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Comparison of two probiotic preparations on growth performance, intestinal microbiota, nutrient digestibility and cytokine gene expression in broiler chickens

Mojtaba Zaghari, Parisa Sarani and Hosna Hajati

Department of Animal Science, College of Agriculture and Natural Resources, University of Tehran, Karaj, Iran

ABSTRACT

This study was done to evaluate the effects of two dietary probiotic preparations (*Bacillus subtilis* and *Bacillus licheniformis*) on growth performance, intestinal microbiota, nutrient digestibility and cytokine gene expression in broiler chickens. A total of 180 male broiler chicks (one-day-old Ross 308 strain, average initial body weight = 40.05 ± 0.12) were used in a completely randomized design (CRD) composed of 3 treatments and 6 replicates. Experimental diets included: (1) basal diet (without additive), (2) basal diet plus 0.5 g/kg diet *B. subtilis* preparation (1×10^9 CFU/g), (3) basal diet plus 0.5 g/kg diet *B. licheniformis* preparation (1×10^9 CFU/g). The results showed that supplementation of *B. licheniformis* improved ($P < 0.05$) broilers' body weight gain (BWG), feed conversion ratio (FCR), and production efficiency factor (PEF). Adding *B. licheniformis* caused the lowest ($P < 0.01$) feed cost per kilogram weight gain and the highest ($P < 0.05$) return of investment (ROI). Probiotic treatments significantly decreased ($P < 0.01$) ileal pH of the broilers. Probiotic treatments improved ($P < 0.01$) apparent metabolizable energy (AME) and total tract digestibility of protein. It was concluded that although both probiotic bacteria improved AME and total tract protein digestibility, *B. licheniformis* was superior to *B. subtilis* in improving broiler chickens' BWG (2580.70 vs. 2427.45 g) and their PEF (418.95 vs. 374.49).

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Bacillus subtilis; *Bacillus licheniformis*; probiotics; gene expression

Introduction

Regarding critical problems of using antibiotics in chicken diets, such as antibiotic resistance and residue in meat and eggs, nowadays, poultry nutritionists are searching to find functional dietary additives as antibiotic alternatives to improve birds' growth performance and immunity and prevent necrotic enteritis and other diseases (Khan and Naz 2013; Yadav et al. 2016). It is well known that probiotics are nonpathogenic living microorganisms which can promote the health status of the consumer if they are consumed in an appropriate amount (FAO/WHO 2002). Probiotic microorganisms must have the ability to adhere to epithelial cells and tolerate the gastrointestinal secretions, such as digestive enzymes, bile, and gastric acid (Khan and Naz 2013). It was reported that probiotics can promote the immune responses and decrease the population of pathogenic microorganisms in the body of poultry (Ubeda and Pamer 2012; Vieira et al. 2013). Attia et al. (2011) reported that probiotic improved the growth of broiler chickens and their immune responses and livability. Also, it was shown that probiotics have beneficial effects on energy and protein utilization in poultry (Attia et al. 2011; Houshmand et al. 2012; Ayasan, 2013). These days, many species of *Bacillus* bacteria are used as probiotic supplements in animal diets. *Bacillus* species are facultative anaerobe gram-positive bacteria that have endospores (Cutting, 2011). The characteristic of spore production by these bacteria is an advantage for them, which allows the bacteria to maintain their livability for a long time under high temperature or pressure than non-producing spore bacteria. Also, they can tolerate the hard and acidic environment of the stomach and reach safely to the end of the digestive system (Cutting 2011). *Bacillus* spp. have the ability to produce different antioxidants (Latorre et al. 2017), and probiotic supplementation can help to reduce oxidative stress in broilers (Shah et al. 2019a). In a study conducted by Khan et al. (2014b), adding probiotic (Protexin®, Hilton Pharma, Holland) to Hubbard broiler breeders' diet increased the level of paraoxonase (a member of anti-oxidant defence system) in serum of the birds. In addition, *Bacillus* spp. can produce antimicrobials (Urdaci et al. 2004), such as bacteriocins and bacteriocin like inhibitory materials (e.g. Subtilin, Coagulin), and antimicrobial substances, which contain a high amount of peptides and polyketides e.g. Bacilysin, Difficidin, Macrolactin, and Macrolactin (Belih et al. 2015). Also, *Bacillus* spp. have the potential to improve immune response and they can be considered as probiotic microorganisms (Lee et al. 2010a, 2010b). Some of *Bacillus* species, which are used as probiotics in humans and animals, include *Bacillus coagulans, Bacillus clausii, Bacillus cereus, B. Subtilis, and B. licheniformis* (Cutting 2011; Inci and Ayasan, 2019). Upadhaya et al. (2019) reported that *B. subtilis* supplementation improved BW and FCR of broiler chickens during grower and finisher periods. The term *licheniformis* means the ability of producing *Lichenicidine*. The

CONTACT Hosna Hajati h.hajati@ut.ac.ir Department of Animal Science, College of Agriculture and Natural Resources, University of Tehran, Karaj PO Box 3158777871, Iran

Lichenicidine is a two-peptide lantibiotic, which has antimicrobial effects against all *Listeria monocytogenes*, methicillin-resistant *Staphylococcus aureus*, and vancomycin-resistant *enterococcus* strains, and has been noticed by some lantibiotic producers. A few investigations have been done on the effects of *B. licheniformis* in poultry, and little information is available about the effect of *B. licheniformis* on nutrient metabolism in chickens (Liu et al. 2012). Adding *B. licheniformis* spores to diet as probiotic improved the performance of broiler chickens challenged with necrotic enteritis under commercial-like condition (Knap et al. 2010). Also, another probiotic bacterium, *B. subtilis,* was used in broilers' diet by some researchers (Lee et al. 2010b; Lee et al. 2015) to evaluate the effect of *B. subtilis* on immunity and *Eimeria* infection in broilers. This bacterium inhibited pathogenic bacteria proliferation, helped gut integrity, and improved the performance of broilers challenged with *Eimeria* spp. and *Clostridium perfringens* (Lee et al. 2010b; Lee et al. 2015). Bai et al. (2016) reported that supplementation of *B. subtilis* ($2–4 \times 10^{10}$ CFU/kg) improved the average of daily gain, average of daily feed intake (ADFI), and FCR of broiler chickens during the whole period of the experiment (1–42 d). Preparation of proper probiotic microorganism as a growth promoter additive is not an easy task and it needs extensive inquiries. So, before choosing special strains of bacteria as probiotics, in vivo trails are necessary to determine their advantages or disadvantages for the host animal. Hence, the aim of the present study was to compare the effects of *B. subtilis* (1×10^9 CFU/g) and *B. licheniformis* (1×10^9 CFU/g) on growth performance, intestinal microbiota, nutrient digestibility, IL6, IL10, or interferon gamma gene expression in broiler chickens.

Materials and methods

Broiler chickens and house management

A total of 180-day-old male broiler chicks (Ross 308) were purchased from a local hatchery (Morghdasht Hatchery, Karaj, Iran). The birds were weighed (average initial weight = 40.05 ± 0.12) and divided into 18 litter pens (1m× 0.67 m) in a completely randomized design with three treatments, 6 replicates, and 10 birds per replicate for 4 wk. The pens had wood shavings as bedding on a concrete floor. At the end of the fourth week, 60 male broiler chicks transferred to individual metabolic cages to measure the digestibility of nutrients. The birds were raised in accordance with the guidelines of the Institutional Animal Care and Ethics Committee of the Iranian Council of Animal Care (Care ICoA, 1995). The initial brooding temperature was set at 32 ± 1°C during the first week of age, then gradually reduced 3°C weekly to reach 22°C, the final temperature was kept until the end of the experiment. The relative humidity was kept around 55–60% during the whole period of the experiment. Cross-ventilation was run at this study, and the lighting programme was as 23 h light: 1 h dark.

Experimental diets

The experimental diets were formulated according to Ross 308 nutrient specifications (2014, with some modification according to Zaghari et al. 2017) during starter (1–10 d), grower (11–24 d), and finisher (25–44 d) periods (Table 1). The main ingredients of the diets were corn and soybean meal. Dietary treatments included: 1 – basal diet (without additive), 2 – basal diet plus 0.5 g/kg diet *B. subtilis* (1×10^9 CFU/g), 3 – basal diet plus 0.5 g/kg diet *B. licheniformis* (1×10^9 CFU/g). All diets were given in mash form and prepared weekly. Each probiotic preparation was first mixed with 1 kg of the experimental diet, then mixed with the whole amount of feed. The feed of each experimental unit was kept in a separate bucket. Feed and water was provided ad libitum throughout the experimental period (1–44 d). The probiotic strains used in the present study were provided from a commercial company (Biorun Company, Tehran, Iran). The probiotic preparations were single-strain probiotic composed of spore-forming bacteria including *B. subtilis CH201* at 1×10^9 CFU/g, and *B. licheniformis CH200* at 1×10^9 CFU/g. At the end of the experiment, 3 samples of each diet used in different period of rearing were examined to see the livability of *B. subtilis* and *B. licheniformis* in those diets. Diet samples were weighed and 1:4 wt/vol dilutions were used with sterile 0.9% saline. Then, 10-fold dilutions of each were prepared in a sterile 96-well Bacti flat-bottom plate. The bacteria livability was studied by using two culture medium, nutrient agar, and brain heart infusion agar. The plates were incubated at 37°C for 18 h and bacterial counts were expressed as Log_{10}

Table 1. Ingredients and nutrient composition of basal diets[a].

Item	Starter (1–10d)	Grower (11–24d)	Finisher (25–44d)
Ingredient (%)			
Corn	50.84	54.86	59.82
Soybean meal	41.86	37.93	32.73
Corn oil	3.09	3.40	3.96
Dicalcium Phosphate	1.72	1.53	1.35
Calcium carbonate	1.07	0.98	0.90
Salt	0.25	0.25	0.24
Sodium bicarbonate	0.15	0.15	0.16
Vitamin–mineral premix[b]	0.25	0.25	0.25
Mineral premix[c]	0.25	0.25	0.25
DL-methionine	0.24	0.20	0.18
L-lysine HCl	0.16	0.11	0.10
L-Threonine	0.09	0.05	0.03
Calculated Nutrient content			
AME (Kcal/Kg)	2900	3000	3100
Crude protein %	22.23	20.81	18.89
Available phosphorus %	0.46	0.42	0.38
Calcium %	0.93	0.84	0.76
Na %	0.15	0.15	0.15
DCAB meq/Kg	256	244	221
Lys (dig) %	1.24	1.11	0.99
Met (dig) %	0.55	0.49	0.45
Met + Cys (dig) %	0.92	0.84	0.77
Thr (dig) %	0.83	0.75	0.66
Analyses Nutrient content			
Dry matter (DM, %)	89.4	89.4	89.3
Crude protein %	22.20	20.78	18.88
Crud fat %	4.88	5.18	5.79
Crude fibre %	4.11	3.90	3.65

[a]The bacterial strain was added at the starter, grower and finisher basal diets at 0.5 g/kg diet.

[b]Vitamin premix supplied the followings per kg of diet: vitamin A, 9000 IU; vitamin D_3, 2000 IU; vitamin E, 36 mg; vitamin K_3, 2 mg; vitamin B_1, 1.75 mg; vitamin B_2, 6.6 mg; vitamin B_6, 2.94 mg;
vitamin B_{12}, 0.015 mg; nicotinic acid, 29.7 mg; folic acid, 1 mg.

[c]Mineral premix supplied the followings per kg of diet: calcium pantothenate, 9.8 mg; choline chloride, 250 mg; Mn, 99.2 mg; Zn, 84.7 mg; Cu, 10 mg; Fe, 50 mg; Se, 0.2 mg; I, 0.99 mg.

Table 2. Presence of the bacteria in the feed at the end of the experiment (44 d).

	Culture medium	
	Nutrient agar	Brain heart agar
Bacillus subtilis (CFU/g)	4×10^7	1.13×10^7
Bacillus licheniformis (CFU/g)	1.29×10^9	6.2×10^8

CFU/g of tissue (Habib et al. 2015). The results of evaluating the livability of probiotic strains in diets showed that *B. licheniformis* had higher livability than *B. subtilis* (Table 2).

Growth performance

Growth performance parameters, such as BW, BWG, feed intake (FI), were measured for the birds in each pen, weekly. After adjusting for mortality, adjusted FCR of each pen was also recorded weekly. In addition, the PEF was calculated for the entire duration of the experiment based on the following formula: PEF = [live ability (%) ×live weight (kg)]/ [age (d) × FCR] × 100 according to Marcu et al. (2013).

Economic indicators

The feed cost per kilogram weight gain was calculated for the whole period of the experiment, as multiplying FCR by the average cost of the diets. Return on investment (ROI) was used to compare speculation's benefit in the experimental groups. For the calculation of ROI the benefit of an investment was divided by the cost of the investment (Zaghari et al. 2017).

Relative weight of digestive organs

At 44 d, 20 birds from each experimental group were selected according to the average BW within the group, weighted individually, and killed by cervical dislocation. Then, liver, proventriculus, and gizzard were separated from the carcass. The gallbladder was separated from the liver and the content of proventriculus and gizzard removed, then the liver, proventriculus, and gizzard were weighed. The organ weights were expressed as a percentage of live BW.

Measurement of gastrointestinal tract pH

At 44 d, just after GIT removal from 20 birds per treatment, the pH of the proventriculus, gizzard, ileum, and cecum contents was measured by a pH-meter (Testo 206 pH-meter, Germany). The pH of proventriculus and gizzard was measured by placing the electrode of the pH-meter in an incision that was made in the centre of the organ. For measuring the pH of ileum digest content, the electrode of the pH-meter was placed in an incision that was made 4 cm proximal to the ileocecal junction. For measuring the pH of ceca, both ceca were opened and their pH was measured (Teuchert 2014).

Ileal and cecal microflora

After slaughter at 44 d, the contents of ileum and ceca were collected immediately in sterile glass containers. Conventional microbiological techniques using selective agar media were used for the microbial analysis. One gram of the sample was taken into sterile test tubes and diluted 1:10 in 9 ml Ringer's diluent (pH 6.8~7.2) and homogenized. Then, 1 mL from dilutions was spread on appropriate selective agar media for enumeration of total aerobic bacteria, *Lactobacillus*, *Yeast*, and *Mold, Enterobacteriaceae, Coliform, Clostridium perfringens*. Bacterial colonies were counted by the pour plate method. Selective agar media were used for enumeration of *Lactobacillus* bacteria (MRS agar, Merck, Darmstadt, Germany), total aerobic bacteria (TSA agar, Merck, Darmstadt, Germany), Yeast and Mold (YGC agar, Merck, Darmstadt, Germany), *Enterobacteriaceae* (VRBD agar, Merck, Darmstadt, Germany), *Coliform bacteria* (VRBD agar, Merck, Darmstadt, Germany), and *Clostridium perfringens* (SIA agar, Merck, Darmstadt, Germany). The average number of colonies was multiplied by the reciprocal of the dilution factor and expressed as CFU/g of the content (Torlak et al. 2013; Menconi et al. 2014).

Total tract and ileal nutrient digestibility

At d 29, in order to investigate the nutrient digestibility, 60 male broilers with similar average BW were divided into individual metabolic cages with 0.15 m^2 area. Measurement of apparent digestibility of nutrients was done by collecting both total excreta and ileal digesta. Apparent digestibility coefficients of nutrients were determined by using acid insoluble ash (AIA, Celit 281, Merck, Darmstadt, Germany) as an analytical marker. All experimental diets were mixed with 1% AIA for 2 d before collecting excreta. During the 2 d collection period, excreta from each cage were collected 2 times daily. Feed residue, feathers, and scales in the excreta trays were carefully removed and stored in sealed bags at −20°C. At the end of the 2 d collection period, all birds were killed by cervical dislocation. The carcasses were subsequently opened and the entire gastrointestinal (GI) tract was exposed carefully. The ileum was ligated and then separated from the rest of the GI tract and subsequently the ileal content was immediately stored in sealed bags at −20°C. After drying for 72 h at 50°C in an oven, the feed, excreta, and ileal samples were finely ground and analysed for crude protein (CP), gross energy (GE), calcium (Ca), and phosphorus (P). All analyses were performed by analysing each sample in triplicate. Determination of CP was performed by the AOAC method (AOAC, International. 2000. Official Methods of Analysis of AOAC International. 16th ed. AOAC International, Gaithersburg, MD) using an auto analyzer (KJETEK auto 1030 analyzer unit). The GE was also measured by adiabatic bomb calorimeter (IKA-Calorimeter C400). Total P was determined by a spectrophotometer (Envisense UV-2100 spectrophotometer) at the wavelength of 400 nm, and Ca was measured by an atomic absorption spectrophotometer (Shimadzu AA-670) at the wavelength of 422 nm. After determining the amount of nutrients and marker in feed samples, excreta, and ileal sample, apparent nutrient digestibility coefficients and AME (Kcal/ kg) were calculated using the following formulas (Stefanello et al., 2016).

$$\text{Digestibility (\%)} = 100 - [100 \times (M_i \times E_o / M_o \times E_i)]$$

$$\text{AME (Kcal/ kg)} = GE_i - [GE_o \times (M_i / M_o)]$$

M_i: Concentration of acid insoluble ash in the diet
M_o: Concentration of acid insoluble ash in the excreta and ileal digesta
E_i: Concentration of CP, GE, Ca, P in the diet
E_o: Concentration of CP, Ca, P in the excreta and ileal digesta and GE in the ileal digesta
GE_i: GE (Kcal/kg) in the diet
GE_o: GE (Kcal/kg) in the excreta

Isolation of RNA, reverse transcription, and real-time PCR

At 42 d, blood samples were collected from the brachial vein from 12 birds in each treatment. The samples were collected into Eppendorf tubes with ethylene-diamine-tetra-acetic acid (EDTA) and were centrifuged, and their plasmas were stored at −80°C until analysis. Total RNA was extracted from blood plasma samples using Trizol (GeneAll, South Korea), and alcoholic sediment RNA samples were treated with DNase I (Fermentase, USA) to remove contaminating genomic DNA. The concentration of RNA was determined using a Nano-drop 2000 spectrophotometer (Thermo Scientific, USA). The purity of RNA was verified at an optical density ratio of 260–280 nm. Two microgram of RNA was reverse-transcribed to cDNA in the presence of reverse transcription enzyme (Thermo, USA) using the Oligo-dT and random primers. The primers included interleukin 10 (IL10), interleukin 6 (IL6), Interferon gamma (INF-y), and glyceraldehyde 3 phosphate dehydrogenase (GAPDH) as internal control. Their sequence was found in national centre of biotechnological database (NCBI). Real-time quantitative polymerase chain reaction (qRT-PCR) was performed in triplicate reaction using both forward and reverse primers, cDNA, SYBR Green (Takara, Japan). The qRT-PCR was performed using step one thermal cycler (96Grad, PeQlab). During the PCR reaction, samples were subjected to an initial denaturation phase at 94°C for 30 secs, followed by 40 cycles of denaturation at 95°C for 3 secs and annealing and extension at 60°C for 30 sec. The sequences of the primers used in the present study are shown in Table 3. The results from qRT-PCR were analysed using the step one real-time PCR (ABI, USA). Average gene expression relative to the GAPDH, as endogenous control for each sample, was calculated using the $2^{-\Delta\Delta Ct}$ method, where ΔΔCT was calculated by the following formula: ΔΔCT = Ct target gene − Ct GAPDH (Livak and Schmittgen, 2001).

Bacterial penetration into the blood

Blood samples collected from 12 birds per treatment at 42 d were used to investigate the probiotic bacteria penetration into the blood. About 0.1 CC of blood was cultured on blood agar culture medium, then the plates were incubated at 35 ± 2°C under aerobic conditions for 24–48 h, then the presence of bacteria on the medium was evaluated (Brouillette et al. 2004).

Statistical model and analysis

The statistical design was completely randomized design using the following model: $X_{ij} = \mu + Ti + eij$; where X_{ij} is the observation, μ is the overall mean, Ti is the effect of the treatment, (i = 5), and eij is the residual error. The data of the present study were analysed by the GLM procedure of SAS software (SAS 2004). Variation of the data was shown as standard error of means (SEM). Differences among treatments were analysed by Duncan's multiple range tests. Probability values as $P < 0.05$ were considered significant. Also, microbial loads were analysed after $\log_{10}$ transforming.

Results and disscussion

Growth performance

Supplementation of *B. licheniformis* increased ($P < 0.05$) FCR of broiler chicks during the first week of rearing. Also, adding dietary *B. licheniformis* improved ($P < 0.05$) BWG, FCR of the broilers during the fifth week. The PEF of the broilers fed with *B. licheniformis* was higher ($P < 0.05$) than other groups in the whole period of rearing (Table 4). However, the FI of the broilers in different experimental group was not different ($P>0.05$).

It is well known that gut health is a key point for animal performance due to its critical importance on nutrient digestion, absorption and metabolism, incidence of enteric diseases, and immune responses (Yegani and Korver 2008; Hamasalim 2016). Previous studies have shown that probiotics improved broilers' growth performance and promoted greater populations of symbiotic microbiota in the intestine of broilers (Mountzouris et al. 2007; Latorre et al. 2017; Rhayat et al. 2017). In a recent study, Shah et al. (2019b) found that supplementation of *Lactobacillus* bacteria increased intestinal villus' height and absorptive capacity in broiler chickens that led to higher final BW of the birds. In our study, adding dietary *B. licheniformis CH200*, improved BWG, FCR, and PEF with no effect on FI during the whole period of the experiment

Table 3. Chicken GAPDH and cytokine primer sequences.

Gene[a]	Primer sequence[b] (5`-3`)	Tm	Ref	Amplicon
IL-10	F:CGGGAGCTGAGGGTGAA	58	NM_001004414.2	272
	R:GTGAAGAAGCGGTGACAGC	60		
IFN-γ	F: CAACTTGTTTGTTCTGTCTGTCATC	60	NM_205149.1	185
	R:TTCTCATTTCTCTCTGTCCAGTTCT	60		
IL6	F:CCAGAAATCCCTCCTCGCCAATC	60	NM_204628.1	110
	R:CCCTCACGGTCTTCTCCATAAACG	60		
GAPDH	F:CTTTGGCATTGTGGAGGGTC	60	NM_204305.1	128
	R:ACGCTGGGATGATGTTCTGG	60		

[a]GAPDH = Glyceraldehyde-3-phosphate dehydrogenase; IL = interleukin; IFN = interferon.
[b]F = Forward primer; R = Reverse primer.

Table 4. Effect of *B. subtilis* and *B. licheniformis* on body weight gain, feed intake, and feed conversion ratio of broilers.

	Treatment[A]				
	C	B.S	B.L	SEM[1]	*P*-Value
First week					
BWG (g/bird)	122.06	116.65	116.30	2.041	0.114
FI (g/bird)	143.83[ab]	138.65[b]	149.25[a]	1.93	0.005
FCR (g/g)	1.17[b]	1.18[b]	1.28[a]	0.02	0.013
Second week					
BWG (g/bird)	212.30	216.01	216.13	4.99	0.828
FI (g/bird)	304.00	296.68	296.48	10.03	0.835
FCR (g/g)	1.42	1.36	1.37	0.042	0.584
Third week					
BWG (g/bird)	297.30	297.21	303.60	5.78	0.676
FI (g/bird)	441.70	430.68	437.33	4.72	0.281
FCR (g/g)	1.48	1.44	1.43	0.029	0.498
Forth week					
BWG (g/bird)	421.77	417.65	450.40	10.42	0.0843
FI (g/bird)	736.20	721.68	765.72	12.16	0.0605
FCR (g/g)	1.74	1.72	1.70	0.049	0.847
Fifth week					
BWG (g/bird)	546.50[b]	553[b]	644.90[a]	19.40	0.0025
FI (g/bird)	989.75	940.75	989.15	24.963	0.289
FCR (g/g)	1.77[a]	1.70[a]	1.53[b]	0.035	0.0001
Sixth week					
BWG (g/bird)	614.05	619.90	653.90	11.04	0.2866
FI (g/bird)	1122.75	1108.15	1141.25	15.02	0.3032
FCR (g/g)	1.86	1.80	1.75	0.048	0.302
Whole period					
BWG (g/bird)	2427.45[b]	2407.20[b]	2580.70[a]	51.81	0.0420
FI (g/bird)	3735.61	3641.68	3768.02	43.50	0.1121
FCR (g/g)	1.54[a]	1.51[ab]	1.46[b]	0.020	0.0207
PEF[2]	374.49[b]	377.51[b]	418.95[a]	12.487	0.0246

[a-b]Means within rows with no common letters are significantly different at $P < 0.05$.
[A]C, Control; *B.S, Bacillus subtilis; B.L, Bacillus licheniformis*.
[1]Standard error of means.
[2]production efficiency factor.

Table 5. Effect of *B. subtilis* and *B. licheniformis* on the feed cost per kilogram weight gain and return on investment (1–44 d).

Treatment[A]	Feed cost per kg gain ($)	Return on investment (ROI)
C	0.48[b]	1.24[b]
B.S	0.64[a]	1.26[b]
B.L	0.45[c]	1.36[a]
SEM[1]	0.004	0.033
P-Value	0.0001	0.0238

[a-b-c]Means within a column with no common letters are significantly different at $P < 0.05$.
[A]C, Control; *B.S, Bacillus subtilis; B.L, Bacillus licheniformis*.
[1]Standard error of means.

(1–44 d). However, *B. subtilis CH201* had no effect on birds' growth performance traits. This is in agreement with the results of Abudabos et al. (2017) who reported that dietary *B. subtilis* did not change BW and FCR of broiler chickens compared with the control group. In contrast to our results, Jayaraman et al. (2017) reported that using *B. subtilis PB6* improved BW and FCR of broiler chickens. The reasons for the inconsistencies may be due to the broilers' strain, house condition, probiotic livability, or administration dosage. In agreement with our result, Liu et al. (2012) reported that *B. licheniformis* improved BWG and FCR in broiler chickens. Hong et al. (2005) found that *Bacillus* bacteria improved immune function and increased the level of endogenous antimicrobial peptides in the gut, which led to higher growth performance. Gerard et al. (2008) reported that adding different species of *Lactobacillus* could stimulate gut-associated immunity and enhanced the growth performance of broiler chickens. Zhang et al. (2012, 2013) found that the BWG was increased by the administration of a *Bacillus*-based probiotic (10^5 or 10^8 CFU/kg) in broilers' diet. It is clear that FCR is an important parameter which can influence the economic profits of poultry farms, so improving birds' FCR by adding dietary probiotic is a valuable finding which may lead to the reduction of the feed cost per kg meat production, parallel to get the expected weight gain or even higher (Jahromi et al. 2016). Additional studies have demonstrated the potential of *B. licheniformis* in improving the broilers' FCR (Mountzouris et al. 2007; Awad et al. 2009; Karimi Torshizi et al. 2010). Yeo and Kim (Yeo and kim 1997) suggested that the mechanisms by which probiotics improve FCR can include changing the intestinal microbiota, promoting the growth of nonpathogenic bacteria with the ability of producing lactic acid and hydrogen peroxide, decreasing the growth of gut harmful microbiota, or increasing nutrient digestion and utilization in the body of the probiotic consumer. In contrast, several other studies reported no changes in the performance of broilers that consumed probiotic (Knap et al. 2011; Shams Shargh et al. 2012; Afsharmanesh et al. 2013). There are many variables that can affect broilers' response to dietary additives. Some of these variables are the house environment, management practice, diet composition, strains of probiotics, technique of producing and administration, level of consumption, bird's strain and age, level of barn cleanliness, and persistence of strain in the bird (Mountzouris et al. 2007; Lee et al. 2010b; Molnar et al. 2011).

Economic indicators

The results showed that adding dietary *B. licheniformis* decreased ($P < 0.01$) average feed cost ($) per kg gain compared to other treatments (Table 5). Also, supplementation of *B. licheniformis* increased ($P < 0.05$) ROI.

There is some evidence that certain probiotic bacteria can improve the economic efficiency of broiler production. In a previous study, Panda (2006) reported that *Lactobacillus sporo* genes improved the net income and economic efficiency of broiler chickens compared to the control group. Bonsu et al. (2012) reported that a multi-strain probiotic (*Lactobacilli* 1×10^8 cfu/g, *Bacillus* 1×10^{12} cfu/g, and *Saccharomyces cerevisiae* 1×10^5 cfu/g) improved the economic benefit of Cobb hybrid broilers. They related this economic improvement to the positive influence of probiotic on the broilers' performance. Also, Jadhav et al. (2015) reported that using probiotic in poultry diet has several positive effects on the birds' gut health state and performance. Jadhav et al. (2015) concluded that probiotic supplementation is so useful for increasing economic efficiency of broiler production. In the present study, the economic profits improved by applying *B. licheniformis*. It decreased FCR of the broiler chickens that lead to lower feed cost per kg gain of the broiler chickens and it also increased ROI. However, *B. subtilis* increased feed cost per kg gain compared to other treatments and it did not change ROI compared to the control group. Zaghari et al. (2017) indicated that birds fed with *B. subtilis* had the lowest feed cost per kg gain which is not seen in the present study. This contrast in findings could be related to the difference in the rearing condition and level of probiotic supplementation.

Table 6. Effect of *B. subtilis* and *B. licheniformis* on the relative weight of digestive organs and gastrointestinal tract pH in broilers.

	Relative weight organ[1]			gastrointestinal tract pH			
Treatment[A]	proventriculus	gizzard	Liver	proventriculus	gizzard	ileum	ceca
C	0.356	1.522	1.796	3.116	2.802	6.964[a]	6.531
B.S	0.346	1.436	1.792	3.168	2.848	6.600[b]	6.461
B.L	0.339	1.411	1.847	3.109	2.879	6.297[b]	6.612
SEM[2]	0.011	0.035	0.051	0.128	0.113	0.127	0.059
P-Value	0.565	0.082	0.699	0.939	0.890	0.002	0.209

[a-b]Means for probiotic main effect within a column with different superscripts are different at $P < 0.05$.
[A]C, Control; *B.S, Bacillus subtilis; B.L, Bacillus licheniformis*.
[1]Expressed as percent of live weight.
[2]Standard error of means.

Relative digestive organ weight and pH gastrointestinal tract

The relative weights of proventriculus, liver, and gizzard were not affected ($P > 0.05$) by adding *B. subtilis* or *B. licheniformis* (Table 6). Supplementation of *B. subtilis* or *B. licheniformis* decreased ($P < 0.01$) ileal pH content of broiler chickens compared to the control group (Table 6).

The range of digestive tract pH must be proper for the probiotic activity and the probiotic should be stable in the digestive system (Svihus 2014). In the present study, adding probiotics decreased the pH of ileum in broiler chickens. It has been demonstrated that *B. subtilis* spores create an anaerobic environment in the gut which is helpful for the lactic acid producing bacteria (Hoa et al. 2000). The lactic acid bacteria produce lactic acid, reduce the intestinal pH, and prevent the growth of harmful bacteria (Spring et al. 2000). Thus, changing the pH may result in shifting intestinal microbiota profile (Ptak et al. 2015). Probiotics have the ability to produce digestible proteins, vitamin, digestive enzymes, such as amylase and protease. Also, they can reduce the intestinal pH by producing lactic acid which helps for nutrient digestion and absorption.

Ileal and cecal microflora

Adding dietary *B. subtilis* or *B. licheniformis* had not significant ($P > 0.05$) effect on the population of Total aerobic bacteria, *Lactobacillus*, *Coliforms*, *Enterobacteriaceae*, *Clostridium perfringens, Yeast* and *Mold* in the ileum or ceca of the broilers (Tables 7 and 8). In the present study, probiotic bacteria did not affect the ileal or cecal microbial population. Cengiz et al. (2015) reported that adding dietary probiotic (*Lactobacillus acidophilus, Lactobacillus casei, Enterococcus faecium, and Bifidobacterium thermophilus*) did not change the population of total aerobes, *Salmonella*, and *Lactobacilli* in gut. There are some reasons for this event such as lack of success of probiotics to: (1) survive from transiting through stomach to intestine, or (2) adhere physically and colonization on the gut surfaces (Jin et al. 1998). Regarding the results of the present study, it seems that under normal condition (healthy birds fed on corn-soybean meal diet), probiotics have little effect on the microbial population of the intestine.

Apparent ileal and total tract nutrient digestibility

The apparent ileal digestibility of CP, GE, Ca, and P was not affected by dietary *B. subtilis* or *B. licheniformis* ($P > 0.05$, Table 9). However, AME and total tract digestibility of CP were significantly higher ($P < 0.01$) in probiotic fed groups than those of the control birds.

It is interesting to note that probiotics may be considered as ' live enzyme factory' because of their potential in producing amylase, protease, and lipase (Dhama and Singh 2010). So, probiotics can enhance the digestion and absorption of carbohydrate, proteins, and lipids and thus contribute in improving the efficiency of feed consumed (Dhama and Singh 2010). Also, it was recognized that *Bacillus* spp. produced cellulase and xylanase (Kogut and Arsenault 2016).

In this study, adding *B. subtilis* or *B. licheniformis* to broilers' diet improved apparent digestibility of protein and ME compared to the control group. In agreement with our findings, a recent study has shown that supplementing *B. subtilis* improved total tract digestibility of DM, CP, and ME in broiler chickens (Reis et al. 2017). As mentioned before, the ileal pH of broilers fed with probiotics was lower than control birds which can help the absorption of protein and minerals like Copper, Ca, Iron, Manganese, and Magnesium (Raghuwanshi et al. 2015). Also, other researchers have confirmed that *B. subtilis* improved protein digestibility (Knap et al. 2011; Mahmoud et al. 2017). The positive effect on protein digestibility can be related to probiotics' mechanisms of action, which regulates the intestinal

Table 7. Effect of *B. subtilis* and *B. licheniformis* on microflora in the ileum of broilers (log CFU/g).

Treatment[A]	C	*B.S*	*B.L*	SEM[1]	*P*-Value
Lactobacillus	7.083	7.404	7.673	0.223	0.184
Total aerobic bacteria	6.358	6.220	6.845	0.209	0.603
Coliforms	5.389	5.911	5.845	0.488	0.713
Enterobacteriaceae	5.481	6.173	6.109	0.390	0.392
Clostridium perfringens.	2.268	2.046	2.113	0.261	0.819
Yeast and *Mold*	3.581	3.088	3.888	0.242	0.088

[A]C, Control; *B.S, Bacillus subtilis; B.L, Bacillus licheniformis*.
[1]Standard error of means.

Table 8. Effect of *B. subtilis* and *B. licheniformis* on ceca microflora in broilers (log CFU/g).

Treatment[A]	C	*B.S*	*B.L*	SEM[1]	*P*-value
Lactobacillus	8.339	8.313	8.355	0.223	0.990
Total aerobic bacteria	7.827	7.781	7.960	0.273	0.877
Coliforms	7.534	7.596	7.381	0.238	0.799
Enterobacteriaceae	7.604	7.924	7.785	0.184	0.458
Clostridium perfringens.	3.672	3.827	3.289	0.401	0.665
Yeast and *Mold*	3.846	4.425	4.281	0.273	0.331

[A]C, Control; *B.S, Bacillus subtilis; B.L, Bacillus licheniformis*.
[1]Standard error of means.

Table 9. Apparent Ileal and total tract nutrient digestibility in broilers.

Treatment[A]	C	B.S	B.L	SEM[1]	*P*-value
Ileal digestibility %					
CP	74.62	75.94	76.00	1.130	0.8300
GE	65.64	69.00	72.42	2.121	0.1325
Ca	19.28	20.72	27.63	1.130	0.3830
P	35.12	36.16	39.74	3.673	0.6607
Total tract digestibility %					
CP	56.70[b]	63.31[a]	60.58[a]	1.260	0.0030
Ca	20.88	20.94	29.10	2.447	0.0658
P	30.47	35.51	31.51	2.185	0.2762
AME (Kcal/kg)	2820.59[b]	2992.88[a]	2970.19[a]	21.153	0.0002

Note: Means within rows with no common letters are significantly different at $P < 0.05$.
[A]C, Control; *B.S, Bacillus subtilis; B.L, Bacillus licheniformis*.
[1]Standard error of means.

microbial population, reduces digestive disorders, inhibits intestinal harmful microorganisms, and improves the feed efficiency in body (Abaza et al. 2008). In addition, some researchers have reported that probiotics increased enzyme secretion from pancreas and intestinal mucosa (Chen et al. 2009). On the other hand, it was reported that using *B. subtilis* spore (GalliPro®) at 0.2 g/kg as a feed additive reduced broilers' requirements of amino acids, CP, and consequently their feed cost (Zaghari et al. 2015).

Cytokine gene expression

The gene expressions of IL6, INF-gamma, and IL10 are shown in Figure 1(A, B, C). The IL6 gene expression tended ($P = 0.056$) to be higher in *B. licheniformis* group than the control group. Dietary supplementation of probiotics did not affect the relative gene expression of INF-gamma ($P = 0.3753$), or IL10 ($P = 0.1204$).

Immunomodulation property of probiotic organisms is exerted through their effect on T helper cells in a strain-specific manner (Fong et al. 2016). Also, they can activate various immune cells (Fong et al. 2016). Probiotics can also modulate intestinal barriers and stimulate the production of mucins, defensins, chemokines, and anti- and pro-inflammatory cytokines (Kogut and Swaggerty 2012). The mode of action of probiotics is not completely known yet; however it is obvious that these microorganisms affect birds' microflora by their immunomodulatory characteristics (Hardy et al. 2013). On the other hand, cytokines are proteins or peptides secreted by specific cells which play an important role in immunity and regulation of inflammatory responses. In fact, probiotics have stabilizing effects in the intestine by stimulating the secretion of the anti- and pro-inflammatory cytokines, increasing the number of lymphocytes in the lamina propria and intraepithelial cells, and prevents the pathogenic bacteriás growth and illness of the bird (Dhama and Singh 2010). Cytokines produced by Th2 cells, like interleukin 10 and interleukin 4, are very important in the progress of humoral immune responses (production of antibodies), and the cytokines secreted by Th3 or regulatory T cells are critical in controlling the immune responses (Palamidi et al. 2016). Interleukin 6, produced by T cells, monocytes, and macrophages, acts as both pro- and anti-inflammatory cytokines, and also helps differentiate Th17 cells (Waititu et al. 2014). Increasing the IL-6 gene expression helps define the population of heterophiles that can eliminate pathogens (Hong et al., 2006). In this study, *B. licheniformis* stimulated the immune system by enhanced IL6 expression in broilers' plasma. The effect of probiotics on the immune system of broiler chickens varies in different environmental conditions. In the study of Cao et al. (2013), using dietary *Enterococcus faecium* in broiler chickens stimulated immune response as the birds fed *E. faecium* had a greater level of IL-4 in their jejunal mucosa. Several studies on human and animal have also provided the evidence that certain species of probiotics can stimulate innate immunity (Amit-Romach et al. 2010; Weiss et al. 2010) and humoral immune responses (Nermes et al. 2011; Khan et al. 2014a). Exploration of the effects of *Bacillus* strains supplementation on performance, microbiota establishment, nutrient digestibility, and immunity status may provide a better understanding of how different probiotics can be commercially used in order to deliver an alternative to the use of antibiotics. However, there is no much information about the enzymes or substances secreted from *B. Licheniformis* or *B. subtilis* in broilers' intestine, so further investigations are needed to clarify the exact functions of the probiotics in broilers.

Bacterial penetration into the blood

The results of the bacterial detection in the blood showed that no bacteria have entered the blood stream of the broiler chickens.

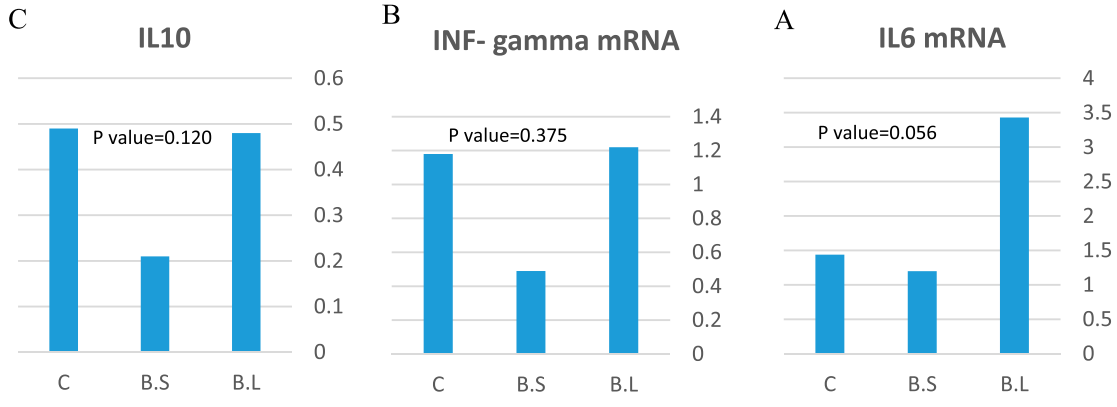

Figure 1. Effect of *B. subtilis* and *B. licheniformis* on the gene expression of IL6 (A), INF- gamma (B), and IL10 (C) in the blood plasma ($n = 36$) of broilers at 42 d of age. Each bar on the graph shows a treatment mean ± SEM.

Translocation of probiotic bacteria from the intestine to the blood and the following bacteremia is one of the critical issues that should be considered when probiotics are supplemented in diet (Lopetuso et al. 2017). It was reported that certain strains of bacteria have high potential to adhere on intestinal mucosa which increase the possibility of bacterial penetration to the blood. This happening is even more possible in hosts who have epithelial barrier dysfunction (De Marco et al. 2018). It is well known that the health state of the birds depends on some certain factors such as integrated intestinal barrier (Wu et al. 2019). Integrated intestinal barrier can protect the bird against the entrance of antigens and pathogenic bacteria. As described by Ohland and MacNaughton (2010), the intestinal barrier includes epithelial cells, epithelial junctions, and mucous layer which contain immunoglobulin A and antimicrobial peptides. Previous research studies have shown that using probiotic can help to improve intestinal barrier function (Zareie et al. 2006; Wu et al. 2019). In addition, Wu et al. (2019) have found that adding *Paenibacillus polymyxa* to broilers' diet could up-regulate gene expression of *claudin*-1, *occluding,* and *mucin*-2 as the genes related to intestinal barrier. In the present study, lack of *Bacillus subtilis* and *Bacillus licheniformis* in broilers' blood confirmed that probiotic bacteria only function in the birds' digestive tract and do not penetrate into the animals' circulation system. So, there is no likelihood of complications from high microbial count and septicemia arising from the presence of these bacteria and bacteriocin produced by them in the blood of broiler chickens.

Conclusions

Adding 0.5 g *B. Licheniformis* preparation to broilers' diet improved BWG and PEF of the birds. The experimental diets did not have any significant effect on the relative weight of the proventriculus, gizzard, and liver of the broilers. Supplementation of *B. subtilis* or *B. licheniformis* had no significant effect on the population of microflora in the ileum or ceca of the broilers. Adding *B. Licheniformis* to broilers' diet improved AME and total tract protein digestibility of the broiler chickens; however, dietary treatments did not have any significant effect on ileal digestibility of CP, GE, Ca, and P. The expressions of IL6, IL10, or interferon gamma genes were not changed among the different groups. Regarding our results, we recommend using 0.5 g *B. licheniformis* preparation (1×10^9 CFU/g) per kg of diet of broiler chickens during 1–42 d.

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