IMMUNOLOGY, HEALTH AND DISEASE

The effects of dietary *Bacillus subtilis* supplementation, as an alternative to antibiotics, on growth performance, intestinal immunity, and epithelial barrier integrity in broiler chickens infected with *Eimeria maxima*

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ABSTRACT The objective of this study was to investigate the effects of dietary *Bacillus subtilis* supplementation on growth performance, jejunal lesion scores, oocyst shedding, and cytokine and tight junction protein expression in broiler chickens infected with *Eimeria maxima*. A total of 196 male day-old Ross 708 broilers were given a nonexperimental diet until 14 D of age. Then, all chickens were randomly assigned to one of seven dietary treatments: 2 basal diets (**CON** and **NC**); CON + virginiamycin (**AB1**); CON + bacitracin methylene disalicylate (**BMD**; **AB2**); CON + *B. subtilis* 1781 (**PB1**); CON + *B. subtilis* 747 (**PB2**); or CON + *B. subtilis* 1781 + 747 (**PB3**). At day 21, all chickens except those in the CON group were orally inoculated with *E. maxima* oocysts. At 7 D after *E. maxima* infection, the body weight gains of chickens fed PB2 and PB3 increased ($P = 0.032$) as much as those in chickens fed AB2. The body weight gain and feed efficiency of chickens fed PB2 were significantly increased ($P < 0.001$), and PB2 chickens showed ($P = 0.005$) the lowest lesion scores after *E. maxima* infection. Chickens fed PB2 showed ($P < 0.05$) lower mRNA expression of IL-1β in infected chicken groups. Chickens in the AB1, AB2, PB1, PB2, and PB3 groups showed ($P < 0.05$) greater mRNA expression of junctional adhesion molecule 2 in jejunal tissue, whereas occludin expression increased ($P < 0.05$) in the jejunal tissue of chickens fed AB2 or PB2. Dietary *B. subtilis* supplementation significantly improved the growth performance of young chickens to a level comparable with that induced by virginiamycin or BMD without *E. maxima* infection. After infection with *E. maxima*, dietary virginiamycin and BMD significantly enhanced the epithelial barrier integrity, and the dietary *B. subtilis* 747 showed significantly enhanced growth performance, intestinal immunity, and epithelial barrier integrity. Together our results indicated that certain strains of *B. subtilis* provide beneficial effects on the growth of young broiler chickens and have the potential to replace antibiotic growth promoters.

Key words: *Bacillus subtilis*, chicken, *Eimeria maxima*, intestinal immunity, gut health

2020 Poultry Science 99:725–733
https://doi.org/10.1016/j.psj.2019.12.002

INTRODUCTION

The United Nations estimates that there will be more than 9 billion people on the planet by the year 2050 (Roberts, 2011); thus, the world population will be 32% higher than that in 2006. In addition, the meat consumption per person per year is predicted to increase by 26% in the same period and will primarily comprise chicken consumption (FAO, 2010; OECD-FAO, 2010). Because of increasing concerns regarding antimicrobial resistance (Gadde et al., 2017b), growing consumer preference for antibiotic-free meat products will influence future directions in poultry and livestock production (Godfray et al., 2010; Shepon et al., 2018; Sander et al., 2019). As of 2017, about 40% of boiler feed in the U.S. was already antibiotic free under "No Antibiotics Ever" programs (Rennier, 2017).

Consumer awareness of antimicrobial resistance and food safety and increasing understanding of the interaction of nutrients, intestinal microbiota, and the immune system in maintaining good gut health have resulted in

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the limited use of antibiotic growth promoters (**AGP**) and anticoccidials in animal agriculture and in a paradigm shift in the use of feed additives by commercial companies (Lee et al., 2011a,b; Yadav and Jha, 2019). Therefore, there will be an increasing need to understand how intestinal microbiota and the immune system can be modulated by dietary nutraceuticals and natural feed additives as alternatives to antibiotics in controlling enteric diseases (Ganguly, 2013; Chan et al., 2015; Gadde et al., 2017b). Notably, *Eimeria* spp. are the etiologic agents of avian coccidiosis, an intestinal disease responsible for an estimated annual economic loss of more than $3 billion worldwide (Lillehoj and Trout, 1996; Shirley and Lillehoj, 2012). Increasing implementation of antibiotic-free poultry production system in the U.S. is making the control of some enteric pathogens such as coccidiosis-causing *Eimeria* species and NE-inducing *Clostridium perfringens* (***C. perfringens***) strains challenging. Because coccidiosis is a primary risk factor for NE, it will be more desirable if alternatives to antibiotics can reduce *Eimeria* as well as *C. perfringens*. (Gallucci and Matzinger, 2001; Peek and Landman, 2011). There is currently a wide range of feed additives available through the feed industry, including acidifiers, prebiotics, probiotics, phytochemicals, enzymes, osmoregulators, nucleotides, and zinc oxide (Gadde et al., 2017b; Lin et al., 2017).

Many strains of *Bacillus subtilis* have been selected as probiotics on the basis of their *in vitro* inhibitory effects on chicken pathogenic bacteria (Fritts et al., 2000; Li et al., 2016; Nhung et al., 2017; Grant et al., 2018). Dietary supplementation with *B. subtilis* has been shown not only to improve growth performance and to beneficially alter the gastrointestinal microflora to decrease colonization by chicken pathogenic *E. coli* and *C. perfringens* but also to have a protective role against chicken coccidiosis (Knap et al., 2010; Lee et al., 2015). Therefore, this study was conducted to investigate the effects of dietary *B. subtilis* supplementation on posthatch growth, intestinal immunity, and epithelial barrier integrity in broiler chickens infected with *Eimeria maxima* during their early growth phase. To evaluate host immune function during coccidiosis, we also investigated growth performance, lesion scores, oocyst shedding, jejunal cytokines, and tight junction (**TJ**) proteins in broiler chickens infected with *E. maxima*.

MATERIALS AND METHODS

All experiments were approved by the Beltsville Agricultural Research Center Institutional Animal Care and Use Committee.

Chickens and Experimental Design

A total of 196 male day-old Ross 708 broilers were obtained from a local hatchery (Longenecker's Hatchery, Elizabethtown, PA) and were randomly housed in Petersime starter brooder cage units (Zulte, Belgium) and provided with normal feed (not the experimental diet) until they were 14 D old. All chickens were weighed and allocated to 7 dietary treatments in a randomized complete block design at 14 D of age. The dietary treatments included a basal diet based on corn and soybean meal (**CON**), a second basal diet similar to CON (**NC**), CON + virginiamycin (Phibro Animal Health, Teaneck, NJ) at 20 g/ton (22 ppm) (**AB1**), CON + bacitracin methylene disalicylate (**BMD**; Zoetis, Durham, NC) at 50 g/ton (55 ppm) (**AB2**), CON + *B. subtilis* 1781 (**PB1**), CON + *B. subtilis* 747 (**PB2**), and CON + *B. subtilis* 1781 + 747 (**PB3**). *B. subtilis* strains were obtained from Church & Dwight Co., Inc. (Waukesha, WI). The dose of *B. subtilis* in the treatment was a total of 1.5×10^5 CFU/g feed. For PB3 (2-strain combination), each strain composed 50% of the total CFU count (each strain at 7.5×10^4 CFU/g feed). At the beginning of the study, each treatment contained 4 cages with 7 chickens (Figure 1). Each cage was 0.65 m in width and 0.75 m in length (14 chickens/m^2). All cages were kept in the same room. Each cage was considered an experimental unit. The chickens were given ad libitum

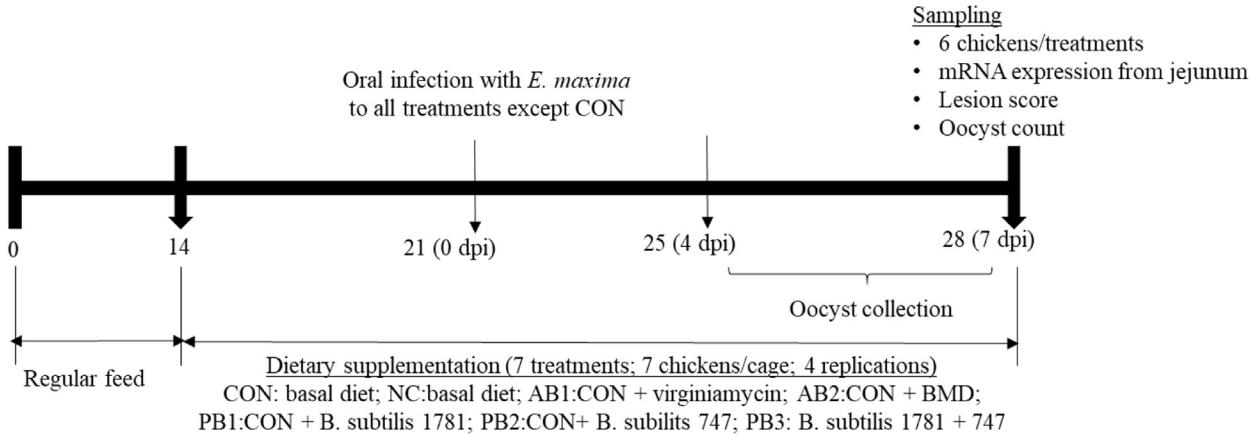

Figure 1. Schematic outline of the experimental design. Dpi: days postinfection. Abbreviations: CON: basal diet; NC: basal diet; AB1; diet supplemented with virginiamycin at 20 g/ton (22 ppm); AB2: diet supplemented with BMD at 50 g/ton (55 ppm); PB1: diet supplemented with *B. subtilis* 1781; PB2: diet supplemented with *B. subtilis* 747; PB3: diet supplemented with *B. subtilis* 1781 + 747.

access to water and feed throughout the study. Figure 1 shows the experimental schedules.

Body Weight and Feed Intake Measurement

Feed additions were weighed and recorded. The feeders were shaken once per day. The chickens and feed were weighed at 21 and 28 D of age for computation of growth performance. Dead chickens were removed and weighed daily to calculate mortality and adjust the growth performance data.

Oral Infection With E. maxima

All chickens except those in the CON group were infected by oral gavage at 21 D of age with 1.0×10^4 oocysts of *E. maxima* Beltsville strain 41 A/chicken, as previously described (Lillehoj et al., 2016; Oh et al., 2018).

Collection of Intestinal Samples

Six chickens were randomly selected from each treatment and used for collection of intestinal samples at day 28. The chickens were euthanized by cervical dislocation, and the intestines were removed immediately. From each chicken, a small section of the jejunum without contents was collected aseptically and stored in RNAlater (Applied Biosystems, Foster City, CA) at −20°C for further use.

Coccidia Lesion Score

Lesion scores from the jejunum in chickens euthanized for sample collection at day 28 were determined on a scale from 0 (none) to 4 (high) by 4 independent observers in a blinded fashion, as previously described (Johnson and Reid, 1970).

Fecal Oocyst Shedding

Fecal oocysts were collected daily between days 25 and 28 (4 and 7 D postinfection [**dpi**]). Oocyst numbers were determined as previously described (Lee et al., 2011a,b), using a McMaster chamber as per the formula: total oocysts/chicken = [oocyst count × dilution factor × (fecal sample volume/counting chamber volume)]/number of chickens per cage.

Isolation of RNA and Reverse Transcription

Total RNA was isolated from the jejunum samples stored in RNAlater by using TRIzol (Invitrogen, Carlsbad, CA) in accordance with the manufacturer's recommendations. Approximately 50 mg of jejunal tissue was homogenized in 1 mL of TRIzol using a handheld homogenizer (TissueRuptor; Qiagen Inc., Valencia, CA). Chloroform was added to the homogenized sample. The sample was centrifuged at 12,000 × *g* for 15 min at 4°C to allow phase separation. RNA present in the colorless upper aqueous phase was then precipitated with 100% isopropanol (Sigma-Aldrich Corp., St. Louis, MO). The RNA pellet was then washed with 75% ethanol (Sigma-Aldrich Corp.), air-dried, and resuspended in RNase-free water. The quantity of RNA was assessed using a NanoDrop (ND-1000) spectrophotometer (NanoDrop products, Wilmington, DE) according to the absorbance at 260 nm. RNA purity was evaluated as per the OD260/OD280 ratio. The eluted RNA was stored at −80°C until further use. Total RNA (1 μg) was then reverse transcribed to cDNA using a QuantiTect reverse transcription kit (Qiagen Inc., Valencia, CA). Briefly, the RNA sample was incubated with genomic DNA wipeout buffer at 42°C for 2 min to remove any genomic DNA contamination. Reverse transcription (RT) of the genomic DNA–depleted sample was then carried out by the addition of Quantiscript Reverse Transcriptase, Quantiscript RT buffer, and RT primer mix (Qiagen Inc.). The reaction was carried out in a thermal cycler (Mastercycler EP Gradient S; Eppendorf, Hauppauge, NY); the cycling conditions were 42°C for 30 min, followed by inactivation of reverse transcriptase at 95°C for 3 min. The cDNA samples were divided into aliquots and stored at −20°C.

Gene Expression Analysis by quantitative Real-Time PCR

The oligonucleotide primer sequences used for quantitative real-time PCR (qRT-PCR) are listed in Table 1. The various cytokines and intestinal TJ proteins whose differential expression was evaluated in the jejunum included IL-1β, IL-2, and IL-6; interferon (**IFN**)-γ; junctional adhesion molecule (**JAM**) 2; and occludin. Glyceraldehyde-3-phosphate dehydrogenase (**GAPDH**) was used as the reference gene. Amplification and detection were carried out using a Stratagene M x 3000P qPCR system (Agilent Technologies Inc., Santa Clara, CA) and RT^2 SYBR Green qPCR master mix (Qiagen). Each sample was analyzed in triplicate, and nonspecific primer amplification was assessed through the inclusion of no-template controls. Standard curves were generated with $\log_{10}$ diluted RNA, and the levels of individual transcripts were normalized to those of GAPDH in the Q-gene program (Muller et al., 2002).

Statistical Analysis

Data for each response were analyzed using Mixed Model (PROC MIXED) in SAS (SAS Inst. Inc., Cary NC). The design was a randomized complete block design. Each cage was considered an experimental unit. Each cage unit was the block factor. The results are given as least squares means and pooled SEM. Probability values less than 0.05 were considered significantly different. In cases in which the overall effect was significant in growth performance, means were compared in a pairwise manner (PDIFF option). For other results, the PDIFF option was used to compare significance between groups.

Table 1. Quantitative real-time PCR oligonucleotide primer sequences.

Type	Target gene	Primer sequence (5′-3′)	PCR product size (Kb)
Reference	GAPDH	F-GGTGGTGCTAAGCGTGTTAT R-ACCTCTGCCATCTCTCCACA	264
Proinflammatory	IL-1β	F-TGGGCATCAAGGGCTACA R-TCGGGTTGGTTGGTGATG	244
	IL-6	F-CAAGGTGACGGAGGAGGAC R-TGGCGAGGAGGGATTTCT	254
Th1	IL-2	F-TCTGGGACCACTGTATGCTCT R-ACACCAGTGGGAAACAGTATCA	256
	IFN-γ	F-AGCTGACGGTGGACCTATTATT R-GGCTTTGCGCTGGATTC	259
Tight junction proteins	JAM2	F-AGCCTCAAATGGGATTGGATT R-CATCAACTTGCATTCGCTTCA	59
	Occludin	F-GAGCCCAGACTACCAAAGCAA R-GCTTGATGTGGAAGAGCTTGTTG	68

Abberviations: F, forward primer; R, reverse primer.

RESULTS

Growth Performance

The initial body weight of chickens measured before treatment did not show significant differences ($P = 0.247$) among the treatment groups (Table 2). At day 21, the body weights of chickens on dietary AB1 (875 g), AB2 (888 g), PB2 (873 g), and PB3 (885 g) ($P = 0.063$) tended to be higher than those of chickens (827 g) fed the basal diet. As expected, infection with *E. maxima* decreased ($P = 0.038$) the body weights of chickens (average 1,050 g) at day 28 (7 dpi) regardless of dietary treatment, relative to the weights of uninfected chickens (1,175 g). From day 15 to 21 (before infection), the body weight gains in chickens fed diets supplemented with AB1 (402 g), AB2 (395 g), or PB2 (391 g) increased ($P = 0.032$) beyond that of CON-fed chickens (345 g) (Table 2). After infection, from day 22 to 28, chickens infected with *E. maxima* showed ($P < 0.001$) lower body weight gains (average 187 g) than uninfected chickens (346 g). However, chickens fed PB2 showed ($P < 0.05$) greater body weight gains (205 g) than infected chickens (169 g) fed NC (Table 2). Overall, *E. maxima* infection decreased ($P < 0.05$) the body weight gains of chickens (average 546 g) regardless of dietary treatment, as compared with those (692 g) in uninfected chickens fed CON. Chickens fed AB2 (587 g) and PB2 (580 g) showed ($P < 0.05$) greater body weight gain than infected chickens (493 g) fed NC (Table 2). The feed intake during the experimental period did not differ among different treatments (Table 2). Before infection, from day 14 to 21, the feed efficiency of broiler chickens was not affected ($P > 0.05$) by the treatments. From day 22 to 28 (after infection), the feed efficiency of infected chickens (average 0.255), regardless of dietary supplements, decreased below that of uninfected chickens

Table 2. Growth performance of chickens fed diet supplemented with antibiotics or probiotics.

Treatments	CON	NC	AB1	AB2	PB1	PB2	PB3	SEM	*P*
BW, g									
Initial	488	493	471	492	513	496	492	10.0	0.247
Day 21	827	845	875	888	842	873	885	14.2	0.063
Day 28	1,175	1,008	1,047	1,079	1,059	1,058	1,053	28.5	0.038
BWG, g									
Day 15 to 21	345^{c}	$356^{b,c}$	402^{a}	395^{a}	$366^{a,b}$	391^{a}	$384^{a,b}$	11.6	0.032
Day 22 to 28	346^{a}	169^{c}	$177^{b,c}$	$188^{b,c}$	$202^{b,c}$	205^{b}	$182^{b,c}$	11.6	0.001
Overall	692^{a}	493^{c}	$539^{b,c}$	587^{b}	$534^{b,c}$	580^{b}	$545^{b,c}$	21.9	0.001
FI, g									
Day 15 to 21	606	673	596	568	607	584	667	46.8	0.628
Day 22 to 28	702	634	574	611	637	630	608	27.3	0.138
Overall	1,307	1,306	1,170	1,180	1,244	1,214	1,275	68.1	0.659
FE									
Day 15 to 21	0.569	0.546	0.690	0.696	0.604	0.673	0.583	0.05	0.244
Day 22 to 28	0.494^{a}	0.267^{c}	$0.309^{b,c}$	$0.311^{b,c}$	0.318^{b}	$0.325^{b,c}$	$0.300^{b,c}$	0.02	0.001
Overall	0.529	0.387	0.468	0.500	0.430	0.479	0.429	0.03	0.140

[a–c]Means in the same row with different superscripts differ ($P < 0.05$).

The dose of *B. subtilis* strain in treatment was a total of at 1.5×10^5 CFU/g feed. For PB3 (2-strain combination), each strain composed 50% of the total CFU count (each strain represents 7.5×10^4 CFU/g feed). All chickens except those fed CON were infected by oral gavage at day 21 with 1.0×10^4 oocysts/bird of *E. maxima*.

Abberviations: AB1, diet supplemented with virginiamycin at 20 g/ton (22 ppm); AB2, diet supplemented with BMD at 50 g/ton (55 ppm); BW, body weight; BWG, body weight gain; CON, basal diet; FE, feed efficiency; FI, feed intake; NC, basal diet; PB1, diet supplemented with *B. subtilis* 1781; PB2, diet supplemented with *B. subtilis* 747; PB3: diet supplemented with *B. subtilis* 1781 + 747; *P*: *P* value; SEM: standard error of the mean.

(0.494) fed CON (Table 2). However, among infected chickens, chickens fed PB2 had ($P < 0.05$) greater feed efficiency (0.318) than infected chickens (0.267) fed NC. Over the experimental period, the feed efficiency did not differ among treatments.

Coccidia Lesion Score and Fecal Oocyst Shedding

The *E.* maxima infection increased ($P = 0.005$) the lesion score in the jejunum of chickens at 7 dpi (Figure 2A). Among infected chickens, those fed PB2 had ($P < 0.05$) lower lesion scores (1.4) than chickens (average 2.2) fed other diets. The mean fecal oocyst shedding number per chicken is presented in Figure 2B. Chickens in the uninfected group (CON) excreted no fecal oocysts, but infection with *E. maxima* increased ($P < 0.05$) fecal oocyst shedding regardless of treatment group at 7 dpi. AB1 and AB2 did not decrease fecal oocyst appearance, whereas the fecal oocyst shedding in chickens fed a diet supplemented with PB1 (3,152,645 oocyst/chicken), PB2 (2,870,218 oocyst/chicken), and PB3 (4,236,793 oocyst/chicken) decreased ($P < 0.05$) below that of chickens (6,037,032 oocyst/chicken) fed NC.

Intestinal Transcript Levels of Proinflammatory and Th1 Cytokines

In jejunal tissue, infection with *E. maxima* increased ($P < 0.05$) the transcript levels of IL-1β (average 3.54×10^{-4}; Figure 3A) and IL-6 (average 9.41×10^{-4}; Figure 3B) regardless of dietary supplementation. Among treatment groups, chickens (2.06×10^{-4}) fed PB2 showed lower ($P < 0.05$) transcript levels of IL-1β than chickens (4.38×10^{-4}) fed NC.

In jejunal tissue, infection with *E. maxima* increased ($P < 0.05$) the levels of IL-2 (average 1.02×10^{-4};

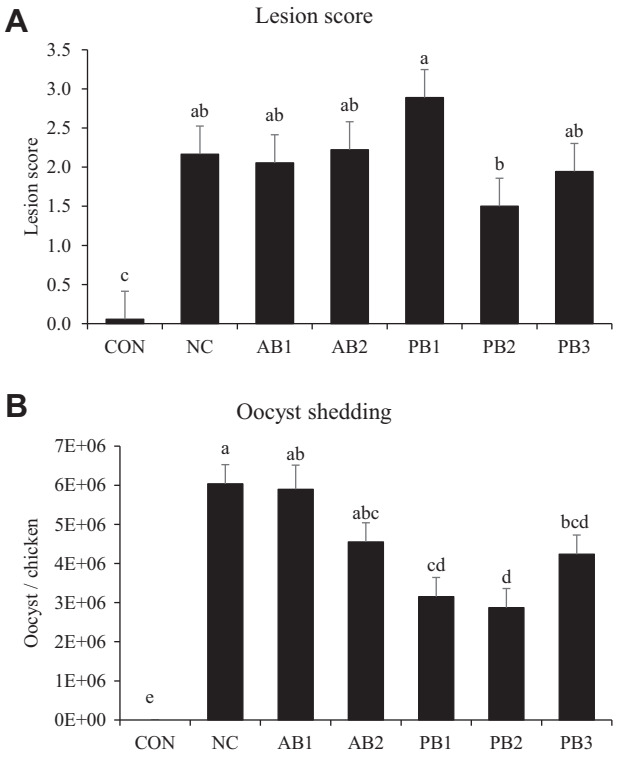

Figure 2. Lesion score and oocyst shedding of chickens fed diet supplemented with antibiotics or probiotics during infection with *E. maxima*. (A) Lesion score, (B) oocyst shedding. The dose of *B. subtilis* in treatment was 1.5×10^5 CFU/g feed. For PB3 (2-strain combination), each strain composed 50% of the total CFU count (each strain represents 7.5×10^4 CFU/g feed). All chickens except those fed CON were infected by oral gavage at day 21 with 1.0×10^4 oocysts/chicken of *E. maxima*. Bars with no common letter differ significantly ($P < 0.05$). The data were collected at day 28 (7 D postinfection) and were analyzed using Proc Mixed Procedure in SAS. Each bar represents the mean ± SEM (n = 6). Transcript levels of the cytokines were measured using quantitative RT-PCR and normalized to GAPDH transcript levels. Abbreviations: CON: basal diet; NC: basal diet; AB1: diet supplemented with virginiamycin at 20 g/ton (22 ppm); AB2: diet supplemented with BMD at 50 g/ton (55 ppm); PB1: diet supplemented with *B. subtilis* 1781; PB2: diet supplemented with *B. subtilis* 747; PB3: diet supplemented with *B. subtilis* 1781 + 747; RT-PCR: real-time PCR.

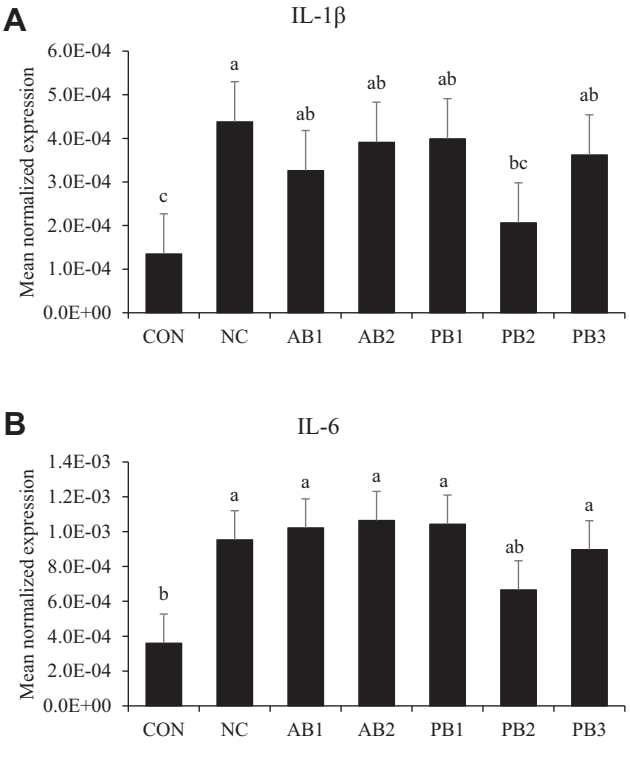

Figure 3. Transcripts of proinflammatory cytokines in the jejunum of chickens fed diet supplemented with antibiotics or probiotics during infection with *E. maxima*. (A) IL-1β, (B) IL-6. The dose of *B. subtilis* in treatment was 1.5×10^5 CFU/g feed. For PB3 (2-strain combination), each strain composed 50% of the total CFU count (each strain represents 7.5×10^4 CFU/g feed). All chickens except CON were infected by oral gavage at day 21 with 1.0×10^4 oocysts/bird of *E. maxima*. Bars with no common letter differ significantly ($P < 0.05$). Each bar represents the mean ± SEM (n = 6). The data were collected at day 28 (7 D postinfection) and were analyzed using Proc Mixed Procedure in SAS. Transcript levels of the cytokines were measured using quantitative RT-PCR and normalized to GAPDH transcript levels. Abbreviations: CON: basal diet; NC: basal diet; AB1: diet supplemented with virginiamycin at 20 g/ton (22 ppm); AB2: diet supplemented with BMD at 50 g/ton (55 ppm); PB1: diet supplemented with *B. subtilis* 1781; PB2: diet supplemented with *B. subtilis* 747; PB3: diet supplemented with *B. subtilis* 0.1781 + 747.

Figure 4A) and INF-γ (average 3.8×10^{-4}; Figure 4B) regardless of dietary supplementation. Among the treatment groups, chickens fed PB2 had lower transcript levels of IL-2 and INF-γ than chickens fed NC.

Transcript Levels of Tight Junction Proteins

In jejunal tissue, infection with *E. maxima* did not affect transcript levels of JAM2 (Figure 5A) and occludin (Figure 5B). Chickens fed a diet supplemented with antibiotics (AB1: 8.19×10^{-2}, and AB2: 1.09×10^{-1}) and probiotics (PB1: 9.37×10^{-2}, PB2: 8.60×10^{-2}, and PB3: 1.08×10^{-1}) had greater ($P < 0.05$) transcript levels of JAM2 than did chickens fed both basal diets (CON: 4.40×10^{-2} and NC: 3.94×10^{-2}). The transcript levels of occludin in the jejunum in chickens fed AB2 (7.65×10^{-2}) and PB2 (7.41×10^{-2}) were higher ($P < 0.05$) than those in chickens fed other diets.

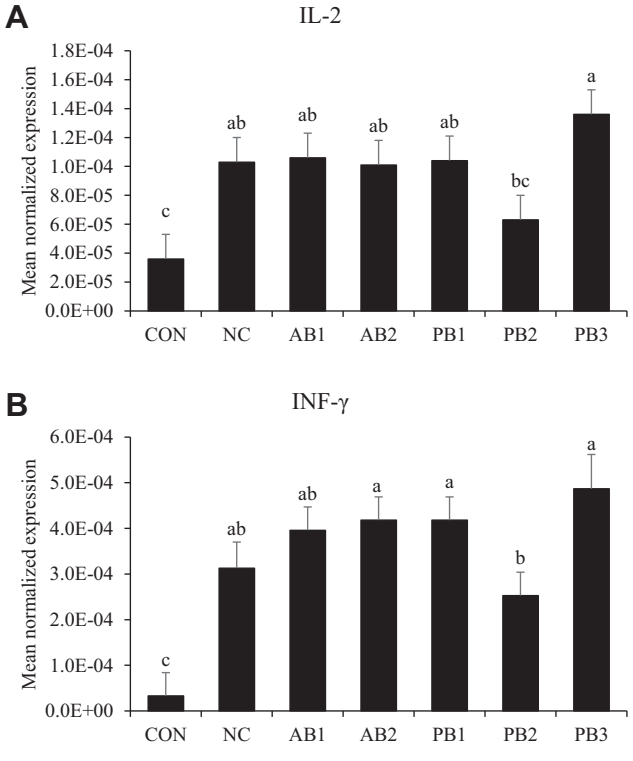

Figure 4. Transcripts of Th1 in the jejunum of chickens fed diet supplemented with antibiotics or probiotics during infection with *E. maxima*. (A) IL-2, (B) INF-γ. The dose of *B. subtilis* in treatment was 1.5×10^5 CFU/g feed. For PB3 (2-strain combination), each strain composed 50% of the total CFU count (each strain represents 7.5×10^4 CFU/g feed). All chickens except those fed CON were infected by oral gavage at day 21 with 1.0×10^4 oocysts/bird of *E. maxima*. Bars with no common letter differ significantly ($P < 0.05$). Each bar represents the mean ± SEM (n = 6). The data were collected at day 28 (7 D postinfection) and were analyzed using Proc Mixed Procedure in SAS. Transcript levels of the cytokines were measured using quantitative RT-PCR and normalized to GAPDH transcript levels. Abbreviations: CON: basal diet; NC: basal diet; AB1: diet supplemented with virginiamycin at 20 g/ton (22 ppm); AB2: diet supplemented with BMD at 50 g/ton (55 ppm); PB1: diet supplemented with *B. subtilis* 1781; PB2: diet supplemented with *B. subtilis* 747; PB3: diet supplemented with *B. subtilis* 0.1781 + 747.

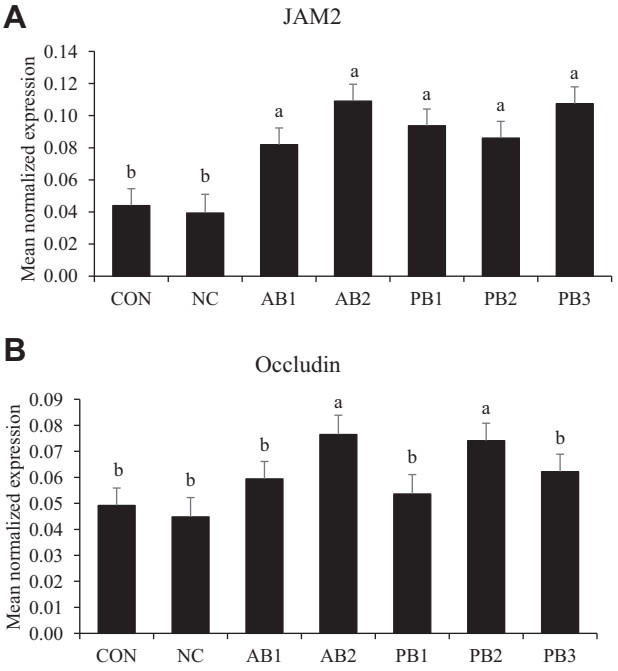

Figure 5. Transcripts of tight junction proteins in the jejunum of chickens fed diet supplemented with antibiotics or probiotics during infection with *E. maxima*. (A) JAM2, (B) occluding. The dose of *B. subtilis* in treatment was 1.5×10^5 CFU/g feed. For PB3 (2-strain combination), each strain composed 50% of the total CFU count (each strain represents 7.5×10^4 CFU/g feed). All chickens except those fed CON were infected by oral gavage at day 21 with 1.0×10^4 oocysts/bird of *E. maxima*. Bars with no common letter differ significantly ($P < 0.05$). Each bar represents the mean ± SEM (n = 6). The data were collected at day 28 (7 D postinfection) and were analyzed using Proc Mixed Procedure in SAS. Transcript levels of the tight junction proteins were measured using quantitative RT-PCR and normalized to GAPDH transcript levels. Abbreviations: CON: basal diet; NC: basal diet; AB1: diet supplemented with virginiamycin at 20 g/ton (22 ppm); AB2: diet supplemented with BMD at 50 g/ton (55 ppm); PB1: diet supplemented with *B. subtilis* 1781; PB2: diet supplemented with *B. subtilis* 747; PB3: diet supplemented with *B. subtilis* 0.1781 + 747.

DISCUSSION

This study was conducted to investigate the effects of *B. subtilis* 1781 and 747 on growth performance, intestinal immunity, and epithelial barrier integrity in broiler chickens infected with *E. maxima* compared with antibiotics. The doses of *B. subtilis* 1781 and 747 used in this study were based on the recommended level of *Bacillus*-based probiotics for the poultry industry and would cost approximately $2 per ton of feed for use under commercial conditions (Gadde et al., 2017b). This study included virginiamycin and BMD, 2 well-established AGP widely used in the poultry industry. The growth-promoting effects of these 2 antibiotics as AGP have already been demonstrated in numerous studies (Combs and Bossard, 1963; Miles et al., 1984; Engberg et al., 2000; Gadde et al., 2018). In the present study, the effects of AGP was validated, and AGP improved body weight gain in chickens fed a diet supplemented with virginiamycin and BMD for 14 to 21 D during the noninfection period. Chickens fed a diet supplemented with *B. subtilis* 1781, 747, or a combination of 1781 and

747 also showed greater body weight gain than did chickens fed a basal diet. Notably, the degree of improvement in body weight gain in chickens fed a diet supplemented with these *B. subtilis* strains was not different from that of chickens fed a diet supplemented with virginiamycin or BMD. This finding is largely consistent with those of Gadde et al. (2017a), who have reported that chickens fed a diet supplemented with *B. subtilis* 1781 grow better than chickens fed a basal diet. In addition, the beneficial growth performance of noninfected chickens fed a diet supplemented with *B. subtilis* has already been documented (Lee et al., 2010; Aliakbarpour et al., 2012; Jeong and Kim, 2014).

Eimeria spp. contributes to an estimated $3 billion annual loss worldwide, and 7 distinct species infect avian intestinal mucosa (Lillehoj and Trout, 1996; Shirley and Lillehoj, 2012). In the present study, infection with *E. maxima* significantly decreased the body weight gain of chickens below that of noninfected chickens. Virginiamycin and BMD supplementation was not efficacious against coccidiosis because they are not anticoccidial medications. The results of this work thus showed beneficial effects of dietary *B. subtilis* supplementation to young chickens infected with *E. maxima* through its action on innate immunity by decreasing proinflammatory response and enhancing gut integrity by reducing intestinal damages caused by coccidiosis. In chickens that were fed *B. subtilis*–supplemented diet, the body weight gains of infected chickens fed *B. subtilis* 747 improved beyond that of infected chickens on a basal diet, whereas *B. subtilis* 1781 or a combination of 1781 + 747 did not affect body weight gain in infected chickens. Lee et al. (2015) have also demonstrated that only two of nine tested *B. subtilis* strains improved body weight gain in chickens infected with *E. maxima*. The beneficial effect on body weight gain was effective in improving feed efficiency in infected chickens fed a diet supplemented with *B. subtilis* 747. The reasons why certain strains of *B. subtilis* show beneficial effects on coccidiosis-infected chickens need further studies.

The body weight gain and lesion score are commonly used as clinical measurements for evaluating the severity of coccidiosis (Zhu et al., 2000). In the present study, chickens infected *with E. maxima* exhibited high lesion scores, thus indicating severe extensive destruction of the gut epithelium in the area of Meckel's diverticulum, whereas infected chickens fed a diet supplemented with *B. subtilis* 747 showed lower lesion scores at 7 dpi. Infection with *E. maxima* increased the fecal oocyst output of chickens; however, *B. subtilis* 747 supplementation markedly decreased the fecal oocyst output. Therefore, in infected chickens, the growth-promoting effect of *B. subtilis* 747 supplementation was supported by the results of the lesion score and oocyst shedding.

Eimeria infection activates chickens' innate and acquired immune response, which involves the secretion of various chemokines and cytokines (Lillehoj, 1998). Cytokines, small immune-regulatory peptides aid in cell-to-cell communication during immune responses. IL-1β is an important proinflammatory cytokine that is produced mainly by activated macrophages and plays an important role in the innate immune responses through recruitment of inflammatory cells (Hong et al., 2006). IL-6, produced by T cells, monocytes, and macrophages, functions as both a proinflammatory and anti-inflammatory cytokine, and also promotes Th17-cell differentiation (Waititu et al., 2014). Increased IL-6 expression has also been proposed to aid in defining populations of heterophils that are more capable of responding to and eliminating pathogens (Swaggerty et al., 2004; Hong et al., 2006). In the present study, chickens infected with *E. maxima* showed increased expression of IL-1β and IL-6 at 7 dpi, regardless of antibiotic or probiotic supplementation. Among the infected chickens, *B. subtilis* 747 decreased the expression of IL-1β and IL-6. In addition to the changes in expression of various proinflammatory cytokines, this study also investigated the alterations in IL-2 and IFN-γ levels. Chickens infected with *E. maxima* showed increased expression of IL-2 and INF-γ, regardless of antibiotic or probiotic supplementation. Among the infected chickens, IL-2 and INF-γ expressions were downregulated in chickens fed diets supplemented with *B. subtilis* 747. If an immune response occurs, cytokines or chemokines are released in sufficient amounts to suppress the immune responses (Klasing, 2007). Klasing, (2007) has reported that a cytokine storm induces metabolic changes, including increased protein degradation in skeletal muscle, thereby diverting nutrients from the muscle and other tissues, so that they are made available for the increased demands of leukocytes and the production of protective proteins. Ultimately, these responses decrease growth performance and directly influence the success of poultry production. In practice, under equalized feed intake, a vigorous acute-phase immune response in chickens has been estimated to account for approximately 10% of nutrient use (Klasing, 2007). Jiang et al., (2010) have reported that lipopolysaccharide-challenged chickens (1 mg lipopolysaccharide per kg of body weight at 14, 16, 18, and 20 D of age) show a 22% decrease in body weight gain during challenge; 59% of the loss is accounted for by decreased feed intake, and the remaining 41% is attributed to immune response–related factors (Broom and Kogut, 2018).

Many factors related to disease and stress can damage intestinal epithelial integrity, thus decreasing nutrient absorption, increasing pathogenic invasion and inflammatory disease, and consequently decreasing growth performance (Yegani and Korver, 2008). Therefore, the intestinal epithelium serves as a physical barrier against invading pathogens and intraluminal toxins (Ulluwishewa et al., 2011; Song et al., 2014). It is composed of a single layer of columnar epithelial cells that are tightly bound by intercellular junctional complexes. These junctional complexes maintain the integrity of the epithelial barrier by regulating paracellular permeability and are composed of TJs, gap junctions, adherens junctions, and desmosomes (Gadde et al., 2017a). Tight junctions include 4 integral transmembrane proteins (occludin, claudin, JAM, and

tricellulin) that interact with cytosolic scaffold proteins, which in turn bind the actin cytoskeleton (Ulluwishewa et al., 2011; Lee et al., 2015). Junctional adhesion molecule-2 and occludin play important roles in the assembly and maintenance of TJs and the regulation of intestinal permeability, as evidenced by increased paracellular permeability to macromolecules in knockout mice (Al-Sadi et al., 2011; Lee et al., 2015). In the present study, the expression of JAM2 was elevated in all the supplemented groups regardless of infection with *E. maxima*, and occludin was elevated in the chickens fed a diet supplemented with BMD and *B. subtilis* 747. Gadde et al. (2017a) have suggested that increased TJ protein expression in chickens fed a diet supplemented with probiotics improves intestinal barrier function and provides optimal gut health.

Overall, dietary *B. subtilis* supplementation significantly improved the growth performance of noninfected chickens during the posthatch growth period similar to AGP supplementation. After infection with *E. maxima*, dietary virginiamycin and BMD supplementation enhanced epithelial barrier integrity, whereas *B. subtilis* 747 improved the growth performance, intestinal immunity, and epithelial barrier integrity of chickens in this study. Together, our results indicated that dietary *B. subtilis* supplementation has the potential to replace antibiotics fed to broiler chickens.

ACKNOWLEDGMENTS

This work was supported by ARS CRIS Project 8042-32000-107-00D.

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