickens (Knap et al., 2010; Zhou et al., 2016).

Solid-state fermentation (SSF) has been widely used to scale-up production of value-added products as it has low capital investment and is environment-friendly (Hölker and Lenz, 2005; Krishna, 2005; el-Bendary, 2006). Several fermentation parameters are known to affect the growth of probiotics, and only few studies have investigated these parameters for *Bacillus licheniformis* growth in SSF (Kiers et al., 2000; Zhao et al., 2008). The conditions of SSF for *Bacillus licheniformis* growth and the effects of fermented products on broilers under *Clostridium perfringens* challenge have not been widely studied.

The purpose of this study was to investigate the growth parameters of *Bacillus licheniformis* in SSF and the effects of *Bacillus licheniformis*-fermented products on *Clostridium perfringens*-challenged broilers. The results provide valuable information about the growth of *Bacillus licheniformis* in SSF and its effect on *Clostridium perfringens*-induced NE in broilers.

Material and Methods

Research on animals was conducted according to the institutional committee on animal use (IACUC Approval No. 104-14). All experiments were conducted in Yilan, Taiwan (latitude 24°46'00" N and longitude 121°45'00" E). The experimental period was carried out between July 1 and December 15, 2015.

Bacillus licheniformis was purchased from the Food Industry Research and Development Institute (Hsinchu, Taiwan). After thawing, *Bacillus licheniformis* was inoculated into an Erlenmeyer flask containing tryptic soy broth (Sigma-Aldrich, St. Louis, MO, USA) and incubated at 30 °C for 18 h, being shaken at 160 rpm.

The procured substrates such as wheat bran, soybean meal, yeast, fish meal, brown sugar, and glucose were ground to fine powder. *Bacillus licheniformis* SSF was optimized by investigating the effect of the following treatments on the bacterial count (colony forming unit – cfu) production, using different concentrations of carbon sources (glucose and brown sugar), concentrations of nitrogen sources (soybean meal and yeast), initial moisture contents (40-70%), and SSF period (two, four, and six days with two-day intervals). Potassium dihydrogen phosphate was added to the fermented substrate to increase the biomass yield by SSF. Each substrate or combined substrates were mixed with water to give the required initial moisture contents in a space bag and autoclaved at 121 °C for 30 min. The cooled substrates were inoculated with 4% (v/w) inoculum, mixed carefully under sterile conditions, and incubated at 30 °C in a chamber with free oxygen and relative humidity above 80%. The fermented products were dried at 50 °C for two days and homogenized by mechanical agitation. The fermented powder was then stored at 4 °C prior to analysis.

The fermented powder was diluted serially in 0.85% NaCl and plated on tryptic soy agar (TSA; Sigma-Aldrich, St. Louis, MO, USA), which was incubated for 18 h at 30 °C. Bacterial growth was counted and expressed as cfu/g. For determination of spores, fermented powder was diluted in 0.85% NaCl and then heated at 80 °C for 10 min before plating on TSA. After incubation at 30 °C for 18 h, colonies formed were counted and expressed as cfu/g. The counts of surviving cells were determined according to Pieniz et al. (2014). For heat-resistant analysis, fermented powder was diluted in 0.85% NaCl and incubated at different temperatures (80, 90, and 100 °C) for 5, 10, and 15 min, respectively. Counts of surviving cells were determined by plating on TSA. The survival percentage (%±SD) of strains to heat was calculated as follows: % survival = (viable count after exposure to heat/viable count without exposure to heat) × 100. For acid-resistant analysis, the fermented powder was diluted in 0.85% NaCl and the viability examined at low pH (pH 2.0, 3.0, and 4.0 prepared in 0.85% NaCl containing 0.1% peptone). The suspensions were incubated at 30 °C for 3 h. Counts of surviving cells were determined by plating on TSA. The survival percentage (%±SD) of strains at different pH values was calculated as

follows: % survival = (viable count after exposure to acid/viable count without exposure to acid) × 100. For bile salt-resistant analysis, fermented powder was diluted in PBS containing different concentrations of oxgall (0.1, 0.2, and 0.3%) and plated on TSA. The plates were incubated at 30 °C for 18 h. Cell count was compared with that of the control agar plates (without oxgall). The survival percentage (%±SD) of strains at different concentrations of bile salts was calculated as follows: % survival = (viable count after exposure to bile salts/viable count without exposure to bile salts) × 100.

Antimicrobial activity of *Bacillus licheniformis* SSF products were analyzed using an agar-well diffusion assay. The necrotic enteritis beta toxin-like (NetB)-positive *Clostridium perfringens* (ATCC 13124) and *Staphylococcus aureus* (BCRC10780) were used as indicators of bacterial pathogen for the determination of antimicrobial activity. The fermented powder was diluted in 0.85% NaCl and transferred into a well in Gifu anaerobic medium agar (GAM agar; Sigma-Aldrich, St. Louis, MO, USA) containing *Clostridium perfringens*. The plates were incubated under anaerobic conditions at 37 °C for 24 h and then examined for zones of inhibition. The control discs were impregnated with ampicillin and enramycin. The fermented powder was diluted in 0.85% NaCl and transferred into a well in the lysogeny broth agar (LB agar; Sigma-Aldrich, St. Louis, MO, USA) containing *Staphylococcus aureus*. The plates were incubated at 37 °C for 24 h and then examined for zones of inhibition. The control discs were impregnated with ampicillin.

A total of 48 one-day-old male broilers (Avian) were purchased from a local commercial hatchery. All the broilers were randomly divided into four groups with three replicates. Each replicate was assigned to a cage (four chicks per cage of 68 × 66 × 33 cm dimension). The four groups (n = 12 per group) were: basal diet (control) plus oral administration of *Clostridium perfringens* (1×10^8 cfu/mL), basal diet plus oral administration of *Clostridium perfringens* (1×10^8 cfu/mL) and 2 g/kg of bacitracin methylene disalicylate (BMD), basal diet plus oral administration of *Clostridium perfringens* (1×10^8 cfu/mL) and 3 g/kg of four-day fermented product (4DF; 1.2×10^6 cfu/g spore count), and basal diet plus oral administration of *Clostridium perfringens* (1×10^8 cfu/mL) and 3 g/kg of six-day fermented product (6DF; 1.2×10^6 cfu/g spore count). The basal diets were formulated based on the National Research Council recommendations (NRC, 1994) (Table 1). Feed and water were offered *ad libitum*. Birds were housed in stainless-steel and temperature-controlled batteries for five weeks. The temperature was

Table 1 - Nutrient composition of basal diet

Ingredient (g kg^{-1} of dry matter)	Control	BMD	4DF	6DF
Corn, yellow	511.8	511.8	511.8	511.8
Soybean meal (36.7% CP)	350	350	350	350
Fish meal	100	100	100	100
$CaCO_3$ (38%)	20	20	20	20
$CaHPO_4$	10	10	10	10
Salt	4	4	4	4
Choline (50%)	0.2	0.2	0.2	0.2
Vitamin premix[1]	1	1	1	1
Mineral premix[2]	1	1	1	1
Methionine (99.5%)	2	2	2	2
Fermented product			3	3
Antibiotics (10% BMD)		2		
Calculated composition				
Crude protein (%)	23.04	23.04	23.04	23.04
Metabolizable energy (kcal/kg)	3350	3350	3350	3350
Ca (%)	1.6	1.6	1.6	1.6
P (%)	0.8	0.8	0.8	0.8
Lysine (%)	1.38	1.38	1.38	1.38
Methionine + cystine (%)	1	1	1	1

CP - crude protein; BMD - bacitracin methylene disalicylate; 4DF - four-day fermented product; 6DF - six-day fermented product.
[1] Supplied per kg diet: retinol, 6000 IU; cholecalciferol, 900 IU; tocopherol, 30 IU; menadione, 3 mg; riboflavin, 6 mg; pantothenic acid, 18 mg; niacin, 60 mg, cobalamin, 30 µg.
[2] Supplied per kg diet: Cu, 20 mg; Zn, 100 mg; Fe, 140 mg; Mn, 4 mg; Se, 0.1 mg; I, 0.2 mg.

set at 32 °C on the first day, gradually reduced to 24 °C by the third week, and then maintained at 24 °C until the end of the experiment. The lighting schedule was 22L:2D throughout the experiment. Birds were orally inoculated with 1 mL (1×10^8 cfu/mL) of an overnight culture of *Clostridium perfringens* on 18, 19, and 20 days of age. The individual body weight, average daily gain, average daily feed intake, and feed conversion ratio (FCR) was recorded every week. Broilers were sacrificed by cervical dislocation at 22 and 35 days of age. The small intestine, spleen, and bursa of Fabricius were excised and analyzed.

Six birds from each group (two birds per replicate) were randomly selected, sacrificed, and examined for degree of *Clostridium perfringens*-induced necrotic lesions. The duodenum, jejunum, and ileum sections of the chick intestine were examined for lesions. Lesion scores were observed and recorded according to previous study (Keyburn et al., 2008), wherein 0 is normal and 1 to 6 indicate increasing severity of infection: 1 = thin or friable walls; 2 = focal necrosis or ulceration (1-5 foci); 3 = focal necrosis or ulceration (6-15 foci); 4 = focal necrosis or ulceration (16 or more foci); 5 = patches of necrosis of 2-3 cm long; and 6 = diffuse necrosis typical of field cases.

The small intestine of six birds per group was analyzed at three different locations: 2 cm after the gizzard (duodenum), before Meckel's diverticulum (jejunum), and before the ileo-cecal transition (ileum). These samples were fixed in 10% (w/v) neutral-buffered formalin solution (Sigma, St. Louis, MO, USA) at 4 °C. Tissue was sectioned at 5-µm thickness (three cross-sections from each sample) and stained with hematoxylin and eosin. The villus length and crypt depth of each segment was measured randomly on 30 villi in one bird by using Olympus CKX41 microscope (Olympus Corporation, Tokyo, Japan) with a digital video camera. The images were analyzed using stereological image software, Cast Image System (Version 2.3.1.3, Visiopharm Albertslund, Hørsholm, Denmark).

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 transcriptase-PCR was performed using *MiniopticonTM Real-Time PCR detection system* (Bio-Rad, Hercules, CA, USA) and KAPA *SYBR* FAST qPCR Kit (*Kapa Biosystems*, Boston, MA, USA). Polymerase-chain reaction was performed by 40 cycles at 95 °C for 30 s, 58-60 °C for 60 s, and 72 °C for 30 s. β-actin mRNA was determined as the internal control gene. The mRNA expression of each gene (Table 2) was normalized to the β-actin mRNA expression in the same sample. Threshold cycle (C_t) values were obtained, and relative gene expression was calculated using the formula $(1/2)^{Ct\ target\ genes - Ct\ \beta\text{-}actin}$.

Table 2 - Primer sequences for quantitative reverse transcription-PCR

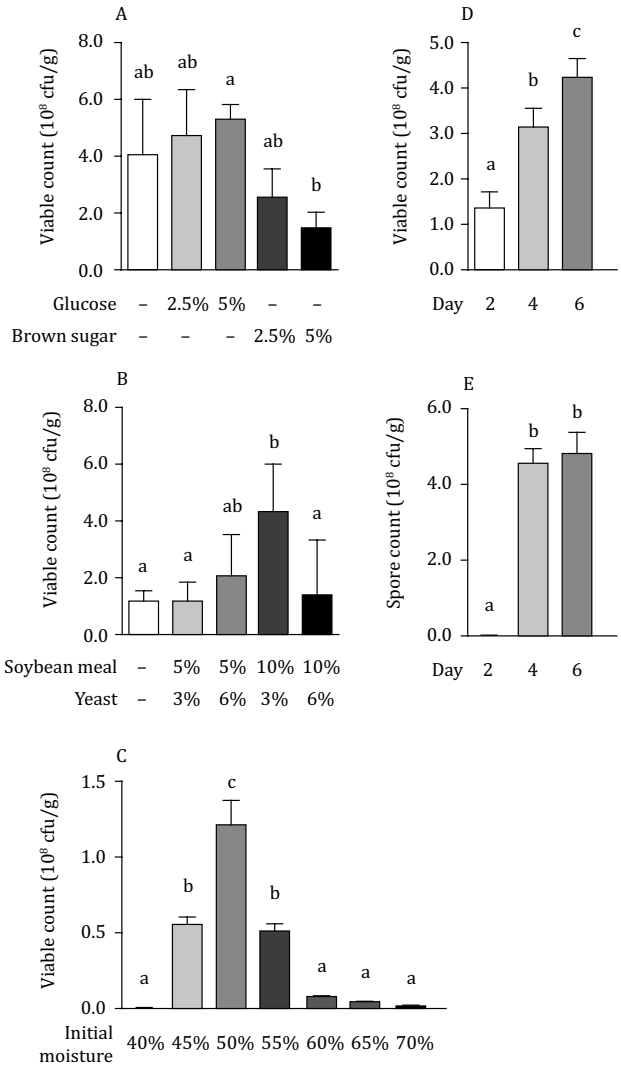
Gene	Primer sequences (5′-3′)	Annealing temperature (°C)
iNOS	F: AGGCCAAACATCCTGGAGGTC R: TCATAGAGACGCTGCTGCCAG	60
COX-2	F: AACACAATAGAGTCTGTGACGTCTT R: TATTGAATTCAGCTGCGATTCGG	60
IFN-γ	F: ACACTGACAAGTCAAAGCCGCACA R: AGTCGTTCATCGGGAGCTTGGC	55
IL-1β	F: CAGCCTCAGCGAAGAGACCTT R: CACTGTGGTGTGCTCAGAATCC	60
IL-4	F: TGTGCCCACGCTGTGCTTACA R: CTTGTGGCAGTGCTGGCTCTCC	60.9
IL-10	F: AGCAGATCAAGGAGACGTTC R: ATCAGCAGGTACTCCTCGAT	60.9
β-actin	F: CATCACCATTGGCAATGAGAGG R: GGTACATTGTGGTACCACCAGAC	60

PCR - polymerase chain reaction; *iNOS* - inducible nitric oxide synthase; *COX-2* - cyclooxygenase-2; *IFN-γ* - interferon-γ; *IL-1β* - interleukin-1β; *IL-4* - interleukin-4; *IL-10* - interleukin-10; F - forward; R - reverse.

All experimental data were analyzed by ANOVA using the GLM procedure of SAS (Statistical Analysis System, version 9.2) in a completely randomized design. Duncan's new multiple range test was used to evaluate differences between means. Each broiler formed the experimental unit. P-values of less than 0.05 were considered statistically significant.

Results

Bacillus licheniformis SSF was optimized by studying the result of different treatments on cfu. Although the changes in *Bacillus licheniformis* biomass were not statistically significant, a trend of increased bacterial growth was observed with the supplementation of glucose compared with the control group (Figure 1A). In contrast, brown sugar supplementation tended to reduce the bacterial growth (Figure 1A). In the nitrogen resource analysis, 10% soybean meal in combination with 3% yeast supplementation revealed the highest bacterial growth compared with other treatments (Figure 1B) (P<0.05). The increased biomass production of *Bacillus licheniformis* in SSF was observed

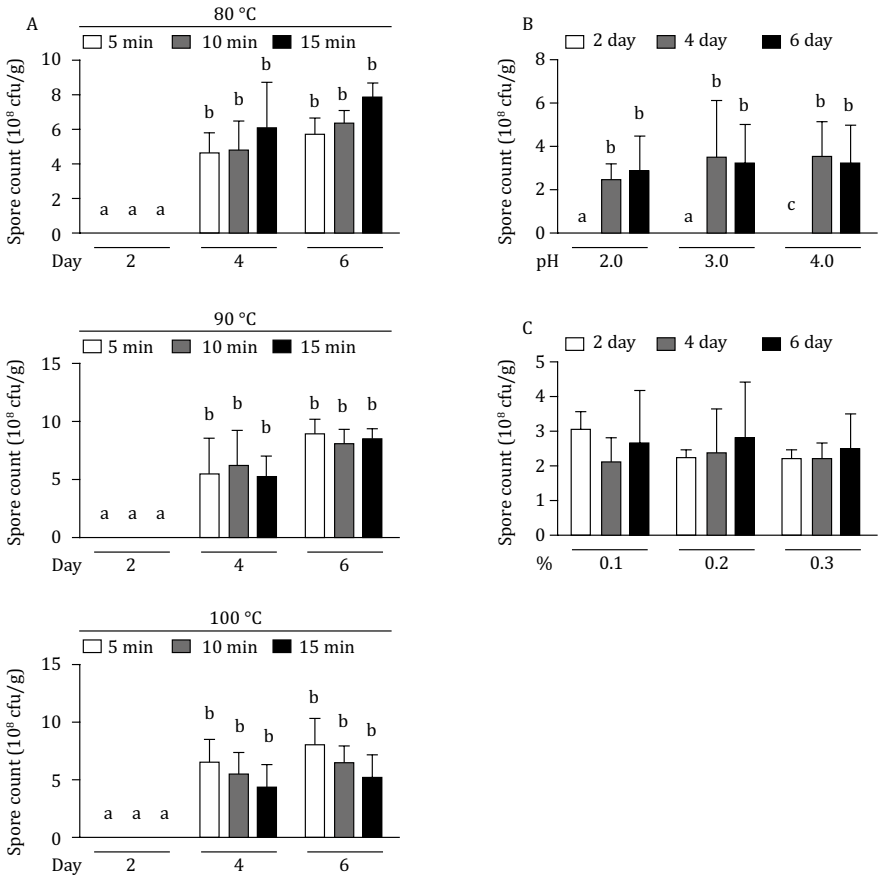


(A) Effect of different carbon sources (glucose and brown sugar) on bacterial count of *Bacillus licheniformis* in SSF. (B) Effect of different nitrogen sources (soybean meal and yeast) on bacterial count of *Bacillus licheniformis* in SSF. (C) Effect of different initial moisture (40-70%) on bacterial count of *Bacillus licheniformis* in SSF. (D) Effect of different fermentation duration (two, four, and six days) on bacterial count of *Bacillus licheniformis*. (E) Effect of different fermentation duration (two, four, and six days) on spore production of *Bacillus licheniformis*.
Values were expressed as mean ± standard deviation (n = 3).
Means with different letters are significantly different (P<0.05).

Figure 1 - Optimization of growth parameters of *Bacillus licheniformis* by solid-state fermentation (SSF).

at the initial moisture content of 50% (Figure 1C) (P<0.05). The *Bacillus licheniformis* biomass in SSF was positively correlated with extended incubation period (Figure 1D) (P<0.05). A similar result was also observed in spore production, but no significant difference was found between four and six days of SSF (Figure 1E). Together, these findings demonstrate that the optimal parameters for *Bacillus licheniformis* in SSF are 5% glucose and 10% soybean meal in combination with 3% yeast at the initial moisture content of 50% with extended incubation period.

Heat-resistant analysis showed that fermented products produced by four and six days of SSF were highly resistant to heat compared with fermented products produced by two days of SSF (Figure 2A) (P<0.05). A similar result was also observed in acid-resistant analysis. Fermented products produced by four and six days of SSF were resistant to the acidic environment compared with fermented products produced by two days of SSF (Figure 2B) (P<0.05). However, no significant difference was found in bile salt-resistant analysis among treatments (Figure 2C). The fermented products from two days of SSF exhibited potent antimicrobial activity against *Staphylococcus aureus* (Figure 3A). The antimicrobial effects were further increased in fermented products from four and six days of SSF (Figure 3A). In addition to *Staphylococcus aureus*, fermented products from six days of SSF also showed antimicrobial activity against *Clostridium perfringens* compared with enramycin (Figure 3B) and ampicillin (Figure 3C). These results demonstrate that the fermented products produced by four



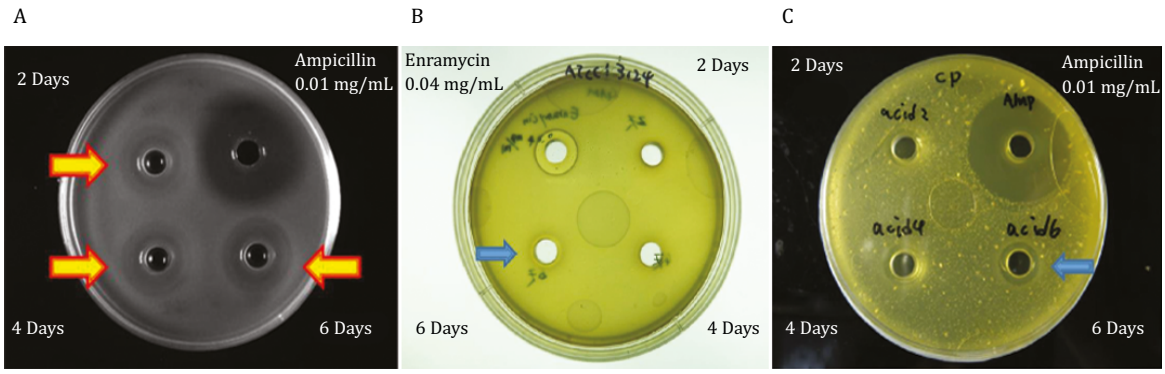
(A) Effect of heat treatments (80, 90, and 100 °C) and different fermentation duration (two, four, six days) on spore production of *Bacillus licheniformis*. (B) Effect of acid treatments (pH 2.0, 3.0, and 4.0) and different fermentation duration (two, four, and six days) on spore production of *Bacillus licheniformis*. (C) Effect of bile salt treatments (0.1, 0.2, and 0.3%) and different fermentation duration (two, four, and six days) on spore production of *Bacillus licheniformis*.
Values were expressed as mean ± standard deviation (n = 3).
Means with different letters are significantly different (P<0.05).

Figure 2 - Determination of tolerance of stress on spore production of *Bacillus licheniformis* solid-state fermentation product.

and six days of SSF are thermostable and acid-tolerant. The six days of *Bacillus licheniformis* SSF products had the highest antimicrobial activity.

Our results demonstrated that *Clostridium perfringens* challenge could induce intestinal necrotic lesions in broilers (data not shown). To investigate what effect the SSF product from *Bacillus licheniformis* has on birds under *Clostridium perfringens* challenge, we offered the broilers a basal diet, a basal diet supplemented with antibiotics (bacitracin methylene disalicylate; BMD), and a basal diet supplemented with four or six days of *Bacillus licheniformis* SSF products. After feeding the diets for five weeks, no significant difference was found in the growth performance according to body weight and food intake among the groups (Table 3). Antibiotic treatment resulted in a significantly reduced FCR between day 1 and day 21 compared with the control group (Table 3) ($P<0.05$). Although it did not reach statistical significance, four and six days of *Bacillus licheniformis* SSF products caused a similar trend in improving FCR in broiler chickens between day 1 and day 21 (Table 3). After 22 days of feeding, four and six days of *Bacillus licheniformis* SSF products efficiently alleviated the intestinal damage caused by *Clostridium perfringens* compared with control and antibiotic treatment (Figure 4A) ($P<0.05$). After feeding the diets for 35 days, no significant difference was found in the intestinal lesion scores among the groups (Figure 4B). These findings demonstrate that the dietary fermented products of *Bacillus licheniformis* could inhibit the *Clostridium perfringens*-induced necrotic lesions in the small intestines of broilers, and these effects are more effective than commercial antibiotics.

Subsequently, we examined the morphology of the small intestine in broilers after SSF product treatment under *Clostridium perfringens* challenge. After 22 days of feeding, results showed that the villus length was significantly increased in the duodenum and jejunum in the groups treated with antibiotics and fermented products compared with the control group (Table 4) ($P<0.05$). The antibiotics significantly reduced the duodenal crypt depth, whereas no significant difference was found in the groups treated with *Bacillus licheniformis* SSF products for four and six days (Table 4). By contrast, four and six days of *Bacillus licheniformis* SSF products remarkably reduced the jejunal crypt depth compared with the control group (Table 4) ($P<0.05$). Similar to antibiotics, four and six days of *Bacillus licheniformis* SSF products significantly increased the villus length:crypt depth ratio (Table 4) ($P<0.05$). After 35 days of feeding, six days of *Bacillus licheniformis* SSF products significantly increased intestinal villus length compared with the control group (Table 5) ($P<0.05$). The antibiotics and SSF product treatments were able to reduce the jejunal crypt depth (Table 5) ($P<0.05$). Furthermore, villus length:crypt depth ratio in the duodenum was elevated after antibiotics and SSF product treatments (Table 5) ($P<0.05$). Taken together, these results indicate that the SSF



(A) Antimicrobial activity of fermented product from different fermentation durations (two, four, and six days) against *Staphylococcus aureus* compared with ampicillin. Three experiments were carried out, and one representative result is shown. (B) Antimicrobial activity of fermented product from different fermentation durations (two, four, and six days) against *Clostridium perfringens* compared with enramycin. Three experiments were carried out, and one representative result is shown. (C) Antimicrobial activity of fermented product from different fermentation durations (two, four, and six days) against *Clostridium perfringens* compared with ampicillin. Three experiments were carried out, and one representative result is shown.

Figure 3 - Assessment of antimicrobial activity of *Bacillus licheniformis* solid-state fermentation product.

products from *Bacillus licheniformis* reveal similar effects on improving morphology of the small intestine in broilers under *Clostridium perfringens* challenge.

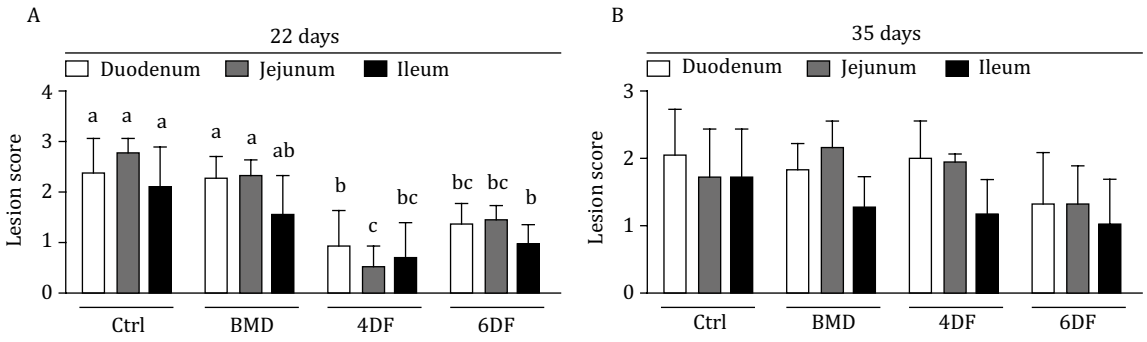
After 22 days of feeding, we found no statistically significant difference in the expression of *iNOS* and *COX-2* genes in the spleen of broilers from the control and SSF product-treated groups (Figure 5A). The expression of *IL-1β* gene of broilers in the six-day SSF product-treated group was greater than in the broilers treated with antibiotics (Figure 5A) (P<0.05). Similarly, six days of SSF products remarkably induced *IFN-γ* mRNA expression in the spleen of broilers compared with the control (Figure 5A) (P<0.05). No significant difference was found in the expression of the *IL-4* and *IL-10* genes in the spleen of broilers between the control and SSF product-treated groups (Figure 5A).

After feeding the diets for 35 days, the expression of *iNOS* gene was elevated in the spleen of boilers from the groups treated with antibiotics and six days of SSF product (Figure 5B) (P<0.05). The antibiotics treatment significantly promoted the *COX-2* mRNA expression (Figure 5B) (P<0.05). However,

Table 3 - Effect of solid-state fermentation product on growth performance of broilers under *Clostridium perfringens* challenge

	Control	BMD	4DF	6DF
Body weight (g)				
1 day	45.07±0.93[1]	44.29±1.51	44.41±1.36	44.64±0.58
21 days	778.33±62.84	910±103.95	841.67±97.51	884.17±52.82
35 days	1570±252.39	1533.3±122.2	1563.3±153	1660±140.8
Average daily gain (g)				
1-21 days	34.92±3.01	41.22±4.89	37.97±4.58	39.98±2.54
21-35 days	48.69±13.97	54.17±15.94	50±5.06	51.43±7.29
Average daily feed intake (g)				
1-21 days	71.59±3.61	64.5±7.45	65.92±1.67	68.06±3.66
21-35 days	135.83±12.16	128.09±15.75	137.62±4.12	141.19±4.12
Feed conversion ratio (daily feed intake/daily gain)				
1-21 days	2.05±0.11a	1.59±0.32b	1.75±0.22ab	1.71±0.19ab
21-35 days	2.92±0.69	2.48±0.72	2.76±0.22	2.79±0.44

BMD - bacitracin methylene disalicylate; 4DF - four-day fermented product; 6DF - six-day fermented product.
[1] Values are expressed as mean ± standard deviation (n = 12).
a-b - Means within a row with different letters are significantly different (P<0.05).



(A) Effect of control (Ctrl), bacitracin methylene disalicylate (BMD), four-day fermented product (4DF), and six-day fermented product (6DF) on intestinal lesion score under *Clostridium perfringens* challenge in broilers at 22 days. (B) Effect of Ctrl, BMD, 4DF, and 6DF on intestinal lesion score under *Clostridium perfringens* challenge in broilers at 35 days.
Values were expressed as mean ± standard deviation (n = 6).
Means with different letters are significantly different (P<0.05).

Figure 4 - Assessment of dietary *Bacillus licheniformis* solid-state fermentation product on intestinal lesion score under *Clostridium perfringens* challenge.

although a slight increase in *COX-2* mRNA expression was observed in the SSF product-treated groups, no statistically significant difference between the control and the SSF product-treated groups were found in the spleen (Figure 5B). The antibiotics and SSF product-treated groups consistently showed increased *IL-1β* and *IFN-γ* mRNA expression in the spleen compared with the control group (Figure 5B; P<0.05). No significant difference was observed in the expression of *IL-4* and *IL-10* genes in the spleen of control and SSF product-treated group (Figure 5B).

Four days of SSF products significantly increased *iNOS* mRNA expression in the bursa of Fabricius (Figure 6A; P<0.05). No significant difference was found in the expression of *COX-2* gene in the bursa of Fabricius between the control and SSF product-treated groups (Figure 6A). The antibiotics- and SSF product-treated groups consistently increased *IL-1β* mRNA expression in the bursa of Fabricius compared with the control group (Figure 6A; P<0.05). The expression of *IFN-γ* gene was remarkably induced in four and six days of SSF product-treated groups (Figure 6A; P<0.05). The expression of *IL-4* gene in the six days of SSF product-treated group was remarkably reduced compared with control group (Figure 6A; P<0.05). No significant difference was found in the expression of *IL-10* gene in the bursa of Fabricius between the control and SSF product-treated groups (Figure 6A).

Table 4 - Assessment of dietary *Bacillus licheniformis* solid-state fermentation product on morphology of small intestine of broiler chickens challenged with *Clostridium perfringens* at 22 days

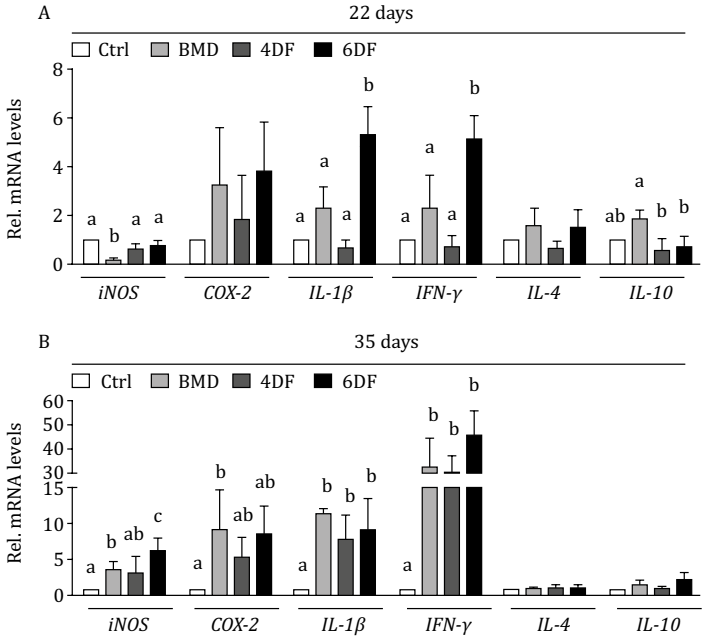
		Control	BMD	4DF	6DF
Villus length (μm)	Duodenum	3974.94±825.80[1]a	8245.76±968.71b	11482.11±818.79c	10828.02±1069.42c
	Jejunum	3979.33±650.62a	9418.04±3468.71b	8888.31±1461.85b	7529.44±2677.54b
	Ileum	3470.01±1396.47ab	4948.36±662.39a	6728±886.42c	2495.73±2164.91b
Crypt depth (μm)	Duodenum	2249.06±451.22a	1696.64±137.47b	1790.84±383.33ab	2107.54±462.85ab
	Jejunum	2245.83±575.87a	1795.24±309.79ab	1438.71±279.4b	1454.78±247.21b
	Ileum	1628.64±287.63ab	1354.62±95.55a	1800.81±369.16b	1712.65±331.22ab
Villus length:crypt depth	Duodenum	1.77267±0.19a	4.87117±0.58b	6.65317±1.42c	5.38417±1.44bc
	Jejunum	1.85483±0.52a	5.138±1.3b	6.1025±1.04b	5.2665±1.21b
	Ileum	2.0845±0.60a	3.65483±0.41b	3.79117±0.4b	2.433±0.78a

BMD - bacitracin methylene disalicylate; 4DF - four-day fermented product; 6DF - six-day fermented product.
[1] Values are expressed as mean ± standard deviation (n = 12).
a-c - Means within a row with different letters are significantly different (P<0.05).

Table 5 - Assessment of dietary *Bacillus licheniformis* solid-state fermentation product on morphology of small intestine of broiler chickens challenged with *Clostridium perfringens* at 35 days

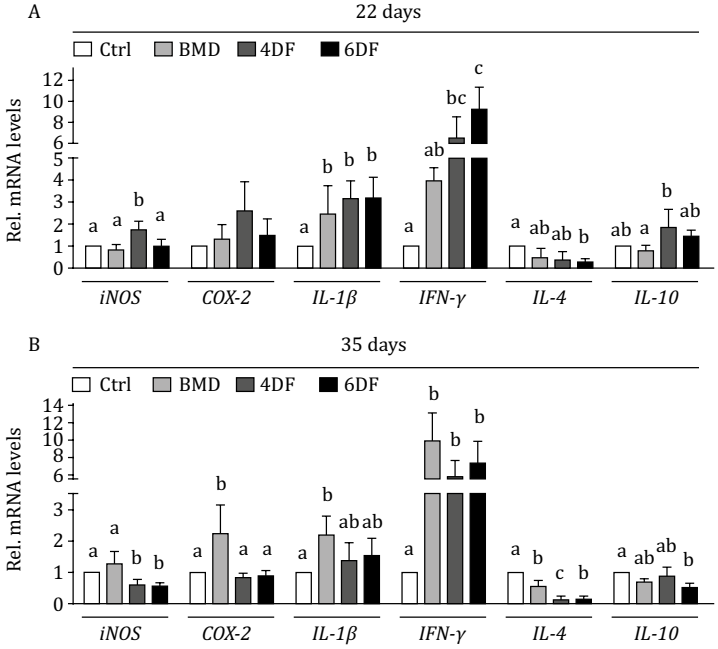
		Control	BMD	4DF	6DF
Villus length (μm)	Duodenum	5551.47±988.83[1]a	11699.92±2306.11b	4734.51±4341.73a	10949.89±970.20b
	Jejunum	3217.23±1535.78a	6808.01±611.15b	9421.9±561.36c	8387.11±1356.65c
	Ileum	5657.58±1185.28a	4811.71±622.69a	5637.28±1574.07a	7910.8±3528.21b
Crypt depth (μm)	Duodenum	1524.39±428.94	1558.49±429.49	1350.97±184.70	1833.17±172.66
	Jejunum	2505.75±470.09a	1553.65±170.24b	1442.09±364.33b	1613.45±444.91b
	Ileum	1674.17±447.19ab	2292.23±1237.95a	1193.41±146.17b	1334.74±435.94ab
Villus length:crypt depth	Duodenum	3.77883±0.76a	7.71367±1.21b	5.47150±1.90c	6.01517±0.78c
	Jejunum	3.393±1.57a	4.42717±0.61a	6.94883±2.04b	5.40517±0.98ab
	Ileum	3.43866±0.39ab	2.71083±1.4a	4.75683±1.42c	5.789±1.45bc

BMD - bacitracin methylene disalicylate; 4DF - four-day fermented product; 6DF - six-day fermented product.
[1] Values are expressed as mean ± standard deviation (n = 12).
a-c - Means within a row with different letters are significantly different (P<0.05).



(A) Effect of control (Ctrl), bacitracin methylene disalicylate (BMD), four-day fermented product (4DF), and six-day fermented product (6DF) on inducible nitric oxide synthase (*iNOS*), cyclooxygenase-2 (*COX-2*), interleukin-1β (*IL-1β*), interferon γ (*IFN-γ*), interleukin-4 (*IL-4*), and interleukin-10 (*IL-10*) mRNA expression in spleens of *Clostridium perfringens*-challenged broilers at 22 days. (B) Effect of Ctrl, BMD, 4DF, and 6DF on *iNOS*, *COX-2*, *IL-1β*, *IFN-γ*, *IL-4*, and *IL-10* mRNA expression in spleens of *Clostridium perfringens*-challenged broilers at 35 days.
Values are expressed as mean ± standard deviation (n = 6).
Means with different letters are significantly different (P<0.05).

Figure 5 - Examination of dietary *Bacillus licheniformis* solid-state fermentation product on mRNA expression in spleens of broiler chickens challenged with *Clostridium perfringens*.



(A) Effect of control (Ctrl), bacitracin methylene disalicylate (BMD), four-day fermented product (4DF), and six-day fermented product (6DF) on inducible nitric oxide synthase (*iNOS*), cyclooxygenase-2 (*COX-2*), interleukin-1β (*IL-1β*), interferon γ (*IFN-γ*), interleukin-4 (*IL-4*), and interleukin-10 (*IL-10*) mRNA expression in bursa of Fabricius of *Clostridium perfringens*-challenged broilers at 22 days. (B) Effect of Ctrl, BMD, 4DF, and 6DF on *iNOS*, *COX-2*, *IL-1β*, *IFN-γ*, *IL-4*, and *IL-10* mRNA expression in bursa of Fabricius of *Clostridium perfringens*-challenged broilers at 35 days.
Values are expressed as mean ± standard deviation (n = 6).
Means with different letters are significantly different (P<0.05).

Figure 6 - Examination of dietary *Bacillus licheniformis* solid-state fermentation product on mRNA expression in bursa of Fabricius of broiler chickens challenged with *Clostridium perfringens*.

Four and six days of *Bacillus licheniformis* SSF products significantly reduced *iNOS* mRNA expression in the bursa of Fabricius (Figure 6B; P<0.05). No significant difference was found in the expression of *COX-2* and *IL-1β* genes in the bursa of Fabricius of the control and SSF product-treated groups (Figure 6B). Similar to the spleen, the antibiotics and SSF products remarkably induced *IFN-γ* mRNA expression in the bursa of Fabricius compared with the control group (Figure 6B; P<0.05). The antibiotics and SSF products consistently attenuated the *IL-4* mRNA expression (Figure 6B; P<0.05). Six days of *Bacillus licheniformis* SSF products were able to inhibit the *IL-10* mRNA expression compared with control group (Figure 6B) (P<0.05). These findings demonstrate that, similar to antibiotics treatment, dietary *Bacillus licheniformis* SSF products exhibit an immunomodulatory role in broilers under *Clostridium perfringens* challenge.

Discussion

In this study, we demonstrated that the ideal culture conditions for *Bacillus licheniformis* yield in SSF are 5% glucose, 10% soybean meal, 3% yeast, and 50% initial moisture content. The *Bacillus licheniformis* SSF product from four and six days of SSF were heat- and acid-resistant. The fermented products were able to inhibit the growth of *Clostridium perfringens* and *Staphylococcus aureus in vitro*. In feeding trial experiments, dietary supplementation of *Bacillus licheniformis*-fermented products significantly improved the morphology of the small intestine and alleviated the intestinal necrotic lesions under *Clostridium perfringens* challenge.

It has been reported that the growth of *Bacillus licheniformis* is significantly increased on soybean-based substrate, and macromolecules of soybean are efficiently degraded to water-soluble low molecular weight compounds during SSF (Kiers et al., 2000). The mixture of wheat bran and rice straw powder could be used as substrates for growth of *Bacillus licheniformis* in SSF (Zhao et al., 2008). Furthermore, the spore yield of *Bacillus licheniformis* is increased by using wheat bran and rice straw powder supplemented with glucose (Zhao et al., 2008). In this study, we also found that supplementation of glucose tended to improve the growth of *Bacillus licheniformis* on wheat bran and soybean meal-based substrates. Extra nitrogen source, such as yeast extract, also elevates the spore production of *Bacillus licheniformis* using wheat bran and rice straw powder substrates (Zhao et al., 2008). In contrast, we found that the concentration of yeast extract was not positively correlated with the growth of *Bacillus licheniformis* in the present study. It has been shown that the optimal initial moisture content for spore yield of *Bacillus licheniformis* is 65% at 37 °C (Zhao et al., 2008). However, we found that the maximum spore yield of *Bacillus licheniformis* was obtained at 30 °C with an initial moisture content of 50%. In addition to the growth of *Bacillus licheniformis*, we provide further evidence that *Bacillus licheniformis* SSF product was heat- and acid-resistant. Whether different incubation temperatures and the combinations of substrate in SSF coordinately affect the growth of *Bacillus licheniformis* and ability of fermented product to resist a harsh environment remain to be further investigated.

Many studies have reported that dietary supplementation of *Bacillus subtilis* can prevent NE in broiler chickens by competitive exclusion of *Clostridium perfringens* in the gastrointestinal tract (La Ragione and Woodward, 2003; Tactacan et al., 2013; Jayaraman et al., 2013; Cheng et al., 2018). It has been shown that *Bacillus licheniformis* isolated from broiler gastrointestinal tract reveals antimicrobial activity against a broad spectrum of pathogens, such as *Clostridium perfringens in vitro* (Barbosa et al., 2005). In the last few decades, several studies have identified that *Bacillus licheniformis* is able to produce bacteriocin-like antimicrobial compounds (Yakimov et al., 1995; Pattnaik et al., 2001; Kayalvizhi and Gunasekaran, 2008; Guo et al., 2012). In the present study, we also demonstrated that the *Bacillus licheniformis* SSF product was able to inhibit the growth of *Clostridium perfringens* and *Staphylococcus aureus in vitro*. Dietary supplementation of *Bacillus licheniformis* spores or virginiamycin in broilers exhibit similar effects on reduction of NE-induced lesion score and mortality (Knap et al., 2010). Consistently, the present data also demonstrate that dietary fermented products from *Bacillus licheniformis* could reduce NE-induced lesion score caused by *Clostridium perfringens* in the small intestines of broilers, and these effects are more effective than

commercial antibiotics. We also provide further evidence that SSF product from *Bacillus licheniformis* can improve the morphology of the small intestine under *Clostridium perfringens* challenge. Taken together, these findings demonstrate that *Bacillus licheniformis* spores or SSF product from *Bacillus licheniformis* are able to ameliorate *Clostridium perfringens*-induced intestinal necrotic lesions in broilers. Whether and how antimicrobial substances are produced from *Bacillus licheniformis* during SSF and fermented product directly inhibits the growth of *Clostridium perfringens in vivo* remains to be investigated in future studies.

T-helper 1 (Th1) and T-helper 2 (Th2) cells regulate distinct immune response pathways (Kidd, 2003). The balance between Th1 and Th2 cells is important for a healthy immune response (Halonen et al., 2009). T-helper 1 cells initiate the cellular immunity to fight pathogens, while Th2 cells drive the humoral immunity and eliminate pathogens by up-regulating antibody production (Kidd, 2003). It has been demonstrated that *IL-1β* could stimulate *IFN-γ* production in natural killer cells (Cooper et al., 2001). Here, we found that *IL-1β* mRNA levels were elevated in spleen and bursa of Fabricius of broilers from SSF product-treated groups under *Clostridium perfringens* challenge, thereby increasing the Th1 cytokine mRNA levels, such as *IFN-γ*. These findings indicate that *Bacillus licheniformis* tends to trigger the cellular immune responses in broiler chickens challenged with *Clostridium perfringens*.

Conclusions

The optimum conditions for *Bacillus licheniformis* in solid-state fermentation is 5% glucose, 10% soybean meal, 3% yeast, and 50% initial moisture content. The fermented products in dietary feed can ameliorate *Clostridium perfringens*-induced intestinal necrotic lesions in broilers. Thus, *Bacillus licheniformis* solid-state fermentation product might provide an alternative source for preventing antibiotic-resistant pathogens in chickens or a substitute for antibiotics to treat *Clostridium perfringens*.

Acknowledgments

This work was supported by the Agricultural Technology Research Institute (10610068), Chung Cheng Agriculture Science and Social Welfare Foundation (106-3), and Ministry of Science and Technology (MOST 107-2321-B-197-002) of Taiwan. The first and second authors contributed equally to this work.

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