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Bacillus licheniformis-Fermented Products Improve Growth Performance and Intestinal Gut Morphology in Broilers under *Clostridium perfringens* Challenge

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Necrotic enteritis caused by *Clostridium perfringens* has reemerged as a severe poultry disease worldwide since the ban on the routine use of antibiotics in animal feed. Probiotics are considered alternatives to antibiotic growth promoters. *Bacillus* species are the most common microorganisms used as probiotics in the feed industry. The current study investigated the growth and surfactin levels of *Bacillus licheniformis* in solid-state fermentation using different substrates and evaluated the effects of *B. licheniformis*-fermented products on growth performance and intestinal morphology in broilers exposed to *C. perfringens*. The highest viable biomass and surfactin concentration of *B. licheniformis*-fermented products was observed at 2% molasses and 20% soybean meal supplementation during fermentation ($P<0.05$). *B. licheniformis*-fermented product-derived surfactin inhibited the growth of *C. perfringens in vitro* in a dose dependent manner ($P<0.05$). *B. licheniformis*-fermented product supplementation (2 g/kg) significantly improved the body weight and average daily gain weight of broilers challenged with *C. perfringens* ($P<0.05$). *B. licheniformis*-fermented products significantly alleviated necrotic lesions and ameliorated intestinal morphology in broilers exposed to *C. perfringens* ($P<0.05$). Collectively, these observations demonstrate that *B. licheniformis*-fermented products improve growth performance and gut morphology in broilers under *C. perfringens* challenge. *B. licheniformis*-fermented products may have the potential to be used as alternatives to antibiotic growth promoters for preventive treatments against *C. perfringens* in broilers.

Key words: *Bacillus licheniformis*, broiler, *Clostridium perfringens*, fermented products, intestinal morphology

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Introduction

Necrotic enteritis is the most common enteric disease caused by *Clostridium perfringens* in poultry. Necrotic enteritis typically occurs in broilers that are 2 to 6 weeks of age and leads to sudden death (Songer, 1996; Timbermont *et al*., 2011). Clinical signs of necrotic enteritis include severe depression, bloody diarrhea, decreased appetite, and ruffled feathers. Lesions of necrotic enteritis are mostly prevalent in the small intestine. Subclinical necrotic enteritis manifests in broilers as poor growth performance without mortality (Van Immerseel *et al*., 2004; Cooper and Songer, 2010; Timbermont *et al*., 2011). Antibiotics in feed have been effective in controlling the prevalence of necrotic enteritis in broilers. However, the occurrence and severity of *C. perfringens*-induced necrotic enteritis has increased over the years due to the banning of the use of antibiotics in food-producing animals (Timbermont *et al*., 2011). Thus, the poultry industry is in urgent need of alternative strategies to prevent necrotic enteritis in broilers.

Probiotics have become the ideal alternative to antibiotic growth promoters. Prevention of *C. perfringens*-induced necrotic enteritis with the use of probiotics or probiotic-derived metabolites has been increasingly studied (Knap *et al*., 2010; Abudabos *et al*., 2013; Cheng *et al*., 2018; Lin *et al*., 2019). *Bacillus licheniformis*, a gram-positive and endospore-forming probiotic strain exhibits *in vitro* antibacterial activity against *C. perfringens* through the production of the antibacterial cyclic lipopeptide surfactin (Thaniyavarn *et al*., 2003; Barbosa *et al*., 2005; Horng *et al*., 2019). Our previous studies have shown that surfactin isolated from *B.*

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licheniformis-fermented products can suppress the growth of *C. perfringens*, *Brachyspira hyodysenteriae*, and *Staphylococcus aureus in vitro* (Horng *et al*., 2019; Lin *et al*., 2019). Furthermore, *B. licheniformis* is able to mitigate *C. perfringens*-induced necrotic enteritis and ameliorate growth performance in broilers (Knap *et al*., 2010; Liu *et al*., 2012; Gong *et al*., 2018; Musa *et al*., 2019).

It has been reported that *B. licheniformis*-fermented products improve growth performance and diarrhea incidence in broilers and weaning piglets, respectively (Hung *et al*., 2019; Chen and Yu, 2020). *B. licheniformis*-fermented products also positively regulate the gut microflora composition of broilers and weaning piglets (Hung *et al*., 2019; Chen and Yu, 2020). It has been demonstrated that probiotics may exclude or reduce the growth of pathogens from the intestine through the alteration of the gut microbiota, thus preventing pathogen infection in broilers (Maki *et al*., 2019). Therefore, we hypothesized that *B. licheniformis*-fermented products might improve growth performance and gut morphology in broilers under *C. perfringens* challenge.

Our recent findings indicated that *B. licheniformis*-fermented products may be produced in solid-state fermentation and its antibacterial activities have been evaluated *in vitro* and *in vivo* (Horng *et al*., 2019; Lin *et al*., 2019). However, the growth performance was not significantly improved by *B. licheniformis*-fermented products in broilers under *C. perfringens* challenge (Lin *et al*., 2019). Compared with a recent study (Chen and Yu, 2020), the insufficient amount of *B. licheniformis* in fermented products may be the main reason for the results previously reported (Lin *et al*., 2019). In addition, we did not measure the surfactin levels in *B. licheniformis*-fermented products in the previous study (Lin *et al*., 2019). Thus, the viable biomass of *B. licheniformis* and antibacterial cyclic lipopeptide levels in *B. licheniformis*-fermented products still need to be optimized for its application in the poultry industry.

Therefore, we aimed to investigate the different substrates for solid-state fermentation for *B. licheniformis* growth and antibacterial cyclic lipopeptide production. We then studied the effect of different concentrations of *B. licheniformis*-fermented products on broilers challenged with *C. perfringens*. The findings provide valuable insights for the use of *B. licheniformis*-fermented products as a possible substitute for antibiotics in the poultry industry.

Materials and Methods

Solid-State Fermentation

B. licheniformis (ATCC® 12713™) was purchased from BCRC (Food Industry Research and Development Institute, Hsinchu, Taiwan). *B. licheniformis* was thawed and inoculated into an Erlenmeyer flask containing tryptic soy broth (TSB; BD Biosciences, Sparks, MD, USA) and then incubated at 30℃ for 18 h in a shaker at 150 rpm. Solid-state fermentation substrates, including glucose, molasses, soybean meal, wheat bran, yeast, fish meal, and potassium dihydrogen phosphate were ground to a fine powder with a grinder. Solid-state fermentation of *B. licheniformis* was optimized by investigating the effect of different concentrations of carbon (glucose and molasses, with 10% soybean meal used as the sole nitrogen source) and nitrogen sources (soybean meal, with 2% molasses used as the sole carbon source) on the bacterial count (colony forming unit, CFU) and surfactin production. Water was added into space bags to bring the initial moisture content of combined substrates up to 50%. The space bags containing solid-state fermentation substrates were autoclaved at 121℃ for 30 min. The sterile fermentation substrates were inoculated with a 4% (v/w) *B. licheniformis* inoculum and cultured at 30℃ with a relative humidity above 80% for 6 days in a chamber. The fermented products were dried in an oven at 50℃ for 2 days and homogenized with a grinder. The fermented powder was then stored at 4℃ until further use.

Determination of Bacterial Count and Surfactin Concentration

The fermented powder was serially diluted in 0.85% NaCl and incubated on tryptic soy agar plates (TSA; BD Biosciences) at 30℃ for 18 h. *B. licheniformis* colonies were counted and expressed as colony forming units per gram (CFU/g). For surfactin extraction, the fermented powder was dissolved in 0.85% NaCl and centrifuged at 12,000×g for 20 min. The supernatant was adjusted to pH 2.0 with concentrated HCl and incubated overnight at 4℃. After centrifugation at 12,000×g for 20 min, the supernatant was discarded and the pellet was then dissolved in distilled water. The crude extracts containing surfactin were dialyzed and lyophilized. The lyophilized extracts were reconstituted in methanol and filtered through a 0.22 μm membrane using a syringe filter. High-performance liquid chromatography was performed to quantify surfactin concentration in the filtrate using the SPD-20A system with a programmable UV detector (20A VP, Shimadzu, Kyoto, Japan) and a reverse phase RP-18 column (LiChrospher 100 RP-18 endcapped, 5 μm, Merck, Temecula, CA, USA). The mobile phase consisted of 3.8 mM trifluoroacetic acid: acetonitrile (20:80, v/v). Surfactin concentration was determined with a UV detector at 210 nm. The flow rate and recorder were set to 1 mL/min and 30 min, respectively. A linear response was obtained over a range of 100−1000 μg/mL of the commercial surfactin standard (Sigma-Aldrich, St. Louis, MO, USA). The slope of the standard curve was used to determine the concentration of surfactin in fermented products.

Antibacterial Activity Determination

The antibacterial activity of *B. licheniformis*-fermented products was analyzed using an agar well diffusion assay. *C. perfringens* was a generous gift from Chung-Hsi Chou (Zoonoses Research Center and School of Veterinary Medicine, National Taiwan University, Taiwan) and was used as a bacterial pathogen indicator for the determination of the antibacterial activity. The detailed sampling and bacterial identification of *C. perfringens* from the field was reported in a previous study (Fan *et al*., 2016). Briefly, *C. perfringens* from intestinal samples of 5-week-old broilers with necrotic enteritis was isolated by streaking on tryptose sulfite cycloserine agar (TSC agar; Sigma-Aldrich) and blood agar plates

and then incubated under anaerobic conditions at 37℃ for 24 hr. The *C. perfringens* toxinotype from field isolates was identified by multiplex PCR reaction and determined as type A and NetB negative. The *B. licheniformis*-fermented product-derived surfactin was serially diluted in 0.85% NaCl (concentration ranging from 7.8 to 250 μg/mL). Surfactin and ampicillin were separately transferred into wells in Gifu anaerobic medium agar (GAM agar; Sigma-Aldrich) containing *C. perfringens* and then cultured under anaerobic conditions at 37℃ for 24 h. Inhibition zones were determined after incubation.

Animal Studies

One-day-old healthy male broiler chicks (Ross 308) were purchased from a local commercial hatchery. On day 1, 60 chicks with an average body weight of 46.58±0.34 g were randomly allocated into 12 cages (4 groups×3 replications, each replication included 5 chicks) in a completely randomized design. Each group was assigned to one of the 4 experimental diets. Broilers were reared in stainless-steel, temperature-controlled cages (190 cm×50 cm×35 cm, width ×length×height) with plastic-coated wire flooring. The experimental diets consisted of: (1) a basal diet plus *C. perfringens* challenge as control; (2) a basal diet plus *C. perfringens* challenge and 0.5 g/kg enramycin from days 1 to 21 and 1.2 g/kg enramycin from days 22 to 35; (3) a basal diet plus *C. perfringens* challenge and 1 g/kg of *B. licheniformis*-fermented products (2.5×10^8 CFU/g of feed); and (4) a basal diet plus *C. perfringens* challenge and 2 g/kg of *B. licheniformis*-fermented products (5.0×10^8 CFU/g of feed).

Table 1. **Basal diet composition**

Item	
Ingredient, g kg^{-1}	
Corn, yellow	511.8
Soybean meal, 36.7% CP	350.0
Fish meal	100.0
$CaCO_3$, 38%	20.0
$CaHPO_4$	10.0
DL-Methionine, 99.5%	2.0
Choline chloride, 50%	0.2
Sodium chloride	4.0
Mineral premix[1]	1.0
Vitamin premix[2]	1.0
Calculated value, g kg^{-1}	
Crude protein	230.0
Analyzed calcium	13.8
Analyzed total phosphorus	7.0
Lysine	13.4
Methionine + Cystine	10.0
ME, kcal/kg	3411

[1] Supplied per kilogram of diet: Cu ($CuSO_4\cdot5H_2O$), 20 mg; Zn (ZnO), 100 mg; Fe ($FeSO_4\cdot H_2O$), 140 mg; Mn ($MnSO_4\cdot H_2O$), 4 mg; Se (Na_2SeO_3), 0.1 mg and I (ethylenediamine dihydriodide), 0.2 mg.

[2] Supplied per kilogram of diet: vitamin A, 6,000 IU; vitamin D_3, 900 IU; vitamin E, 30 IU; vitamin K_3, 3 mg; riboflavin, 6 mg; niacin, 60 mg; pantothenic acid, 18 mg; and vitamin B_{12}, 30 μg.

The diets were formulated to meet or exceed the requirements of broilers according to the National Research Council recommendations (NRC 1994) (Table 1). Fish meal was included in the basal diets to increase the effect of *C. perfringens* challenge (Cooper and Songer, 2010). In the *B. licheniformis*-fermented product-treated groups, the soybean meal in the basal diet was replaced with *B. licheniformis*-fermented products at an equal amount. Feed and water were provided *ad libitum* throughout the experiment. The temperature was set at 32℃ on the first day, gradually reduced to 24℃ by the third week, and then maintained at 24℃ up to the end of the experiment. The lighting schedule was 22L: 2D throughout the experiment. Vaccination of chickens with combined Newcastle disease-infectious bronchitis vaccines was performed on days 4 and 14 via nose drop administration. For the *C. perfringens* challenge, *C. perfringens* (toxinotype A and NetB negative) was grown anaerobically in brain heart infusion (BHI; Sigma-Aldrich, St. Louis, MO, USA) at 37℃ overnight. The detailed sampling and bacterial identification of *C. perfringens* from the field was reported in a previous study (Fan *et al*., 2016). The birds were orally gavaged with 1 mL (1×10^8 CFU/mL) of an overnight culture of *C. perfringens* in BHI at 19, 20, and 21 days of age. Their growth performance, including average body weight, average daily gain weight, average daily feed intake, and feed conversion ratio, were recorded and calculated from days 1 to 35.

Lesion Scoring

On 22 and 35 days of age, three birds from each group (one bird per replicate) were randomly chosen and then euthanized using inhalation of carbon dioxide gas. Each intestinal segment (duodenum, jejunum, and ileum) from each bird was divided into three separate parts and then the lesion score evaluated. Lesion scores were observed and recorded according to a previous study (Al-Sheikhly and Truscott, 1977) (Fig. S1), where 0 is normal and 1 to 3 indicate the increasing severity of the infection. The scores were: 0=no lesions; 1=thin, friable walls, diffuse superficial, and removable fibrin deposit; 2=focal necrosis, ulceration, and non-removable fibrin deposit (1 to 5 foci); 3 =focal necrosis, ulceration, and non-removable fibrin deposit (5 to 15 foci).

Small Intestinal Morphology Analysis

On 22 and 35 days of age, three birds from each group (one bird per replicate) were randomly chosen and euthanized through inhalation of carbon dioxide gas. Three different locations in the small intestine were collected for morphology analysis: 2 cm after the gizzard (duodenum), before the Meckel's diverticulum (jejunum), and 2 cm before the ileo-caecal transition (ileum). These intestinal segments were fixed immediately in 10% (w/v) neutral-buffered formalin solution (Merck, Temecula, CA, USA) at 4℃. Formalin-fixed tissue was dehydrated in xylene, embedded in paraffin, sectioned (5 μm thickness and 6 cross-sections from each sample), and then stained with hematoxylin and eosin. The villus length and crypt depth from each segment were measured randomly in one bird (10 villi per intestinal seg-

ment) using light microscopy with a digital video camera (CKX41 microscope, Olympus Corporation, Tokyo, Japan). The stereological image software (Cast Image System, Visiopharm Albertslund, Hørsholm, Denmark) was used to analyze the images.

Quantitative Reverse Transcription-PCR

On 22 and 35 days of age, three birds from each group (one bird per replicate) were randomly chosen and then euthanized through inhalation of carbon dioxide gas. Total RNA was extracted from spleens using the TRIzol Reagent extraction method (Invitrogen, Carlsbad, CA, USA) and quantified by spectrophotometry. Total RNA was reverse-transcribed using the One-Step RT-qPCR Reagents (Bio-Rad, Hercules, CA, USA) and gene expression (*inos*, *cox2*, *ifnγ*, *il-1β*, *β-actin*) was measured using the MiniopticonTM Real-Time PCR Detection System (Bio-Rad) and the iTaq Universal SYBR Green Supermix (Bio-Rad). The *β-actin* gene was used as a reference to normalize gene expression in the same sample. The primers were as follows: *inos* forward: 5′-AGG CCA AAC ATC CTG GAG GTC-3′, and reverse: 5′-TCA TAG AGA CGC TGC TGC CAG-3′; *cox2* forward: 5′-AAC ACA ATA GAG TCT GTG ACG TCT T-3′, and reverse: 5′-TAT TGA ATT CAG CTG CGA TTC GG-3′; *ifnγ* forward: 5′-ACA CTG ACA AGT CAA AGC CGC ACA-3′, and reverse: 5′-AGT CGT TCA TCG GGA GCT TGG C-3′; *il-1β* forward: 5′-CAG CCT CAG CGA AGA GAC CTT-3′, and reverse: 5′-CAC TGT GGT GTG CTC AGA ATC C-3′; *β-actin* forward: 5′-CAT CAC CAT TGG CAA TGA GAG G-3′, and reverse: 5′-GGT ACA TTG TGG TAC CAC CAG AC-3′.

Ethics Statement

All experiments were conducted according to the approved guidelines of the advisory group on the ethics of animal experiments at the National Ilan University (IACUC, protocol number 107−12).

Statistical Analysis

Data were tested for normality and subjected to a one-way analysis of variance using the general linear models procedure of SAS software package (Version 9.4; SAS Institute, Cary, NC). Linear and quadratic contrasts were used to determine the effects of different concentrations of *B. licheniformis*-fermented product on broilers. A Turkey's honestly significant difference test was carried out with $P<0.05$. Treatments were considered significantly different at $P<0.05$.

Results

Effect of the Fermentation Substrates on B. licheniformis Growth and Surfactin Production

Solid-state fermentation of *B. licheniformis* was optimized by investigating the effect of the combination of substrates on growth and surfactin production. The carbon source analysis showed an increased bacterial growth with the supplementation of 2% molasses compared with the other substrates (Table 2; $P<0.05$). An increasing trend in surfactin levels was observed with the supplementation of 2% molasses compared with 2% glucose (Table 2). However, 2% molasses supplementation increased surfactin levels more than the 1% supplementation (Table 2; $P<0.05$). The nitrogen source analysis showed that a 20% soybean meal supplementation produced the highest bacterial growth and surfactin levels compared with the other substrates (Table 3; $P<0.05$). Furthermore, the agar well diffusion assay showed that surfactin derived from *B. licheniformis*-fermented products had a concentration-dependent inhibitory effect on the growth of *C. perfringens* (Table 4; $P<0.05$).

Effect of the Dietary B. licheniformis-Fermented Product Supplementation on Growth Performance in Broilers Exposed to C. perfringens

The effect of dietary *B. licheniformis*-fermented product supplementation on the growth performance of broilers under *C. perfringens* challenge is shown in Table 5. After the *C. perfringens* challenge, the supplementation of enramycin in the feed of chickens increased their body weight at 21 days of age compared with other treatments ($P<0.05$). The dietary supplementation of 2 g/kg of *B. licheniformis*-fermented products in broilers also increased their body weight at 21 days of age compared with the control group ($P<0.05$). Similar results were also found in broilers at 35 days of age ($P<0.05$). Broilers fed enramycin had a higher average daily weight gain between days 1 and 21 and this was also significant over the whole trial period (days 1 to 35) compared with other treatments ($P<0.05$). The supplementation of 2 g/kg of *B. licheniformis*-fermented products in the feed of chickens also resulted in a significant increase in the average daily weight gain between days 1 and 21 and this was also significant over the whole trial period (days 1 to 35) compared with the control group ($P<0.05$). No significant difference was found in the average daily feed intake among groups. The supplementation of enramycin in the feed of chickens resulted in a significantly reduced feed conversion ratio between days 1 and 21 compared with the control group ($P<0.05$). Similarly, the supplementation of the broiler diet with *B. licheniformis*-fermented products (1 and 2 g/kg) also decreased feed conversion ratio between days 1 and 21 ($P<0.05$). Broilers supplemented with enramycin had a lower feed conversion ratio over the whole trial period (days 1 to 35) compared with other treatments ($P<0.05$). Although the effect was not statistically significant, *B. licheniformis*-fermented products showed a similar trend in ameliorating the feed conversion ratio of broilers between days 22 and 35 and the whole trial period. No chickens died in the experiment.

Effect of the Dietary B. licheniformis-Fermented Product Supplementation on Intestinal Morphology and Splenic Gene Expression in Broilers Exposed to C. perfringens

The effect of dietary *B. licheniformis*-fermented product supplementation on the intestinal lesion score of broilers under *C. perfringens* challenge is shown in Table 6. After 22 days of feeding, enramycin treatment efficiently mitigated the duodenal and jejunal damage caused by *C. perfringens* compared with the control group ($P<0.05$). Similar to the effect of enramycin, the dietary supplementation of 2 g/kg of *B. licheniformis*-fermented products relieved the *C. per-*

Table 2. **Effect of carbon sources on viable count and surfactin level of *B. licheniformis* in fermentation**

	B. licheniformis-fermented products	
	Total viable count (CFU/g)[2]	Surfactin (mg/g)
2% glucose[1]	$6.4\times10^{9}\pm1.2\times10^{9,b}$	5.78 ± 0.78^{ab}
1% molasses	$7.2\times10^{9}\pm0.2\times10^{9,b}$	4.29 ± 0.56^{b}
2% molasses	$1.3\times10^{11}\pm0.5\times10^{10,a}$	6.22 ± 0.69^{a}

[1] The initial concentration of *B. licheniformis* is 5×10^{7} CFU/mL.
[2] Data are expressed as mean±standard deviation ($n=3$).
Different letters indicate a statistically significant difference between groups ($P<0.05$).

Table 3. **Effect of soybean meal concentration on viable count and surfactin level in *B. licheniformis* fermentation**

	B. licheniformis-fermented products	
	Total viable count (CFU/g)[2]	Surfactin (mg/g)
5% soybean meal[1]	$3.1\times10^{11}\pm2.1\times10^{10,c}$	5.72 ± 0.46^{c}
10% soybean meal	$4.7\times10^{11}\pm1.6\times10^{10,b}$	6.48 ± 0.34^{c}
20% soybean meal	$8.3\times10^{11}\pm5.9\times10^{10,a}$	9.75 ± 0.323^{a}
30% soybean meal	$4.3\times10^{11}\pm0.4\times10^{10,b}$	8.30 ± 0.65^{b}

[1] The initial concentration of *B. licheniformis* is 5×10^{7} CFU/mL.
[2] Data are expressed as mean±standard deviation ($n=3$).
Different letters indicate a statistically significant difference between groups ($P<0.05$).

Table 4. **Measurement of the inhibition zone of *B. licheniformis*-fermented product-derived surfactin on *C. perfringens* growth at different concentrations**

Concentration (μg/mL)	Zone of inhibition (cm)[1]
7.8	0.3 ± 0.01^{a}
15.61	0.3 ± 0.01^{a}
31.25	0.4 ± 0.01^{b}
62.5	0.5 ± 0.02^{b}
125	0.7 ± 0.04^{c}
250	0.9 ± 0.02^{d}

[1] Data are expressed as mean±standard deviation ($n=3$).
Different letters indicate a statistically significant difference between groups ($P<0.05$).

fringens-induced necrotic lesions in the duodenum and jejunum of broilers ($P<0.05$). After feeding the diets for 35 days, broilers fed with enramycin and *B. licheniformis*-fermented products (1 and 2 g/kg) had significantly reduced lesion scores in the jejunum compared with the control group ($P<0.05$). Enramycin mitigated the *C. perfringens*-induced necrotic lesions in the ileum of broilers compared with the control group ($P<0.05$). The effect of dietary *B. licheniformis*-fermented product supplementation on the morphology of the small intestine of broilers under *C. perfringens* challenge is shown in Table 7 and Fig. S2. Enramycin and *B. licheniformis*-fermented products (1 and 2 g/kg) had a similar significant effect in improving villus length in the duodenum and jejunum at 21 days of age ($P<0.05$). No significant difference was found in the intestinal crypt depth among groups. The ratio of villus length to crypt depth was increased in the duodenum in broilers fed with enramycin and *B. licheniformis*-fermented products (1 and 2 g/kg) at 21 days of age compared with the control group ($P<0.05$). Although the effect was not statistically significant, *B. licheniformis*-fermented products (1 and 2 g/kg) showed a similar trend in increasing the ratio of villus length to crypt depth in the jejunum at 21 days of age. After 35 days, broilers supplemented with enramycin and *B. licheniformis*-fermented products (1 and 2 g/kg) had longer villus in the jejunum and ileum compared with the other treatments ($P<0.05$). *B. licheniformis*-fermented products (2 g/kg) reduced the jejunal and ileal crypt depth at 35 days of age compared with the other treatments ($P<0.05$). The ratio of villus length to crypt depth was increased in the jejunum and ileum in broilers fed with enramycin and 2 g/kg of *B. licheniformis*-fermented products at 35 days of age compared with the other treatments ($P<0.05$). The effect of dietary *B. licheniformis*-fermented product supplementation on mRNA expression in spleens of broilers under *C. perfringens* challenge is shown in Table 8. Results showed that *inos* mRNA expression was not altered among groups in broilers of 22 days of age. The *cox2* mRNA expression was higher in broilers fed with *B. licheniformis*-fermented products (1 and 2 g/kg) compared with the enramycin group ($P<0.05$). *B. licheniformis*-fermented products (2 g/kg) significantly increased the *ifnγ* and *il-1β* mRNA expression in broilers of 22 days of age compared with the control group ($P<0.05$). Enramycin significantly reduced the *ifnγ* mRNA expression in broilers of the same age compared with the control group ($P<0.05$).

Table 5. **Effect of dietary *B. licheniformis*-fermented product supplementation on the growth performance of broilers challenged with *C. perfringens***

	C[1]	E[2]	L[3]	H[4]	SEM	*P* value
Body weight (g/bird)						
1 d	46.33[5]	46.47	46.90	46.60	0.10	0.373
21 d	759.27[a]	902.67[c]	825.93[b]	850.80[b]	16.46	<0.001
35 d	1682.93[a]	1870.67[c]	1735.84[ab]	1787.03[bc]	22.44	0.003
Average daily gain (g/d/bird)						
1–21 d	33.95[a]	40.77[c]	37.10[b]	38.30[b]	0.78	<0.001
22–35 d	65.98	69.14	64.99	66.87	0.70	0.258
1–35 d	46.76[a]	52.12[c]	48.26[ab]	49.73[bc]	0.64	0.003
Average daily feed intake (g/d/bird)						
1–21 d	52.38	52.83	49.21	51.24	0.78	0.427
22–35 d	156.19	137.78	144.13	151.87	2.97	0.148
1–35 d	93.90	86.81	87.17	91.49	1.39	0.255
Feed conversion ratio						
1–21 d	1.54[a]	1.30[b]	1.33[b]	1.34[b]	0.03	0.005
22–35 d	2.37	1.99	2.23	2.28	0.06	0.123
1–35 d	2.01[a]	1.67[b]	1.81[ab]	1.84[ab]	0.04	0.034

[1] C=Basal diet plus *C. perfringens* challenge.
[2] E=Basal diet plus *C. perfringens* challenge and 0.5 g/kg enramycin from 1 to 21 d and 1.2 g/kg enramycin from 22 to 35 d, respectively.
[3] L=Basal diet plus *C. perfringens* challenge and 1 g/kg *B. licheniformis*-fermented products.
[4] H=Basal diet plus *C. perfringens* challenge and 2 g/kg *B. licheniformis*-fermented products.
[5] Three replicates were performed per group (n=3).
Different letters indicate a statistically significant difference between groups (P<0.05).

Table 6. **Effect of dietary *B. licheniformis*-fermented product supplementation on the intestinal lesion score of broilers challenged with *C. perfringens***

	C[1]	E[2]	L[3]	H[4]	SEM	*P* value
22 d						
Duodenum	2.78[5,a]	1.67[b]	2.22[ab]	1.78[b]	0.12	0.008
Jejunum	2.33[a]	1.56[ab]	2.00[ab]	1.22[b]	0.15	0.039
Ileum	2.22	1.22	1.89	1.44	0.12	0.111
35 d						
Duodenum	2.33	1.78	2.22	1.56	0.11	0.173
Jejunum	2.67[a]	1.89[b]	1.44[b]	1.22[b]	0.12	0.002
Ileum	2.44[a]	1.67[b]	2.22[ab]	1.89[ab]	0.13	0.033

[1] C=Basal diet plus *C. perfringens* challenge.
[2] E=Basal diet plus *C. perfringens* challenge and 0.5 g/kg enramycin from 1 to 21 d and 1.2 g/kg enramycin from 22 to 35 d, respectively.
[3] L=Basal diet plus *C. perfringens* challenge and 1 g/kg *B. licheniformis*-fermented products.
[4] H=Basal diet plus *C. perfringens* challenge and 2 g/kg *B. licheniformis*-fermented products.
[5] Three replicates were performed per group (n=3).
Different letters indicate a statistically significant difference between groups (P<0.05).

After 35 days of feeding, the *inos* mRNA expression was induced in broilers fed with *B. licheniformis*-fermented products (1 g/kg) (P<0.05). *B. licheniformis*-fermented products (1 and 2 g/kg) significantly induced the *il-1β* mRNA expression in broilers of 35 days of age compared with the control group (P<0.05).

Dose-Response Between the B. licheniformis-Fermented Product Concentration, Growth Performance, Lesion Score, and Intestinal Morphology in Broilers Exposed to C. perfringens

The results of linear and quadratic dose-response of dietary *B. licheniformis*-fermented products on broilers challenged with *C. perfringens* are shown in Table 9. The *B. licheniformis*-fermented product concentration was linearly correlated with the body weight, average daily weight gain, and lesion score in broilers under *C. perfringens* challenge (P<0.05). Besides, the concentration of *B. licheniformis*-fermented products was linearly correlated with the villus length and the ratio of villus length to crypt depth in broilers of 22 days of age, particularly in the duodenum and jejunum (P<0.05). The crypt depth and the ratio of villus length to crypt depth were linearly associated with the *B. licheni-*

Table 7. **Effect of dietary *B. licheniformis*-fermented product supplementation on the morphology of small intestine of broilers challenged with *C. perfringens***

		C[1]	E[2]	L[3]	H[4]	SEM	*P* value
22 d							
Villus length (μm)	Duodenum	437.93[5,a]	939.67[bc]	859.40[b]	1099.70[c]	75.84	<0.001
	Jejunum	402.23[a]	880.90[b]	957.40[b]	844.17[b]	72.48	<0.001
	Ileum	396.30[a]	647.23[ab]	714.20[b]	520.43[ab]	44.74	0.031
Crypt depth (μm)	Duodenum	266.90	193.00	163.70	189.83	15.28	0.081
	Jejunum	194.60	168.50	162.17	129.80	14.32	0.415
	Ileum	185.23	155.17	179.30	144.70	9.74	0.421
Villus length: Crypt depth	Duodenum	1.73[a]	4.88[b]	5.34[b]	5.89[b]	0.52	0.004
	Jejunum	2.15	5.33	6.36	7.54	0.77	0.063
	Ileum	2.17	4.21	4.25	3.59	0.34	0.100
35 d							
Villus length (μm)	Duodenum	608.03	878.40	755.97	823.87	69.95	0.402
	Jejunum	455.90[a]	983.63[c]	1013.87[c]	721.27[b]	73.47	<0.001
	Ileum	529.77[a]	799.10[b]	805.13[b]	684.13[b]	38.37	0.001
Crypt depth (μm)	Duodenum	201.03	181.67	156.77	174.67	11.11	0.573
	Jejunum	286.33[a]	198.90[ab]	182.60[ab]	130.00[b]	23.10	0.030
	Ileum	147.50[ab]	139.97[ab]	160.90[a]	100.43[b]	8.58	0.038
Villus length: Crypt depth	Duodenum	3.02	5.51	4.81	4.68	0.54	0.320
	Jejunum	1.69[a]	5.01[b]	5.56[b]	6.11[b]	0.58	0.004
	Ileum	3.59[a]	5.94[b]	5.02[ab]	6.79[b]	0.40	0.013

[1] C=Basal diet plus *C. perfringens* challenge.
[2] E=Basal diet plus *C. perfringens* challenge and 0.5 g/kg enramycin from 1 to 21 d and 1.2 g/kg enramycin from 22 to 35 d, respectively.
[3] L=Basal diet plus *C. perfringens* challenge and 1 g/kg *B. licheniformis*-fermented products.
[4] H=Basal diet plus *C. perfringens* challenge and 2 g/kg *B. licheniformis*-fermented products.
[5] Three replicates were performed per group (n=3).
Different letters indicate a statistically significant difference between groups (P<0.05).

Table 8. **Effect of dietary *B. licheniformis*-fermented product supplementation on mRNA expression in spleens of broilers challenged with *C. perfringens***

	C[1]	E[2]	L[3]	H[4]	SEM	*P* value
22 d						
inos[5]	1.0[6]	0.3	0.8	1.0	0.10	0.057
cox2	1.0[ab]	0.2[a]	1.7[b]	1.6[b]	0.21	0.002
ifnγ	1.0[c]	0.1[d]	1.6[b]	2.1[a]	0.22	<0.001
il-1β	1.0[b]	2.1[ab]	2.9[ab]	3.6[a]	0.35	0.017
35 d						
inos	1.0[b]	0.5[b]	2.2[a]	1.1[b]	0.19	0.001
cox2	1.0[ab]	0.4[b]	1.1[ab]	1.4[a]	0.14	0.036
ifnγ	1.0[ab]	0.8[b]	1.3[ab]	1.4[a]	0.09	0.002
il-1β	1.0[b]	1.5[ab]	2.5[a]	2.5[a]	0.23	0.022

[1] C=Basal diet plus *C. perfringens* challenge.
[2] E=Basal diet plus *C. perfringens* challenge and 0.5 g/kg enramycin from 1 to 21 d and 1.2 g/kg enramycin from 22 to 35 d, respectively.
[3] L=Basal diet plus *C. perfringens* challenge and 1 g/kg *B. licheniformis*-fermented products.
[4] H=Basal diet plus *C. perfringens* challenge and 2 g/kg *B. licheniformis*-fermented products.
[5] *inos*=inducible nitric oxide synthase; *cox2*=cyclooxygenase 2; *ifn*γ=interferon γ; *il-1*β=interleukin 1β.
[6] Three replicates were performed per group (n=3).
Different letters indicate a statistically significant difference between groups (P<0.05).

formis-fermented product concentration in broilers of 35 days of age, particularly in the jejunum and ileum (P<0.05). The quadratic dose-response of dietary *B. licheniformis*-fermented products was also found in the villus length of jejunum and ileum in broilers of 22 and 35 days of age (P<0.05).

Table 9. **Linear and quadratic dose-response of dietary *B. licheniformis*-fermented products on broilers challenged with *C. perfringens***

Item				*P* value	
	Growth performance			Linear	Quadratic
	Body weight			0.007	0.975
	Average daily gain			0.007	0.988
	Average daily feed intake			0.590	0.145
	Feed conversion ratio			0.130	0.197
	Lesion score				
	22 d	Duodenum		0.002	0.783
		Jejunum		0.003	0.326
		Ileum		0.018	0.822
	35 d	Duodenum		0.023	0.258
		Jejunum		0.002	0.029
		Ileum		0.064	0.822
	Intestinal morphology				
B. licheniformis-fermented product concentration[1]	22 d	Villus length	Duodenum	<0.001	0.144
			Jejunum	0.039	0.010
			Ileum	0.382	0.009
		Crypt depth	Duodenum	0.127	0.092
			Jejunum	0.180	0.999
			Ileum	0.216	0.618
		Villus length: Crypt depth	Duodenum	0.003	0.047
			Jejunum	0.018	0.353
			Ileum	0.155	0.068
	35 d	Villus length	Duodenum	0.337	0.844
			Jejunum	0.222	0.001
			Ileum	0.174	0.008
		Crypt depth	Duodenum	0.183	0.029
			Jejunum	0.021	0.615
			Ileum	0.033	0.002
		Villus length: Crypt depth	Duodenum	0.224	0.417
			Jejunum	0.007	0.106
			Ileum	<0.001	0.566

[1] Data were analyzed using results of C (basal diet plus *C. perfringens* challenge), L, (basal diet plus *C. perfringens* challenge and 1 g/kg *B. licheniformis*-fermented products), and H (basal diet plus *C. perfringens* challenge and 2 g/kg *B. licheniformis*-fermented products) group.

Discussion

Solid-state fermentation is considered to be cheaper and more environmentally friendly than submerged fermentation for the production of value-added fermented products (Teng *et al*., 2017; Su *et al*., 2018; Lima-Pérez *et al*., 2019). Our previous study found that the highest viable *B. licheniformis* biomass (4×10^8 CFU/g) was observed at 5% glucose, 10% soybean meal, and 3% yeast after 6 days of solid-state fermentation (Lin *et al*., 2019). Recently, it has been reported that *B. licheniformis* bacterial growth and polysaccharide production was increased in molasses-based submerged fermentation (Gojgic-Cvijovic *et al*., 2019). However, it has not been proven that molasses could be used as the sole carbon source for the optimization of the solid-state fermentation of *B. licheniformis*. Besides, the macromolecules of soybean are efficiently degraded to low molecular weight compounds using *B. licheniformis* during solid-state fermentation (Kiers *et al*., 2000). In this study, we modified the solid-state fermentation conditions for *B. licheniformis* and further demonstrated that replacing 5% glucose with 2% molasses as the sole carbon source and increasing soybean meal concentration from 10% to 20% might increase the number of *B. licheniformis* (8.3×10^{11} CFU/g) after 6 days of solid-state fermentation. The concentration of surfactin in *B. licheniformis*-fermented products was also quantified in the present study. Interestingly, replacing glucose with molasses might increase *B. licheniformis* growth during solid-state fermentation, but the concentration of surfactin in fermented products was not significantly elevated using molasses as the sole carbon source. A previous study reported that the lower growth rate of cell biomass due to nutritional limitations in solid-state fermentation may trigger the synthesis of surfactin (Shaligram and Singhal, 2010). These results imply that *B. licheniformis* growth is not highly correlated with the production of surfactin during solid-state fermentation. Here, *B.*

licheniformis-fermented products also exhibited similar antibacterial activity against *C. perfringens* compared with our previous studies (Horng *et al*., 2019; Lin *et al*., 2019). Collectively, replacing glucose with molasses and increasing the soybean meal concentration during solid-state fermentation may increase the growth of *B. licheniformis*. This fermentation conditions may be suitable for their application in industrial-scale solid-state fermentation in the future.

Over the past decades, it has been demonstrated that the dietary supplementation of probiotics in broilers can provide similar benefits as antibiotics in terms of growth performance and disease prevention (Abudabos *et al*., 2017; Abudabos *et al*., 2020). However, previous studies mainly used probiotics as feed additives for the improvement of broiler production. The application of *B. licheniformis*-fermented products obtained from solid-state fermentation in the poultry industry is rarely investigated. Our previous study showed that *B. licheniformis*-fermented products have similar benefits as antibiotics on the improvement of growth performance and diarrhea incidence in broilers and weaning piglets, respectively (Hung *et al*., 2019; Chen and Yu, 2020). In this study, we further demonstrated that 2 g/kg of *B. licheniformis*-fermented products might ameliorate growth performance and improve gut morphology in broilers challenged with *C. perfringens*. The gut microbiota is important for the optimal growth and disease prevention in broilers. It has been demonstrated that *B. licheniformis*-fermented products also positively regulate the broiler gut microbiota composition (Chen and Yu, 2020). Whether *B. licheniformis*-fermented products ameliorate the growth performance of broilers exposed to *C. perfringens* challenge by altering the gut microflora needs to be further investigated. These findings indicate that the dietary supplementation of *B. licheniformis*-fermented products show beneficial effects in improving growth performance and preventing disease in broilers.

Necrotic enteritis is the most common and serious bacterial disease in broilers. *C. perfringens*-induced necrotic enteritis causes severe necrosis of intestinal mucosa, leading to impaired nutrient utilization and reduced growth performance (Van Immerseel *et al*., 2004; Cooper and Songer, 2010; Timbermont *et al*., 2011). Previous studies reported that dietary *B. licheniformis* supplementation reduce the intestinal lesion score and mortality in broilers exposed to *C. perfringens* (Knap *et al*., 2010; Musa *et al*., 2019; Zhao *et al*., 2019). It has been proposed that *B. licheniformis* may prevent necrotic enteritis by preventing proliferation of *C. perfringens*, promoting the death of *C. perfringens*, or eliminating the effect of the toxins produced by *C. perfringens*. Our recent finding showed that *B. licheniformis*-fermented product-derived antibacterial cyclic lipopeptide surfactin might cause the death of *C. perfringens* and suppress the growth of *C. perfringens in vitro* (Horng *et al*., 2019). Here, we further demonstrated that *B. licheniformis*-fermented products containing surfactin reduced intestinal necrotic lesions and ameliorated gut morphology in broilers challenged with *C. perfringens* to the same level as in the antibiotic-treated group. Compared to previous studies (Knap *et al*., 2010; Musa *et al*., 2019; Zhao *et al*., 2019), the *B. licheniformis*-fermented products generated from solid-state fermentation contain live microorganisms, *B. licheniformis*-derived surfactin, and fermented substrates. Thus, the efficiency of *B. licheniformis*-fermented products on the alleviation of *C. perfringens*-induced necrotic enteritis in broilers may be different compared to previous studies. Whether supplementation of surfactin alone in the diet prevents the mortality and necrotic enteritis in broilers exposed to *C. perfringens* remains to be further investigated. Collectively, these findings indicated that the dietary supplementation of *B. licheniformis*-fermented products exhibited the potential for the prevention of *C. perfringens*-induced necrotic enteritis in broilers.

In a previous study, we determined that the body weight of the non-infected control group and the infected group at 35 days of age was 1.90 kg and 1.76 kg, respectively (Cheng *et al*., 2018). Here, the body weight of the infected group and the enramycin-treated group was 1.68 kg and 1.87 kg at 35 days of age, respectively; however, there is no non-infected control group in the present study. These results partially support that *C. perfringens* challenge cause a negative impact on the broiler body weight, whereas the enramycin treatment alleviates the adverse effect on the body weight. In the present study, the feed conversion ratio of the infected group was higher than in the enramycin-treated group (2.01 versus 1.67). In addition, the enramycin treatment was able to alleviate the intestinal lesion score and improve the intestinal morphology of broilers exposed to *C. perfringens* compared with the infected group. These results may indicate that necrotic enteritis was successfully developed in broilers under the *C. perfringens* challenge.

In conclusion, the highest viable biomass of *B. licheniformis* and surfactin levels were observed at 2% molasses in combination with 20% soybean meal during solid-state fermentation. We demonstrated for the first time that 2 g/kg of *B. licheniformis*-fermented products potentially improved growth performance and intestinal morphology in broilers exposed to *C. perfringens*. Thus, *B. licheniformis*-fermented products may be useful feed additives replacing banned in-feed antibiotics.

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Conflicts of Interest

The authors declare no conflict of interest.

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