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Effect of *Bacillus subtilis*, *Clostridium butyricum* and *Lactobacillus acidophilus* endospores on growth performance, nutrient digestibility, meat quality, relative organ weight, microbial shedding and excreta noxious gas emission in broilers

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ABSTRACT: This study was conducted to evaluate the effects of *Bacillus subtilis*, *Clostridium butyricum* and *Lactobacillus acidophilus* (tri-strain probiotics, TSP) endospores in broilers. TSP can benefit the host animal by increasing nutrient absorption from the gastrointestinal tract and altering the intestinal ecosystem in poultry. A total of 500 day-old ROSS 308 mixed sex broiler chickens with an average initial body weight (IBW) of 45 g ± 0.6 g were used in this 35-day feeding study. Broiler chickens were randomly allotted to one of five dietary treatments: (1) CON (antibiotic free diet), (2) ANT1 (CON + enramycin 5 ppm), (3) ANT2 (CON + avilamycin 5 ppm), (4) TSP1 (CON + 0.1% TSP), and (5) TSP2 (CON + 0.2% TSP) with five replicates per treatment and 20 chicks per pen. Broiler chickens fed on TSP diets exhibited linearly increasing body weight gain (BWG) and decreased feed conversion ratio (FCR) compared to those on the CON diet ($P < 0.05$; Day 21 to Day 35 and Day 1 to Day 35, respectively). Further, dry matter (DM) and nitrogen (N) digestibility were improved ($P < 0.05$) in the TSP treatment at the end of study. The inclusion of TSP reduced (linear, $P = 0.02$) meat lightness (L^*) compared with CON and ANT treatments. Broiler chickens fed with TSP diets had relatively higher (linear, $P < 0.05$) bursa weight than those fed with ANT and CON diets. The supplementation of TSP increased ($P < 0.05$) the ileal and caecal *Lactobacillus* count compared with CON and ANT diets. The ileal and caecal *Escherichia coli* and caecal *Clostridium perfringens* counts were reduced ($P < 0.05$) in the TSP2 group compared with the CON group. Broiler chickens fed with TSP diets exhibited increased ($P < 0.05$) caecal *Bifidobacteria* counts compared with CON and ANT diets. Excreta ammonia (NH_3) gas emission was lower ($P < 0.05$) with the TSP treatment compared with the CON treatment. In conclusion, the supplementation of TSP improved growth performance, nutrient digestibility, meat quality, gut health and reduced noxious gas emission in broilers.

Keywords: ammonia gas emission; body weight gain; bursa weight; *Escherichia coli*; *Lactobacillus*; meat lightness

List of abbreviations

BWG = body weight gain, **FCR** = feed conversion ratio, **DM** = dry matter, L^* = meat lightness, **N** = nitrogen, NH_3 = ammonia, **TSP** = tri-strain probiotics

In the poultry business, antibiotics are used worldwide with the goal of enhancing growth performance and preventing illnesses. However, repeated use of antibiotics in poultry diets results in undesirable outcomes such as the resistance of microorganisms to antibiotics, accumulation of antibiotic residues in animal products and the environement, imbalance of normal microflora and reduction in beneficial intestinal microflora (Hinton et al. 1986; Sinol et al. 2012). This has resulted in severe restrictions or total bans on the employment of antibiotics in animal and poultry

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production in several countries. As a result, the poultry industry must develop alternatives to antibiotics to maintain health and performance under commercial conditions. Probiotics have been increasingly adopted as an alternative to antibiotic growth promoters in poultry diets (Mountzouris et al. 2010; Zhang and Kim 2014). Probiotics, which are live cultures of harmless bacteria or yeast species that equilibrate intestinal microflora to benefit the host (Ferencik et al. 2000; Chen et al. 2013), have been demonstrated to be helpful in maintaining the intestinal ecosystem and enhancing animal health. Probiotics have been reported to improve growth performance and nutrient digestibility, balance intestinal microflora, promote immune function and benefit the intestinal morphology (Li et al. 2009; Sinol et al. 2012; Zhang and Kim 2013). Although, the efficiency of probiotics depends upon various factors such as, selection of strain, administration level, application method, ability of the selected strain to survive at environmental temperatures, long-term storage and viability.

Among a number of bacterial species used as probiotics, spore-forming *Bacillus* spp. has been identified as a suitable probiotic because of the resistance of its spores to harsh conditions and long-term storage at ambient temperature (Sinol et al. 2012; Chen et al. 2013). *Clostridium butyricum* is a butyric acid bacterium that was isolated from soil and healthy animal and human faecal matter (Finegold et al. 1983). *Clostridium butyricum* can produce endospores, which is critical for its capacity to survive at lower pH and relatively greater bile concentrations compared with *Lactobacillus* and *Bifidobacterium* (Kong et al. 2011). In an earlier study the impact of a probiotic complex (*Bacillus subtilis* endospores and *Clostridium butyricum* endospores) in increasing growth performance and meat quality in growing-finishing pigs was confirmed (Meng et al. 2010). However, to the best of our knowledge, the effects of *Bacillus subtilis* DSM 17299, *Clostridium butyricum* and *Lactobacillus acidophilus* endospores in broiler chickens have not been investigated in detail. Thus, this study was designed to assess the effect of *Bacillus subtilis* DSM 17299, *Clostridium butyricum* and *Lactobacillus acidophilus* endospores on growth performance, nutrient digestibility, meat quality, relative organ weight, microbial shedding and excreta noxious gas emission in broiler chickens.

MATERIAL AND METHODS

Source of TSP. The probiotic preparation used in the current experiments was manufactured by a commercial company (Probion, Woogene B & G Co. Ltd., Seoul, Republic of Korea). The product is composed of a mixture of spray-dried spore-forming bacteria, which was guaranteed to contain 2×10^8 viable spores/kg of *Bacillus subtilis* DSM 17299, *Clostridium butyricum* and *Lactobacillus acidophilus*.

Table 1. Diet composition (as-fed basis)

Ingredients (%)	Starter (1–21 day)	Finisher (21–35 day)
Corn grain	57.84	63.49
Corn gluten meal	9.30	5.20
Soybean meal	21.60	21.60
Fish meal (60%)	4.18	2.70
Wheat bran	0.40	0.40
Tallow	3.67	3.73
Limestone	1.60	1.60
TCP	0.50	0.45
L-lysine	0.18	0.10
Salt	0.25	0.25
Vit.-Min. mixture[1]	0.25	0.25
Choline	0.22	0.22
Selenium	0.01	0.01
Calculated composition[2]		
ME (kcal/kg)	3,200	3,200
CP (%)	23.09	20.08
Lys (%)	1.21	1.05
Met + Cys (%)	0.50	0.41
Ca (%)	1.02	0.93
Total P (%)	0.57	0.51
Analysed composition		
CP (%)	23.05	20.05
Lys (%)	1.20	1.04
Met + Cys (%)	0.49	0.41
Ca (%)	1.02	0.92
Total P (%)	0.55	0.50

[1]Vit.-Min. = mixture contains the following amounts of micronutrients per kg: Vitamin A, 1 600 000 IU; Vitamin D3, 300 000 IU; Vitamin K3, 130 mg; Vitamin B2, 1000 mg; Niacin, 2000 mg; Ca-Pantothenate, 800 mg; folic acid, 60 mg; DL-methionine, 6000 mg; Mn, 12 000 mg; Zn, 9000 mg; Fe, 4000 mg; Cu, 500 mg; I, 250 mg; Co, 100 mg; Ca, 7140 mg; BHT, 6000 mg

[2]values for ingredients used in diet formulation were based on broilers requirements in NRC (1994)

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Experimental design, animals and diets. A total of 500 day-old ROSS 308 mixed sex broiler chickens with an average initial body weight (IBW) of 45 g ± 0.6 g were randomly allotted to five treatments with five replicates per treatment and 20 chicks per pen. Broiler chickens were fed with starter (one to 21 days) and finisher (21 to 35 days) diets in the form of mash. All nutrients in diets were formulated to meet or exceed the recommendations of the NRC (1994) for broiler chickens (Table 1). Dietary treatments were (1) CON (antibiotic free diet), (2) ANT1 (CON + enramycin 5 ppm), (3) ANT2 (CON + avilamycin 5 ppm), (4) TSP1 (CON + 0.1% TSP), and (5) TSP2 (CON + 0.2% TSP). It is well accepted that enramycin and avilamycin can be used as antibiotic growth promoters (AGPs) to compare the efficacy of probiotics (Mountzouris et al. 2010; Abudabos 2012). Broiler chickens were raised in a temperature-controlled room with three floors of stainless steel pens of identical size (1.75 × 1.55 m). Room temperature began at 33 °C from Day 1 to Day 3 and was reduced gradually to 24 °C until the end of the experiment; the relative humidity was around 60%. The broiler chickens had free access to diet and water. The Animal Care and Use Committee of Dankook University approved all of the experimental protocols used in the current study.

Chemical analysis. Feed samples were ground to pass through a 1-mm screen, after which they were analysed for DM (method 934.01; AOAC 2000), N (method 968.06; AOAC 2000), calcium (Ca, method 984.01; AOAC 1995), and phosphorus (P, method 965.17; AOAC 1995). Individual AA composition was measured using an AA analyser (Beckman 6300; Beckman Coulter Inc., Fullerton, CA) after 24-h hydrolysis in HCl. For the determination of cysteine (Cys) and methionine (Met), the samples were oxidized with performic acid over night at 0 °C. Performic acid is an oxidizing reagent that converts Cys quantitatively to cysteic acid and Met to Met sulfone (Moore 1963). Nitrogen was determined (Kjectec 2300 Nitrogen Analyser; Foss Tecator AB, Hoeganaes Sweden) and crude protein (CP) was calculated as N × 6.25. Gross energy (GE) was analysed using an oxygen bomb calorimeter (Parr 1600 Instrument Co., Moline, IL, USA).

Sampling and measurements. Birds were weighed on Day 1, Day 21 and Day 35, with birds in each pen being weighed as a group. Mean BW for each treatment was calculated from the pen replicates for each weighing day. The BWG was calculated individually for each period and cumulatively based on pen weights. For the same period, feed intake (FI) of each pen as a group was measured as BW (Day 21 and Day 35) with cumulative averages calculated. The FCR was calculated as (feed intake)/(body weight gain).

At the end of the experiment, apparent total tract digestibility (ATTD) of DM, N and energy (E) were determined using chromic oxide as an indicator (Fenton and Fenton 1979). All broiler chicks were fed diets mixed with 2% Cr_2O_3 for seven days before excreta collection at Week 5. All excreta were pooled by pen and mixed, after which a representative sample was stored in a freezer at −20 °C until analysis. Before chemical analysis, the excreta samples were thawed and dried for 72 h at 50 °C in a forced-air oven (model FC-610, Advantec, Toyo Seisakusho Co. Ltd., Tokyo, Japan), after which they were finely ground to a size that could pass through a 1-mm screen. All feed and excreta samples were then analysed for DM, N and E as described above. Chromium was analysed using UV absorption spectrophotometry (UV-1201, Shimadzu, Kyoto, Japan). The ATTD was then calculated using the following formula:

$$\text{digestibility (\%)} = \{1 - [(Ne \times Cd)/(Nd \times Ce)]\} \times 100$$

where:

Ne = nutrient concentration in excreta (% DM)

Nd = nutrient concentration in diet (% DM)

Cd = chromium concentration in diet (% DM)

Ce = chromium concentration in excreta (% DM)

At the end of experiment, five broiler chickens were randomly selected from each treatment (one bird per pen), euthanised by cervical dislocation and weighed individually. The liver, spleen, bursa, breast, gizzard and abdominal fat were removed by trained personnel and weighed. Relative organ weight (percent of live BW) was calculated. The breast meat Hunter lightness (L^*), redness (a^*) and yellowness (b^*) values were measured using a Minolta CR410 Chroma meter (Konica Minolta Sensing Inc., Osaka, Japan).Yan et al. (2011) described a similar method for calculating meat colour. Cook loss was determined as described previously by Sullivan et al. (2007). Briefly, 5 g of breast meat were heat-treated in plastic bags separately in a water bath (100 °C) for 5 min. Samples were cooled at room temperature. Cooking loss was calculated as (sample weight before cooking – sample weight after cooking)/sample weight before cooking × 100. Drip loss was measured as de-

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scribed by Honikel et al. (1986). Two (2.5 × 2.5 cm) chops were weighed, placed in a drip loss tube (C. Christensen Laboratory, Hillerod, Denmark) and held at 2 °C for 24 h. Then, meat samples were removed, blotted dry on paper towels and reweighed. Differences between sample weights were used to calculate drip loss percent. Duplicate pH values for each sample were measured using a pH meter (Fisher Scientific, Pittsburgh, PA). To determine TBARS, 5 g of breast meat with 20% tricloroacetic acid (TCA), 12.5 ml 2M phosphoric acid were homogenised in one minute at a speed of 14 000 rpm using a homogenizer (AM-8, Nissci Co., Japan) and the extract was titrated by methanol and then homogenised with 5 ml distilled water. Finally, 25 ml of the mixture solution were centrifuged for 15 min at a speed of 1500 rpm at 4 °C. Two ml of the solution were mixed with 2ml 0.005M thiobarbituric acid (TBA) and incubated in a 95 °C water bath for 30 min and then spectrophotometric analysis was performed under 530 nm.

The same slaughtered broiler chickens were used for microbial counts. Ileal and caecal contents were collected into Qorpak glass containers (118 ml) under CO_2, sealed and placed on ice until transported to the laboratory for enumeration of microbial populations. Caecal and ileal samples were assessed for populations of *Lactobacillus*, *Escherichia coli*, *Clostridium perfringens*, and *Bifidobacteria*. One gram of the composite excreta sample from each cage was diluted with 9 ml of 1% peptone broth (Becton, Dickinson and Co., Franklin Lakes, NJ) and then homogenised. Viable counts of bacteria in the caecal and ileal samples were then conducted by plating serial 10-fold dilutions (in 1% peptone solution) onto lactobacilli medium III agar plates (Medium 638; DSMZ, Braunschweig, Germany), MacConkey agar plates (Difco Laboratories, Detroit, MI), Perfringens agar base (Perfringens TSC Agar; Oxoid, Basingstoke, UK) and Wilkins-Chalgren agar (Oxoid, Nepean, Ontario, Canada) supplemented with glacial acetic acid (1 ml/l) and mupirocin (100 mg/l) extracted from antimicrobial discs (Oxoid; Rada et al. 1999) to isolate the *Lactobacillus*, *Escherichia coli*, *Clostridium perfringens* and *Bifidobacteria*, respectively. The lactobacilli medium III agar plates were then incubated for 48 h at 37 °C under anaerobic conditions. The MacConkey and Perfringens agar plates were incubated for 24 h at 37 °C and the Wilkins-Chalgren agar plates were incubated for 72 h at 37 °C. The microflora colonies were counted immediately after removal from the incubator. The concentration of microflora was expressed as log10 colony-forming units per gram of intestinal content.

Excreta NH_3 and hydrogen sulphide (H_2S) contents were measured at day 35 according to the method described by Zhang and Kim (2013). A total of 300 g excreta samples from each pen were collected in 2.6-l plastic boxes, with adhesive tape-sealed holes for pumps. The samples were allowed to ferment for one day at room temperature (25 °C), after which 100 ml of the headspace air were sampled from approximately 2.0 cm above the excreta sample. The concentrations of NH_3 and H_2S were measured within the scope of 5.0–100.0 ppm (No. 3La, detector tube; Gastec Corp. Kanagawa, Japan) and 2.0–20.0 ppm (4LK, detector tube; Gastec Corp.). After collection, the boxes were re-sealed with adhesive plaster to measure the excreta noxious content at Day 3 and Day 5 as aforementioned. Prior to measurement, the excreta samples were manually shaken for approximately 30 s to disrupt any crust formation on the surface of the excreta sample and to homogenise the samples. The excreta volatile fatty acid (VFA) concentration was determined by the method of Erwin et al. (1961). The VFA concentration in the supernatant liquid was determined using a gas chromatograph (VARIAN, CP-3800, CA, USA).

Statistical analysis. Data were statistically analysed by ANOVA using the GLM procedure of SAS/STAT®9.2 (SAS 2008), for a randomised complete block design. Differences among all treatments were separated by Tukey's range test. Before conducting statistical analysis of the *Lactobacillus*, *Escherichia coli, Clostridium perfringens and Bifidobacteria* counts, the value was transformed logarithmically. The linear and quadratic effects of TSP among treatments were analysed using a contrast statement. Orthogonal contrasts were used for the effects of ANT vs. TSP treatments. Variability in the data was expressed as the pooled SE and probability values of less than 0.05 were considered significant.

RESULTS

Growth performance and ATTD of nutrients

In this study, the inclusion of TSP linearly improved BWG and FCR in the grower and overall experimental period compared with the CON diet

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Table 2. Effect of *Bacillus subtilis, Clostridium butyricum* and *Lactobacillus acidophilus* endospores on the growth performance of broilers

	Treatment					SE[1]	*P*-values		ANT vs. TSP
	CON	ANT1	ANT2	TSP1	TSP2		linear	quadratic	
BWG (g/chick)									
Day 1 to 21	880	910	907	990	1001	58	0.39	0.17	0.31
Day 21 to 35	886[b]	882[b]	919[ab]	955[a]	964[a]	17	0.04	0.24	0.12
Day 1 to 35	1.766[b]	1.792[b]	1.816[b]	1.945[a]	1.965[a]	18	0.04	0.31	0.66
FI (g/chick)									
Day 1 to 21	1.095	1.734	1.149	1.221	1.207	65	0.53	0.14	0.84
Day 21 to 35	1.716	1.927	1.709	1.668	1.756	44	0.32	0.78	0.54
Day 1 to 35	2.923	2.996	2.940	3.104	3.144	95	0.33	0.38	0.32
FCR									
Day 1 to 21	1.244	1.253	1.266	1.234	1.206	0.08	0.42	0.61	0.56
Day 21 to 35	1.937[a]	1.965[a]	1.859[ab]	1.828[b]	1.822[b]	0.04	0.01	0.31	0.27
Day 1 to 35	1.655[a]	1.672[a]	1.619[b]	1.596[b]	1.600[b]	0.02	0.02	0.34	0.48

CON = antibiotic free diet; ANT1 = CON + enramycin 5 ppm; ANT2 = CON + avilamycin 5 ppm; TSP1 = CON + 0.1% TSP; TSP2 = CON + 0.2% TSP

[1]pooled SE

[a,b]means in the same row with different superscripts differ ($P < 0.05$)

($P < 0.05$, Table 2). No significant differences were observed in FI throughout the whole experimental period. TSP2 treatment increased DM and N digestibility ($P = 0.03$ and $P = 0.02$, respectively) compared with the CON treatment (Table 3). No differences were observed in E digestibility among the treatments.

Meat quality and relative organ weight

The supplementation of TSP1 and TSP2 linearly increased relative bursa weight compared with CON, ANT1 and ANT2 treatments (51.2, 51.8 vs. 56.2, 53.2 and 55.4; $P = 0.02$; Table 4). Moreover, broilers fed TSP diets showed a higher relative bursa weight compared with other treatments ($P = 0.02$). No significant differences were observed in other parameters ($P > 0.05$).

Microbial shedding and excreta noxious gas emission

The impact of TSP on ileal and caecal microbial shedding is shown in Table 5. In this study, broil-

Table 3. Effect of *Bacillus subtilis*, *Clostridium butyricum* and *Lactobacillus acidophilus* endospores on the nutrient digestibility of broilers

Item (%)	Treatment					SE[1]	*P*-values		ANT vs. TSP
	CON	ANT1	ANT2	TSP1	TSP2		linear	quadratic	
DM	70.48[b]	72.36[b]	71.89[b]	77.43[a]	77.22[a]	1.53	0.03	0.18	0.04
N	68.43[b]	69.21[ab]	68.03[b]	69.49[ab]	71.93[a]	1.23	0.02	0.47	0.41
E	72.84	71.94	74.33	72.69	73.21	1.48	0.36	0.44	0.21

CON = antibiotic free diet; ANT1 = CON + enramycin 5 ppm; ANT2 = CON + avilamycin 5 ppm; TSP1 = CON + 0.1% TSP; TSP2 = CON + 0.2% TSP

[1]pooled SE

[a,b]means in the same row with different superscripts differ ($P < 0.05$)

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Table 4. Effects of *Bacillus subtilis*, *Clostridium butyricum* and *Lactobacillus acidophilus* endospores on meat quality and relative organ weight of broilers

Item	Treatment					SE[1]	*P*-values		ANT vs. TSP
	CON	ANT1	ANT2	TSP1	TSP2		linear	quadratic	
Meat colour									
*L**	56.2[b]	53.2[b]	55.4[b]	51.2[a]	51.8[a]	2.01	0.02	0.32	0.04
*a**	14.9	15.6	15.9	14.2	15.2	1.20	0.19	0.55	0.55
*b**	8.54	8.33	8.92	8.66	7.99	0.78	0.65	0.34	0.29
pH	6.2	6.65	6.21	6.29	6.37	0.18	0.33	0.29	0.17
Drip loss (%)	2.43	2.62	2.21	2.09	1.98	0.33	0.28	0.35	0.25
Cook loss (%)	16.7	17.2	15.7	15.1	15.9	0.51	0.81	0.47	0.96
TBARS (mg/kg)	0.024	0.027	0.025	0.021	0.018	< 0.01	0.06	0.21	0.41
Relative organ weight (%)									
Liver	2.52	2.49	2.66	2.60	2.57	0.13	0.57	0.19	0.42
Spleen	0.058	0.077	0.078	0.065	0.071	< 0.01	0.61	0.29	0.71
Bursa	0.115[b]	0.166[b]	0.143[ab]	0.171[a]	0.182[a]	0.01	0.02	0.31	0.09
Breast	8.34	8.45	8.72	8.64	8.51	0.22	0.33	0.59	0.24
Gizzard	1.67	1.64	1.72	1.59	1.57	0.04	0.54	0.60	0.42
Abdominal fat	1.62	1.54	1.51	1.55	1.59	0.03	0.19	0.77	0.17

CON = antibiotic free diet; ANT1 = CON + enramycin 5 ppm; ANT2 = CON + avilamycin 5 ppm; TSP1 = CON + 0.1% TSP; TSP2 = CON + 0.2% TSP; *L** = indicates lightness; *a** = indicates redness; *b** = indicates yellowness

[1]pooled SE

[a,b]means in the same row with different superscripts differ ($P < 0.05$)

ers fed TSP2 exhibited increased ($P < 0.05$) ileal *Lactobacillus* but reduced *Escherichia coli* counts compared with CON and ANT diets. Moreover, TSP1 and TSP2 diets exhibited increased caecal *Lactobacilus* and *Bifidobacteria* but diminished *Escherichia coli* and *Clostridium perfringens* counts compared with CON diets ($P < 0.05$). The excreta NH_3 was reduced ($P < 0.05$) by TSP2 supplemen-

Table 5. Effects of *Bacillus subtilis*, *Clostridium butyricum* and *Lactobacillus acidophilus* endospores on *Clostridium perfringens* on ileal and caecal microbial shedding in broilers

Item (log_{10} CFU/g)	Treatment					SE[1]	*P*-values		ANT vs. TSP
	CON	ANT1	ANT2	TSP1	TSP2		linear	quadratic	
Ileum									
Lactobacillus	8.55[b]	8.65[b]	8.62[b]	8.83[a]	8.91[a]	0.09	< 0.01	< 0.01	0.03
Escherichia coli	7.79[a]	7.51[b]	7.55[b]	7.41[b]	7.17[c]	0.07	< 0.01	0.03	0.31
Clostridium perfringens	7.64	7.55	7.52	7.41	7.35	0.39	0.44	0.75	0.25
Bifidobacteria	8.37	8.39	8.59	8.71	8.81	0.30	0.11	0.56	0.51
Caecum									
Lactobacillus	9.03[c]	9.47[bc]	9.25[c]	9.71[a]	9.76[a]	0.15	< 0.01	0.03	0.04
Escherichia coli	8.11[a]	7.87[ab]	7.89[ab]	7.67[b]	7.55[b]	0.11	0.01	0.01	0.41
Clostridium perfringens	8.15[a]	7.98[a]	7.95[a]	7.07[b]	7.15[b]	0.25	0.72	0.04	0.61
Bifidobacteria	8.45[b]	8.50[b]	8.61[b]	8.82[a]	8.97[a]	0.09	0.02	0.25	0.55

CON = antibiotic free diet; ANT1 = CON + enramycin 5 ppm; ANT2 = CON + avilamycin 5 ppm; TSP1 = CON + 0.1% TSP; TSP2 = CON + 0.2% TSP

[1]pooled SE

[a,b,c]means in the same row with different superscripts differ ($P < 0.05$)

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Table 6. Effects of *Bacillus subtilis, Clostridium butyricum* and *Lactobacillus acidophilus* endospores on excreta noxious gas emission in broilers

Item	Treatment					SE[1]	*P*-values		ANT vs. TSP
	CON	ANT1	ANT2	TSP1	TSP2		linear	quadratic	
NH_3 (ppm)									
Day 1	36[a]	34[b]	32[ab]	29[b]	30[b]	0.89	< 0.01	0.01	0.41
Day 3	55[a]	56[a]	54[a]	47[b]	49[b]	0.95	0.03	0.07	0.01
Day 5	80[a]	76[ab]	77[ab]	74[b]	70[c]	1.39	0.03	0.26	0.21
H_2S (ppm)									
Day 1	1.7	1.6	1.8	1.5	1.7	0.31	0.60	0.86	0.41
Day 3	2.2	2.4	2.3	2.0	2.1	0.20	0.31	0.63	0.32
Day 5	3.7	3.6	4.0	3.8	3.5	0.42	0.67	0.33	0.68
VFA (μmol/N)									
Propionic acid	45.9	40.1	43.8	45.9	45.2	2.51	0.43	0.66	0.45
Butyrate	24.5	23.1	21.8	23.2	22.0	1.60	0.31	0.74	0.97

CON = antibiotic free diet; ANT1 = CON + enramycin 5 ppm; ANT2 = CON + avilamycin 5 ppm; TSP1 = CON + 0.1% TSP; TSP2 = CON + 0.2% TSP

[1]pooled SE

[a,b,c]means in the same row with different superscripts differ ($P < 0.05$)

tation compared with the CON (Table 6). No significant differences were observed in H_2S and VFA concentrations among the treatments.

DISCUSSION

In the experiments described here, BWG, DM and N digestibility were increased and FCR was reduced; however, dietary supplementation of TSP had no effect on the FI. In agreement with our results, Zhang and Kim (2014) reported that dietary supplementation with 1×10^5 CFU or 2×10^5 CFU/kg of multistrain probiotic significantly improved BWG and reduced FCR in broiler chickens compared with chickens from the control treatment. Likewise, Sinol et al. (2012) reported that inclusion of *Bacillus subtilis* C-3102 in broiler chicken diets resulted in improved FCR (21–42 days) and weight gain (42 days). Zhang et al. (2013) found that BWG was increased by the administration of 10^5 and 10^8 CFU/kg of *Bacillus*-based probiotic. Yang et al. (2012) found that adding 2×10^7 CFU or 3×10^7 CFU/kg of *Clostridium butyricum* to the diet improved the ADG in broiler chickens. Similar positive effects were also reported by other researchers (Talebi et al. 2008; Zhou et al. 2010). However, it has also been reported that probiotics exerted only a minimal effect on growth performance in broiler chickens (Lee et al. 2010; Sinol et al. 2012). The inconsistency might be attributed to the strains of probiotic, technique of preparation, administration dosage, diet composition, bird age and hygienic status (Sinol et al. 2012; Zhang et al. 2012).

Improvement in growth performance and feed efficiency of broiler chickens supplemented with different strains of probiotics (Sinol et al. 2012; Zhang and Kim 2014) are thought to be induced by the cumulative effect of probiotic action including the improvement of feed intake and digestion (Shim et al. 2010), increased digestive enzyme activity and decreased NH_3 production (Chen et al. 2013), maintenance of beneficial microbial populations (Chen et al. 2013) and alteration of bacterial metabolism (Sinol et al. 2012). In this study, DM and N digestibility were increased by dietary supplementation of TSP. The underlying mechanism is related to the fact that increased villus height and villus height-to-crypt depth ratio are directly correlated with an increased epithelial turnover (Sinol et al. 2012), and longer villi are linked with activation of cell mitosis (Samanya and Yamauchi 2002). Shortening of villi and deeper crypts lead to poor nutrient absorption, increased secretion in gastrointestinal tract and reduced performance (Xu et al. 2003). Intestinal morphology including duodenal and ileal villus height and crypt depth and villus height-to-crypt depth ratio are indicative

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of gut health in broiler chickens. Sen et al. (2012) reported that supplementation of *Bacillus subtilis* LS 1-2 in broiler chicken diets resulted in increased villus height and villus height-to-crypt depth ratio in duodenum and ileum at Day 35. Thus, in the present study, improved growth performance in birds fed *Bacillus subtilis* DSM 17299, *Clostridium butyricum* and *Lactobacillus acidophilus* endospores might be due to greater nutrient retention and improved gut health.

In the current study, the inclusion of tri-strain probiotics increased relative weight bursa to the BW compared to the control group. Chen et al. (2013) suggested measuring immune organ weight as a method for evaluating immune status in chickens. Willis et al. (2007) also suggested that the bursa was the primary lymphoid organ in broiler chickens and concluded that probiotics could increase the relative weight of bursa to BW. The colour of broiler chicken meat is important because consumers regard it as a sign of a fresh and high quality product. Froning (1995) had recommended that lower L^* value, showing the level of paleness, could reduce the broiler chicken consumption. Chen et al. (2013) also suggested that broiler chicken size could affect the sensory attributes of breast meat. In this study, broiler chickens that were given probiotics had reduced L^* values compared to those that were given the non-probiotic treatment. Chen et al. (2013) also found similar results after supplementation of probiotics in broiler chickens. In the present study, we hypothesise that the difference in L^* values may be due to the growth rate in broiler chickens. Collectively, our results suggest that the inclusion of probiotics could reduce the lightness value of the breast muscle in broiler chickens and subsequently increase their market price.

Probiotics beneficially affect the host animal by improving intestinal balance (Chen et al. 2013), creating gut micro-ecological conditions that suppress harmful microorganisms like *Clostridium* and *Coliforms* (Shim et al. 2010; Sinol et al. 2012) and by favouring beneficial microorganisms like *Lactobacillus* and *Bifidobacterium*. In our study, the dietary supplementation of *Bacillus subtilis* DSM 17299, *Clostridium butyricum* and *Lactobacillus acidophilus* endospores increased gut *Lactobacillus* and *Bifidobacteria* counts and decreased *Escherichia coli* and *Clostridium perfringens* counts compared with those of other treatments. Suppression of harmful organisms resulted in higher growth and metabolism of beneficial microorganism which might have improved the performance and nutrient retention in the present study. A number of previous studies have demonstrated the potential of probiotics to enhance the growth of beneficial bacteria and suppress potentially pathogenic bacteria in the intestine (Sinol et al. 2012; Zhang and Kim 2013). Ng et al. (2009) suggested that probiotics may influence the intestinal microflora by facilitating antibody production, promoting epithelial barrier integrity, augmenting toll-like receptor signalling and through other mechanisms. Thus, we can confirm the effect of TSP on gut microflora in broiler chickens.

Ammonia is a major aerial pollutant originating from farm animal operations and poultry is one of the principal contributors among domestic animals (Zhang et al. 2013). High concentrations of NH_3 or H_2S can cause hazardous effects to humans and animals (Drummond et al. 1980; Zhang and Kim 2014). It has been suggested that excreta ammonia gas emission is related to nutrient utilisation and the intestinal microbial ecosystem (Ferket et al. 2002). In our study, NH_3 gas emission was reduced by addition of *Bacillus subtilis* DSM 17299, *Clostridium butyricum* and *Lactobacillus acidophilus* to the diet. In agreement with our results, Zhang et al. (2013) found that dietary supplementation with *Bacillus subtilis* UBT-MO2 resulted in 26.5% and 37.9% lower excreta NH_3 concentrations compared with no supplementation. Moreover, Zhang and Kim (2013) also reported that the dietary application of probiotics could reduce the NH_3 contents in the excreta. Accordingly, the reduction of excreta NH_3 may be considered as a manifestation of improved nutrient digestibility and an enhanced gut micro-ecological condition in the current study.

In conclusion, the inclusion of TSP might improve growth performance and nutrient digestibility. Broiler chickens fed with TSP may exhibit increased relative bursa weight and reduced meat lightness. Under the conditions of our study, TSP supplementation could enhance gut health and reduce the noxious toxic gas emission in broiler chickens.

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