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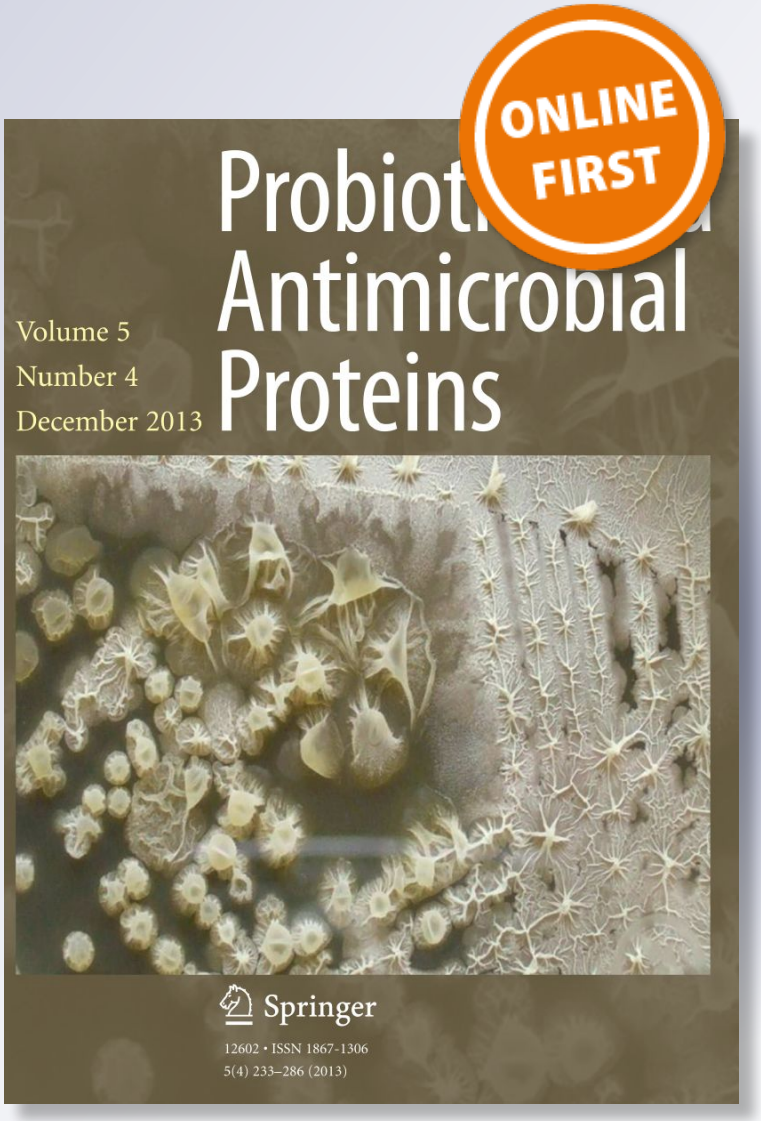


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# The Effects of Direct-fed Microbial Supplementation, as an Alternative to Antibiotics, on Growth Performance, Intestinal Immune Status, and Epithelial Barrier Gene Expression in Broiler Chickens

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**Abstract** The objective of this study was to investigate the effects of *Bacillus subtilis*-based probiotic supplementation in broiler chicken diets on growth performance, feed efficiency, intestinal cytokine, and tight junction (TJ) protein mRNA expression. Zero-day-old broiler chicks ($n = 140$) were randomly assigned to one of five dietary treatments: basal diet (CON); basal diet supplemented with either antibiotic bacitracin methylene disalicylate (BMD); or probiotics, namely, *B. subtilis* strain 1781 (PB1), a combination of *B. subtilis* strain 1104 + strain 747 (PB2), or *B. subtilis* strain 1781 + strain 747 (PB3). Body weight and feed intake were measured at 14 days of age, and the feed conversion ratio (FCR) was calculated. At 14 days of age, ileal samples were collected and used for intestinal cytokine, TJ protein, and mucin gene expression analysis using qRT-PCR. The chickens supplemented with antibiotic (BMD) and *B. subtilis* strain 1781 alone (PB1) had significantly higher body weights compared to controls of the same age. Dietary supplementation with antibiotic (BMD) or probiotics (PB1, PB2, PB3) significantly improved the feed efficiency as evidenced by decreased FCR compared to controls. No differences were observed in the expression of IL1β, IL17F, IFNγ, and MUC2 gene among the different treatment groups. However, elevated expression of IL6 (BMD, PB1, PB2), IL8 (PB2), and TNFSF15 (PB1, PB2, PB3) compared to controls was observed in the ileum. IL2 and IL10 expression was upregulated in chicks in the PB2 and PB3 groups, and IL4 was elevated in the PB1 group. IL13 was elevated in all probiotic-fed groups (PB1, PB2, PB3). Probiotic supplementation was also shown to significantly increase the expression of TJ proteins JAM2, ZO1 (PB2, PB3), and occludin (PB1, PB2). Taken together, *B. subtilis* supplementation altered intestinal immune activity and influenced gut barrier integrity through increased tight junction gene expression.

**Keywords** Chicken · *Bacillus Subtilis* · Probiotic · Gut

## Introduction

Intestinal health plays an important role in successful poultry production and translates directly to improved growth and performance in the birds. Many factors influence gut health, including diet composition, disease status, stress, and feed change. These factors lead to the loss of structural integrity of the intestinal epithelium (decreasing absorptive surface, increasing exposure to luminal antigens), increase in intestinal permeability (translocation of bacteria and their metabolic products into circulation), and increase in inflammatory responses, and they ultimately reduce performance [31]. In recent years, multifactorial diseases causing enteritis (dysbacteriosis) and gut disorders of unknown origin have emerged in broilers as a consequence of the removal of antibiotic growth promoters [24]. Currently, with the increased regulation of antibiotic use, there is a great need for the development of novel alternatives that could positively influence gut health by improving immune responses and barrier function.

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Direct-fed microbials (DFMs), often referred to as probiotics, represent a non-antibiotic nutritional approach to modulate gut function and enhance intestinal health in chickens [3]. They were shown to regulate intestinal epithelial cell function and apoptosis, influence T-lymphocyte populations, modulate cytokine profiles, and enhance antibody secretions [14, 25, 30, 33]. *Bacillus* sp. has been tested as probiotics in commercial poultry applications and have been shown to improve performance, positively modulate intestinal microflora, and inhibit pathogen colonization [12, 14, 18, 20]. Limited research exists on the effects of *B. subtilis* probiotics on alterations in gut immune parameters and the regulation of intestinal tight junction (TJ) protein expression. The present study was conducted with the objective of evaluating the effects of dietary supplementation with novel *Bacillus subtilis* strains isolated from environmental sources, on performance, gut immune response, and epithelial barrier integrity in broilers.

## Materials and Methods

### Birds and Husbandry

One hundred and forty zero-day-old male broiler chicks (Ross/Ross) were obtained from a local hatchery (Longenecker's Hatchery, Elizabethtown, PA) and were randomly allocated to Petersime brooder cages. Cages were equipped with a separate feeder, water trough, and a digitally controlled electrical heat source. The experimental diets in mash form and tap water were provided to the chicks ad libitum. Care and management of the birds followed recommended guidelines [7]. All experimental protocols and procedures were approved by the Small Animal Care Committee of the Beltsville Agricultural Research Center.

### Experimental Design and Diets

Brooder cages with chickens (0 days of age) were randomly assigned to one of the five dietary treatment groups (4 cages/treatment, total of 28 birds/treatment). Based on the treatments assigned, chickens were fed either antibiotic-free basal diets (treatment 1; controls/CON) (composition of basal diets shown in Table 1) or basal diets mixed with either antibiotics or various probiotics (treatment 2–5). The chickens in treatment 2 were given basal diets supplemented with bacitracin methylene disalicylate (BMD) at a 50 g/ton inclusion rate. The birds in the remaining three groups were fed basal diets supplemented with either probiotic *B. subtilis* strain 1781 (treatment 3; PB1), a combination of *B. subtilis* strain 1104 + strain 747 (treatment 4; PB2), or *B. subtilis* strain 1781 + strain 747 (treatment 5; PB3). For all probiotic treatments, the dose included a total of $1.5 \times 10^5$ CFU *Bacillus*/g of feed. For treatments with 2-strain combinations, each strain composed 50% of the total CFU count (each strain represents $7.5 \times 10^4$ CFU *Bacillus*/g of feed). The dose used in this experiment is the recommended level of *Bacillus*-based probiotics for the poultry industry and would cost about $2 per ton of feed for use under commercial conditions.

**Table 1** Ingredient composition of basal diet

| Ingredients | Percentage (%) |
|---|---|
| Corn | 69.01 |
| Soybean meal | 23.99 |
| Soybean oil | 2.75 |
| Dicalcium phosphate | 2.00 |
| Calcium carbonate | 1.40 |
| Salt | 0.35 |
| Poultry vit mix[a] | 0.20 |
| Poultry mineral mix[b] | 0.15 |
| DL-methionine | 0.10 |
| Choline-chloride, 60% | 0.05 |
| Total | 100 |
| Calculated values (DM basis, %) | |
| Crude protein, % | 18.00 |
| Ca, % | 1.19 |
| Available P, % | 0.54 |
| Lys, % | 1.00 |
| Met, % | 0.42 |
| Cys + Met, % | 0.65 |
| TMEn, Kcal/kg | 3585 |

[a] Vitamin mixture provided the following nutrients per kg of diet: vitamin A, 2000 IU; vitamin D3, 22 IU; vitamin E, 16 mg; vitamin K, 0.1 mg; vitamin B1, 3.4 mg; vitamin B2, 1.8 mg; vitamin B6, 6.4 mg; vitamin B12, biotin, 0.17 mg; pantothenic acid, 8.7 mg; folic acid, 0.8 mg; niacin, 23.8 mg

[b] Mineral mixture provided the following nutrients per kg of diet: Fe, 400 mg; Zn, 220 mg; Mn, 180 mg; Co, 1.3 mg; Cu, 21 mg; Se, 0.2 mg

### Probiotic Strains

*B. subtilis* strains 1781, 747, and 1104 were isolated from various environmental sources including water, animal feed, fermented silages, animal manure, and soil. Samples were heat shocked (75 °C for 15 min) to kill the vegetative bacterial populations and inoculated on trypticase soy broth (BD Difco, USA) media to selectively grow the spore-forming bacteria. The resultant strains were identified by genetic isolation and 16S ribosomal RNA sequence identification. DFM 16S rRNA sequences

were compared to existing bacterial type strain 16S rRNA sequences using Bionumerics version 7.1 (Applied Maths). The relevant strains were shown to be members of the *B. subtilis* group per full-length 16S ribosomal DNA (rDNA) sequence comparisons. Strains identified as belonging to *B. subtilis* generally recognized as safe (GRAS) were further tested for safety and functional properties.

All DFM strains used in this study were screened for diarrheal toxin using the Tecra™ *B. cereus* Diarrhoeal Enterotoxin Visual Immunoassay kit (3 M, Maplewood, MN) and demonstrated no toxin production. Antibiotic resistance was assessed by analysis of full-length genome sequences, which demonstrated that there are no transferrable antibiotic resistance genes present in the genomes of any of the *Bacillus DFM* used in this study. The selection of the strains for use in this study was based on their ability to inhibit poultry enteric bacterial pathogens such as *E. coli* and *Clostridium perfringens* in vitro (data not shown).

### Body Weight and Feed Intake Measurement

The average body weight of each group was measured and recorded at 7 and 14 days of age. The feed provided was weighed and recorded throughout the experimental period. The feed intake and feed conversion ratios (FCRs) for each treatment were calculated. Body weight and FCR data were used as criteria to assess the performance differences between the treatments.

### Collection of Intestinal Samples

Six 14 day-old chickens were randomly selected from each group and used for the collection of intestine samples. Birds were euthanized by cervical dislocation and the intestines were removed immediately. A small section of the ileum without contents from each bird was collected aseptically and stored in RNAlater® (Applied Biosystems, Foster City, CA) at −20 °C for further use.

### Isolation of RNA and Reverse Transcription

Total RNA was isolated from the ileum samples stored in RNAlater® using TRIzol (Invitrogen, Carlsbad, CA) following the manufacturer's recommendations. Approximately 50 mg of ileal tissue was homogenized in 1 mL of TRIzol using a hand-held 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 re-suspended in RNase-free water. The quantity of RNA was assessed using a NanoDrop (ND-1000) spectrophotometer (NanoDrop products, Wilmington, DE) by measuring the absorbance at 260 nm. RNA purity was evaluated by measuring the OD260/OD280 ratio (OD = optical density). The eluted RNA was stored at −80 °C until further use. Total RNA (1 μg) was then reverse transcribed to complementary DNA (cDNA) using the QuantiTect® reverse transcription kit (Qiagen Inc., Valencia, CA). Briefly, the RNA sample was incubated with genomic DNA (gDNA) wipeout buffer at 42 °C for 2 min to remove any genomic DNA contamination. Reverse transcription (RT) of the gDNA-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); cycling conditions were 42 °C for 30 min, followed by the 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 qRT-PCR

The oligonucleotide primer sequences used for quantitative real-time PCR (qRT-PCR) are shown in Table 2. The various cytokines and intestinal tight junction proteins whose differential expression was evaluated in the ileum include interleukin (IL)1β, IL2, IL4, IL6, IL8, IL10, IL13, and IL17F; interferon (IFN)γ; tumor necrosis factor superfamily (TNFSF)15; junctional adhesion molecule (JAM)2; occludin; zona occludens (ZO)1; and mucin2 (MUC2). The primer sequences of TJ proteins and MUC2 were adapted from Chen et al. [5]. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the reference gene. Amplification and detection were carried out using the Stratagene Mx3000P qPCR system (Agilent Technologies Inc., Santa Clara, CA) and the $RT^2$ SYBR Green qPCR master mix (Qiagen). Each sample was analyzed in triplicate and non-specific primer amplification was assessed by the inclusion of no template controls. Standard curves were generated using $\log_{10}$ diluted RNA, and the levels of individual transcripts were normalized to those of GAPDH using the Q-gene program [16].

### Data Analysis

Analysis of data was carried out using one-way ANOVA with SAS software [19] (version 9.4, SAS Institute Inc., Cary, NC). Results with a $P$ value ≤0.05 were considered as significantly different. Mean separations were carried out using Tukey's HSD test. All data were expressed as the mean ± SEM for each treatment.

**Table 2** Oligonucleotide primer sequences for qRT-PCR

| Type | Target gene | Primer sequence (5′-3′) | PCR product size (Kb) |
|---|---|---|---|
| Reference | GAPDH | F-GGTGGTGCTAAGCGTGTTAT<br>R-ACCTCTGCCATCTCTCCACA | 264 |
| Proinflammatory | IL1β | F-TGGGCATCAAGGGCTACA<br>R-TCGGGTTGGTTGGTGATG | 244 |
| | IL6 | F-CAAGGTGACGGAGGAGGAC<br>R-TGGCGAGGAGGGATTTCT | 254 |
| | IL8 | F-GGCTTGCTAGGGGAAATGA<br>R-AGCTGACTCTGACTAGGAAACTGT | 200 |
| | IL17F | F-TGAAGACTGCCTGAACCA<br>R-AGAGACCGATTCCTGATGT | 117 |
| | TNFSF15 | F-CCTGAGTATTCCAGCAACGCA<br>R-ATCCACCAGCTTGATGTCACTAAC | 292 |
| Th1 | IL2 | F-TCTGGGACCACTGTATGCTCT<br>R-ACACCAGTGGGAAACAGTATCA | 256 |
| | IFNγ | F-AGCTGACGGTGGACCTATTATT<br>R-GGCTTTGCGCTGGATTC | 259 |
| Th2 | IL4 | F-ACCCAGGGCATCCAGAAG<br>R-CAGTGCCGGCAAGAAGTT | 258 |
| | IL13 | F-CCAGGGCATCCAGAAGC<br>R-CAGTGCCGGCAAGAAGTT | 256 |
| Regulatory | IL10 | F-CGGGAGCTGAGGGTGAA<br>R-GTGAAGAAGCGGTGACAGC | 272 |
| TJ proteins | Occludin | F-GAGCCCAGACTACCAAAGCAA<br>R-GCTTGATGTGGAAGAGCTTGTTG | 68 |
| | ZO1 | F-CCGCAGTCGTTCACGATCT<br>R-GGAGAATGTCTGGAATGGTCTGA | 63 |
| | JAM2 | F-AGCCTCAAATGGGATTGGATT<br>R-CATCAACTTGCATTCGCTTCA | 59 |
| Mucin | MUC2 | F-GCCTGCCCAGGAAATCAAG<br>R-CGACAAGTTTGCTGGCACAT | 59 |

*F* Forward primer, *R* Reverse primer

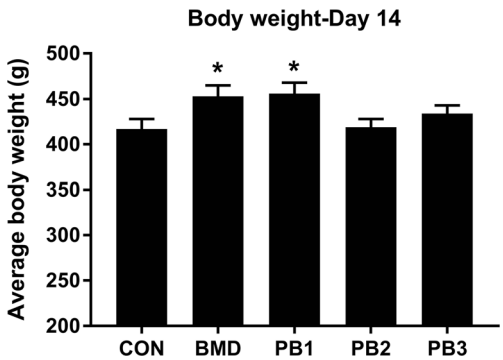


**Fig. 1** Average body weight of broilers at 14 days of age. Chickens were fed basal diets (CON), diets supplemented with antibiotic (BMD), or various strains of *B. subtilis* (PB1, PB2, PB3). The data were analyzed using one-way ANOVA and the means were separated using Tukey's HSD test. The *asterisk* (*) denotes significantly increased body weights compared with controls ($P < 0.05$)

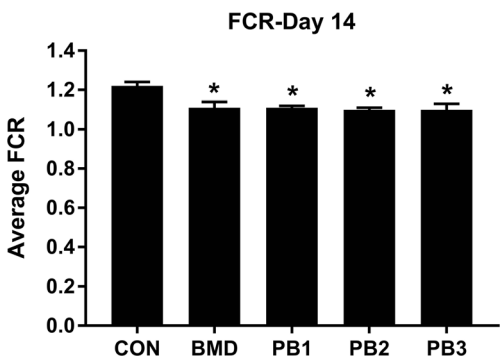


**Fig. 2** Average FCR of broilers at 14 days of age. Chickens were fed either basal diets (CON), diets supplemented with antibiotic (BMD), or various strains of *B. subtilis* (PB1, PB2, PB3). The data were analyzed using one-way ANOVA and the means were separated using Tukey's HSD test. The *asterisk* (*) denotes significantly reduced FCR compared with controls ($P < 0.05$)

## Results

### Body Weight and FCR

The body weight and FCR results at 14 days of age are presented in Figs. 1 and 2, respectively. The birds fed diets with BMD and PB1 showed significantly higher body weights compared to those fed a basal diet (CON) ($P$ = 0.0295). There were no body weight differences in chickens fed diets supplemented with *Bacillus* strain combinations (PB2, PB3). The FCR was found to be significantly improved in all chickens that were administered probiotic or antibiotic treatments compared to the controls ($P$ = 0.0256). Mortality was monitored throughout the course of the study and was found to be 3.6% in CON, 7.1% in PB2, and no mortality was recorded in BMD, PB1, and PB3 groups.

### Effects of Probiotics on Pro-Inflammatory Cytokine Expression

The mean normalized expression of various pro-inflammatory cytokines in the ileum is shown in Fig. 3. No differences were observed in the expression of IL1β ($P$ = 0.1833) and IL17F ($P$ = 0.7123) in any of the treatment groups receiving supplemented diets compared to controls. The levels of IL6 were found to be elevated in birds administered BMD, PB1, and PB2 treatments ($P$ = 0.0123). IL8 expression was significantly increased in the PB2 group compared to controls ($P$ = 0.0061). The birds fed with probiotics (PB1, PB2, PB3) showed significantly increased TNFSF15 ($P$ = 0.0195) expression in the ileum compared to those given non-supplemented basal diets (CON).

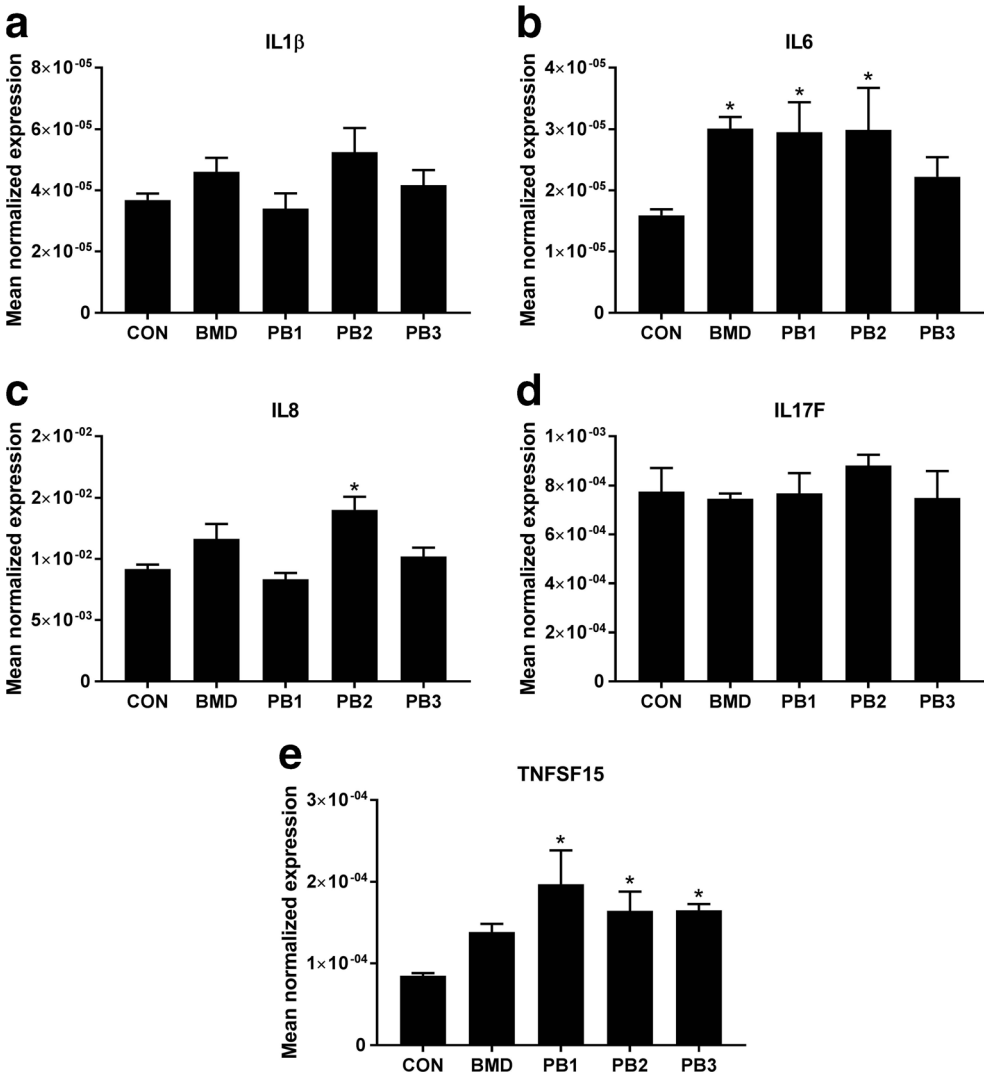


**Fig. 3** Effects of dietary probiotics or antibiotics on the levels of pro-inflammatory cytokine transcripts. **a** IL1β. **b** IL6. **c**. IL8. **d** IL17F. **e** TNFSF15. Chickens were fed either basal diets (CON), diets supplemented with antibiotic (BMD), or various strains of *B. subtilis* (PB1, PB2, PB3). Transcript levels of various cytokines in the ileum were measured using quantitative RT-PCR and normalized to GAPDH transcript levels. The data were analyzed using one-way ANOVA and the means were separated using Tukey's HSD test. Each *bar* represents the mean ± SEM ($n$ = 6). The *asterisk* (*) denotes significantly increased expression compared with controls ($P < 0.05$)

### Effects of Probiotics on Th1 and Th2 Cytokine Expression

The expression levels of various Th1 and Th2 cytokines in the ileum are presented in Fig. 4. IL2 ($P = 0.0035$) and IL10 ($P = 0.0001$) were found to be significantly elevated in PB2 and PB3 treatments compared to controls. The expression of IL4 ($P = 0.0007$) was increased only in *B. subtilis* strain 1781 supplemented birds (PB1). IL13 ($P = 0.0001$) was increased in birds given antibiotic (BMD) or probiotic (PB1, PB2, PB3)-supplemented diets compared to controls. No changes were observed in the expression of IFNγ ($P = 0.5530$) among the various treatment groups.

### Effects of Probiotics on Mucin and Intestinal Tight Junction Protein Expression

The expression of intestinal tight junction protein genes-JAM2 ($P = 0.0004$) and ZO1 ($P = 0.0001$) was significantly increased in the PB2 and PB3 groups, whereas occludin ($P = 0.0008$) was found to be elevated in the PB1 and PB2 groups compared to the CON group. Neither the probiotic nor antibiotic supplementation altered MUC2 ($P = 0.2101$) expression in the ileum at 14 days of age (Fig. 5).

## Discussion

The beneficial effects of *Bacillus*-based (*B. subtilis*, *B. amyloliquefaciens*) probiotic diet supplementation in broiler chickens have been previously documented. Sen et al. [20] showed that broilers fed diets with various levels of *B. subtilis* LS1-2 exhibited significant improvements in growth performance and nutrient retention. The body weights at 42 days of age were shown to be higher in *B. subtilis*-fed chickens compared to those fed a diet without probiotics [2]. Similar increases in body weight gain and feed efficiency were reported with the addition of various *B. subtilis* strains to broiler diets, such as UBT-$MO_2$ [33], C-3102 [9], CH16 [17], and fmbJ [4]. In this study, we investigated the effects of one mono-strain and two multi-strain *B. subtilis*-based probiotics on growth

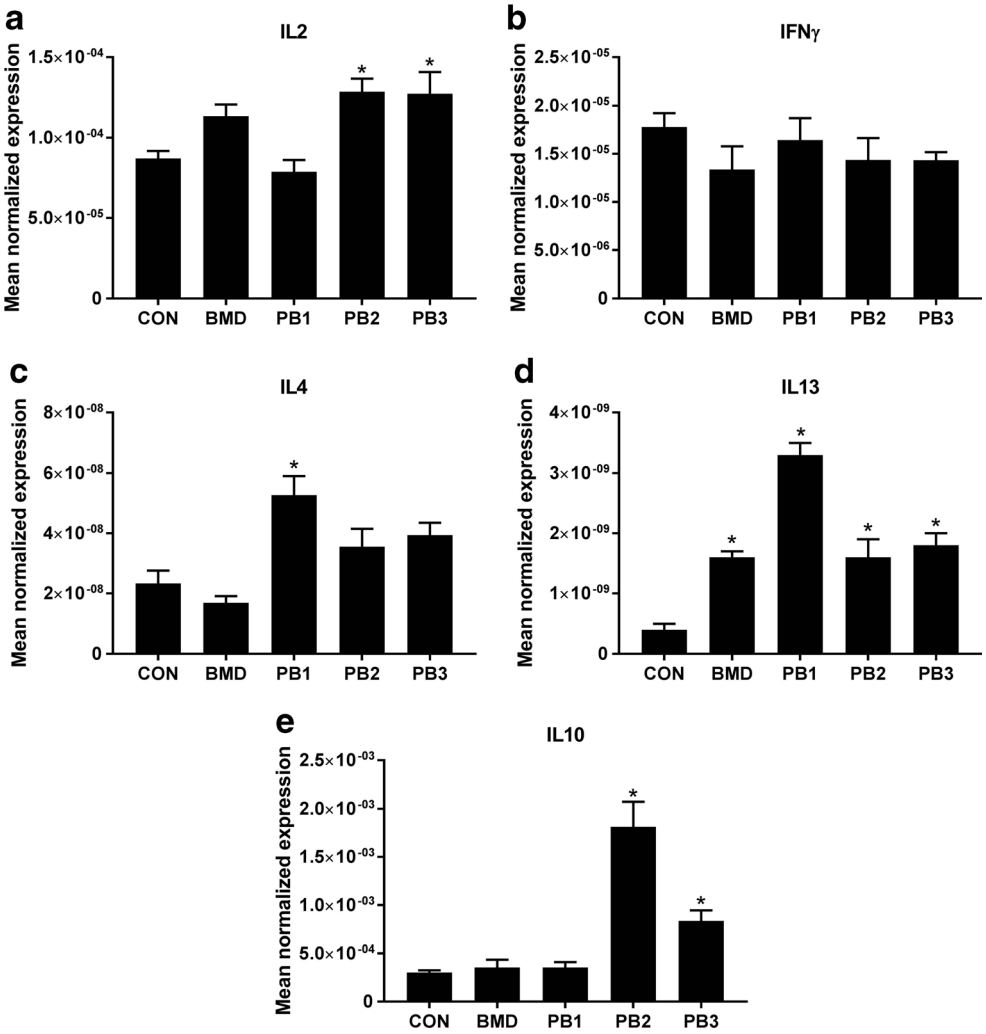


**Fig. 4** Effects of dietary probiotics or antibiotics on the levels of transcripts of Th1 (**a** IL2, **b** IFNγ), Th2 (**c** IL4, **d** IL13) and regulatory cytokines (**e** IL10). Chickens were fed either basal diets (CON), diets supplemented with antibiotic (BMD), or various strains of *B. subtilis* (PB1, PB2, PB3). Transcript levels of various cytokines in the ileum were measured using quantitative RT-PCR and normalized to GAPDH transcript levels. The data were analyzed using one-way ANOVA and the means were separated using Tukey's HSD test. Each *bar* represents the mean ± SEM ($n = 6$). The *asterisk* (*) denotes significantly increased expression compared with controls ($P < 0.05$)

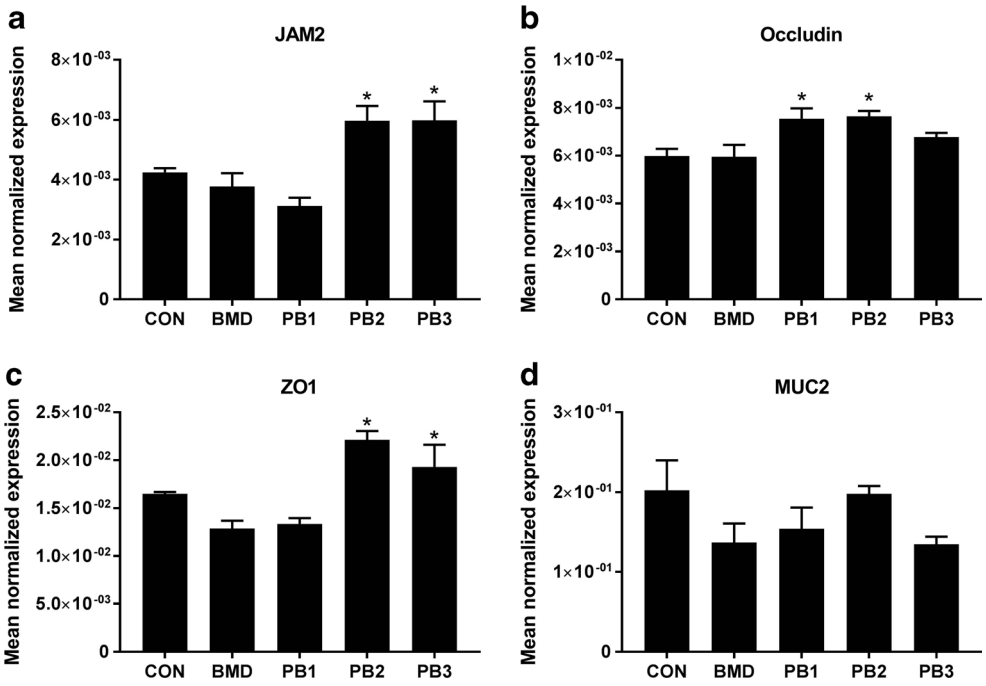


**Fig. 5** Effects of dietary probiotics or antibiotics on the levels of transcripts of TJ proteins (**a** JAM2, **b** occludin, **c** ZO1) and mucin (**d** MUC2). Chickens were fed either basal diets (CON), diets supplemented with antibiotic (BMD), or various strains of *B. subtilis* (PB1, PB2, PB3). Transcript levels of various TJ proteins and mucin in the ileum were measured using quantitative RT-PCR and normalized to GAPDH transcript levels. The data were analyzed using one-way ANOVA and the means were separated using Tukey's HSD test. Each *bar* represents the mean ± SEM ($n = 6$). The *asterisk* (*) denotes significantly increased expression compared with controls ($P < 0.05$)

performance of commercial broilers. Our results, in agreement with previously published reports, show that probiotic (PB1)-supplemented chickens have significantly higher body weights at 14 days of age compared to non-supplemented controls and the increase in body weight observed was similar to that of antibiotic-fed chickens (BMD). The FCR was found to be significantly improved in all the supplemented groups (BMD, PB1, PB2 and PB3) compared to controls. However, there were no differences in the body weight of broilers fed combinations of *B. subtilis* strains (PB2, PB3) compared to birds fed basal diets (CON). The lack of *B. subtilis* supplementation effects on broiler body weight was reported previously [6, 14, 15, 28]. These dissimilarities in results could be attributed to the differences in strains used, administration level, application method, diet composition, and hygiene status [14, 32].

Cytokines are secreted, small immuno-regulatory peptides that aid cell-to-cell communication during immune responses. IL1β is an important pro-inflammatory cytokine that is produced mainly by activated macrophages and plays an important role in the innate immune responses through recruitment of inflammatory cells [8]. IL17F is another pro-inflammatory cytokine, produced by CD4+ T cells, CD8+ T cells, NK cells, and γδ T cells, and plays a role in the recruitment of neutrophils and macrophages [29]. In our experiment, there were no differences in the expression of IL1β and IL17F in any of the probiotic or antibiotic supplemented birds. IL8 (CXCLi2), a chemokine and an important mediator of innate immune defense, was found to be elevated in PB2 birds. TNFSF15, a cytokine involved in the differentiation and proliferation of immune cells, was found to be elevated in all probiotic-fed groups (PB1, PB2, PB3). IL-6, produced by T cells, monocytes, and macrophages, functions as both a pro-inflammatory and anti-inflammatory cytokine and also helps Th17 cell differentiation [28]. Increased IL-6 expression was also proposed to aid in defining populations of heterophils that are more capable of responding to and eliminating pathogens [8, 23]. The dietary supplementation with either *B. subtilis* strain 1781 (PB1), a combination of *B. subtilis* strain 1104 + 747 (PB2), or antibiotic (BMD) significantly increased the ileal IL6 expression in broiler chickens. Lee et al. [14] reported a similar increase in IL6 expression in the intestinal epithelial lymphocytes of chickens fed diets with *B. subtilis* LSSAO1 and 22CP1 strains, whereas no changes were observed in the many other strains tested. In contrast, Waititu et al. [28] showed a decrease in gut IL6 in addition to IL2, IL4 expression in DFM fed birds compared to controls.

In addition to the changes in the expression of various pro-inflammatory cytokines, this study also investigated the alterations occurring in T-helper (Th)1 (IL2, IFNγ), Th2 (IL4, IL13), and regulatory cytokines (IL10) in the gut following *B. subtilis*-DFM supplementation. No differences were observed in IL2 and IFNγ expression. IL4 was found to be upregulated in the PB1 group compared to controls. IL13 expression was significantly increased in all probiotic (PB1, PB2, and PB3) and antibiotic (BMD)-fed broilers compared to those fed basal diets (CON). IL4 and IL13 cytokines play an important role in driving the differentiation of naive Th cells into Th2 cells and regulate antibody-mediated immune responses. IL-10, a pleiotropic cytokine, is involved in the regulation of innate immune reactions and cell-mediated immunity [8]. In this study, IL10 was found to be upregulated in

chickens fed diets with mixtures of probiotic strains (PB2, PB3). Similar increases in IL4, IL10, and IL13 expression upon dietary supplementation with various *B. subtilis* strains were demonstrated by Lee et al. [14].

The effects of *B. subtilis* supplementation on the expression of various intestinal TJ proteins were also investigated. The intestinal epithelium is an integral component of gut mucosal immunity and serves as a physical barrier against invading pathogens and intraluminal toxins [22, 27]. It is composed of a single layer of columnar epithelial cells that are tightly bound together 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 [27]. TJs include four integral transmembrane proteins (occludin, claudin, JAM, and tricellulin) that interact with cytosolic scaffold proteins (ZO), which in turn bind to the actin cytoskeleton [13, 27]. Occludin and JAM2 play an important role in the assembly and maintenance of TJs and the regulation of intestinal permeability as evidenced by increased paracellular permeability to macromolecules in knockout mice [1, 13]. Song et al. [22] reported that the protein levels of occludin and ZO1 were reduced during heat stress in broilers and were associated with increased permeability. They also showed that oral administration of a probiotic mixture improved the protein levels of occludin in broilers independent of heat stress. Similar to these results, in this study, the expression of occludin was found to be elevated in PB1 and PB2 groups and ZO1 and JAM2 were found to be elevated in the PB2 and PB3 groups compared to controls (CON). Increased TJ protein expression in chickens fed probiotic-supplemented diets can translate to increased intestinal barrier function and optimal gut health.

The mucus layer, secreted by goblet cells, covering the intestinal epithelium provides the first line of defense against physical and chemical injuries and invading enteric pathogens [11, 26]. Mucins, a major component of mucus, are large glycoproteins with a highly polymeric protein backbone structure and can be either gel-forming (secretory) or membrane-bound. MUC2, the major secretory mucin, plays a vital role in maintaining the architecture of the mucus layer on the intestinal surface and in preventing microorganisms from approaching the innermost mucus layer [10]. In this study, no differences were observed in the expression of MUC2 in any of the probiotic or antibiotic-fed broilers. Contrary to our results, dietary supplementation with *B. subtilis* was shown to significantly increase the MUC2 mRNA expression in the intestine of broiler chickens [2]. Smirnov et al. [21] reported a similar increase in mucin mRNA expression in the intestine upon supplementation with multi-strain probiotic mixture in broilers. The differences in the expression of cytokines, intestinal tight junction proteins, and mucin observed in this study and studies published previously could be attributed to the differences in the probiotic strains used.

Taken together, this study documented the immunomodulatory activities of *B. subtilis* strains in the ileum coupled with changes in the intestinal TJ proteins. From these results, it can be concluded that supplementation of broiler diets with *B. subtilis* probiotics influences a diverse array of immune gut barrier functions. Further studies characterizing the underlying molecular and signaling mechanisms involved in these alterations should be pursued.

**Compliance with Ethical Standards**

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**Conflict of Interest** Ujvala Deepthi Gadde, Sungtaek Oh, Youngsub Lee, and Hyun S. Lillehoj declare that they have no conflict of interest. Ellen Davis, Noah Zimmerman, and Tom Rehberger are employees of Agro Biosciences Inc. which developed the probiotic strains used in this study.

## References

1. Al-Sadi R, Khatib K, Guo S, Ye D, Youssef M, Ma T (2011) Occludin regulates macromolecule flux across the intestinal epithelial tight junction barrier. Am J Physiol-Gastr L 300:G1054–G1064
2. Aliakbarpour HR, Chamani M, Rahimi G, Sadeghi AA, Qujeq D (2012) The *Bacillus subtilis* and lactic acid bacteria probiotics influences intestinal mucin gene expression, histomorphology and growth performance in broilers. Asian-Austral J Anim 25:1285–1293
3. Applegate TJ, Klose V, Steiner T, Ganner A, Schatzmayr G (2010) Probiotics and phytogenics for poultry: myth or reality? J Appl Poult Res 19:194–210
4. Bai K, Huang Q, Zhang J, He J, Zhang L, Wang T (2016) Supplemental effects of probiotic Bacillus subtilis fmbJ on growth performance, antioxidant capacity, and meat quality of broiler chickens. Poult Sci. doi:10.3382/ps/pew246
5. Chen J, Tellez G, Richards JD, Escobar J (2015) Identification of potential biomarkers for gut barrier failure in broiler chickens. Front Vet Sci 2:14
6. Dersjant-Li Y, Awati A, Kromm C, Evans C (2014) A direct fed microbial containing a combination of three-strain *Bacillus* sp. can be used as an alternative to feed antibiotic growth promoters in broiler production. J Appl Anim Nutr 2:e11
7. FASS (2010) Guide for the care and use of agricultural animals in research and teaching, 3rd edn. Federation of Animal Science Societies, Champaign
8. Hong YH, Lillehoj HS, Lillehoj EP, Lee SH (2006) Changes in immune-related gene expression and intestinal lymphocyte subpopulations following *Eimeria maxima* infection of chickens. Vet Immunol Immunopathol 114:259–272
9. Jeong JS, Kim IH (2014) Effect of *Bacillus subtilis* C-3102 spores as a probiotic feed supplement on growth performance, noxious gas emission, and intestinal microflora in broilers. Poult Sci 93:3097–3103

10. Jiang Z, Applegate TJ, Lossie AC (2013) Cloning, annotation and developmental expression of the chicken intestinal MUC2 gene. PLoS One 8:e53781
11. Kim YS, Ho B (2010) Intestinal goblet cells and mucins in health and disease: recent insights and progress. Curr Gastroenterol Rep 12:319–330
12. La Ragione RM, Woodward MJ (2003) Competitive exclusion by Bacillus subtilis spores of Salmonella enterica serotype Enteritidis and *Clostridium perfringens* in young chickens. Vet Microbiol 94: 245–256
13. Lee SH (2015) Intestinal permeability regulation by tight junction: implication on inflammatory bowel diseases. Intestinal Res 13:11–18
14. Lee KW, Lee SH, Lillehoj HS, Li GX, Jang SI, Babu US, Park MS, Kim DK, Lillehoj EP, Neumann AP, Rehberger TG, Siragusa GR (2010) Effects of direct-fed microbials on growth performance, gut morphometry, and immune characteristics in broiler chickens. Poult Sci 89:203–216
15. Lee KW, Lillehoj HS, Jang SI, Lee SH (2014) Effects of Salinomycin and *Bacillus subtilis* on growth performance and immune responses in broiler chickens. Res Vet Sci 97:305–309
16. Muller PY, Janovjak H, Miserez AR, Dobbie Z (2002) Processing of gene expression data generated by quantitative real-time RT-PCR. BioTechniques 32:1372–1374 **1376, 1378–1379**
17. Nguyen ATV, Nguyen DV, Tran MT, Nguyen LT, Nguyen AH, Phan TN (2015) Isolation and characterization of Bacillus subtilis CH16 strain from chicken gastrointestinal tracts for use as a feed supplement to promote weight gain in broilers. Lett Appl Microbiol 60:580–588
18. Park JH, Kim IH (2014) Supplemental effect of probiotic *Bacillus subtilis* B2A on productivity, organ weight, intestinal *Salmonella* microflora, and breast meat quality of growing broiler chicks. Poult Sci 93:2054–2059
19. SAS Institute Inc (2013) Base SAS® 9.4 procedures guide: statistical procedures, Second edn. SAS Institute Inc., Cary
20. Sen S, Ingale SL, Kim YW, Kim JS, Kim KH, Lohakare JD, Kim EK, Kim HS, Ryu MH, Kwon IK, Chae BJ (2012) Effect of supplementation of *Bacillus subtilis* LS 1-2 to broiler diets on growth performance, nutrient retention, caecal microbiology and small intestinal morphology. Res Vet Sci 93:264–268
21. Smirnov A, Perez R, Amit-Romach E, Sklan D, Uni Z (2005) Mucin dynamics and microbial populations in chicken small intestine are changed by dietary probiotic and antibiotic growth promoter supplementation. J Nutr 135:187–192
22. Song J, Xiao K, Ke YL, Jiao LF, Hu CH, Diao QY, Shi B, Zhou XT (2014) Effect of probiotic mixture on intestinal microflora, morphology, and barrier integrity of broilers subjected to heat stress. Poul Sci 93:581–588
23. Swaggerty CL, Kogut MH, Ferro PJ, Rothwell L, Pevzner IY, Kaiser P (2004) Differential cytokine mRNA expression in heterophils isolated from *Salmonella*-resistant and –susceptible chickens. Immunology 113:139–148
24. Teirlynck E, Guseem MDE, Dewulf J, Haesebrouck F, Ducatelle R, Van Immerseel F (2011) Morphometric evaluation of "dysbacteriosis" in broilers. Avian Pathol 40:139–144
25. Thomas CM, Versalovic J (2010) Probiotics-host communication: modulation of signaling pathways in the intestine. Gut Microbes 1: 148–163
26. Tsirtsikos P, Fegeros K, Balaskas C, Kominakis A, Mountzouris KC (2012) Dietary probiotic inclusion level modulates intestinal mucin composition and mucosal morphology in broilers. Poult Sci 91:1860–1868
27. Ulluwishewa D, Anderson AC, McNabb WC, Moughan PJ, Well JM, Roy NC (2011) Regulation of tight junction permeability by intestinal bacteria and dietary components. J Nutr 141:769–776
28. Waititu SM, Yitbarek A, Matini E, Echeverry H, Kiarie E, Rodriguez-Lecompte JC, Nyachoti CM (2014) Effect of supplementing direct-fed microbials on broiler performance, nutrient digestibilities, and immune responses. Poult Sci 93:625–635
29. Weaver CT, Hatton RD, Mangan PR, Harrington LE (2007) IL-17 family cytokines and the expanding diversity of effector T cell lineages. Annu Rev Immunol 25:821–852
30. Wolfenden RE, Pumford NR, Morgan MJ, Shivaramaiah S, Wolfenden AD, Pixley CM, Green J, Tellez G, Hargis BM (2011) Evaluation of selected direct-fed microbial candidates on live performance and *Salmonella* reduction in commercial turkey brooding houses. Poult Sci 90:2627–2631
31. Yegani M, Korver DR (2008) Factors affecting intestinal health in poultry. Poult Sci 87:2052–2063
32. Zhang ZF, Zhou TX, Ao X, Kim IH (2012) Effects of β-glucan and *Bacillus subtilis*, on growth performance, blood profiles, relative organ weight and meat quality in broilers fed maize-soybean meal based diets. Livest Sci 150:419–424
33. Zhang ZF, Cho JH, Kim IH (2013) Effects of *Bacillus subtilis* UBT-MO2 on growth performance, relative immune organ weight, gas concentration in excreta, and intestinal microbial shedding in broiler chickens. Livest Sci 155:343–347