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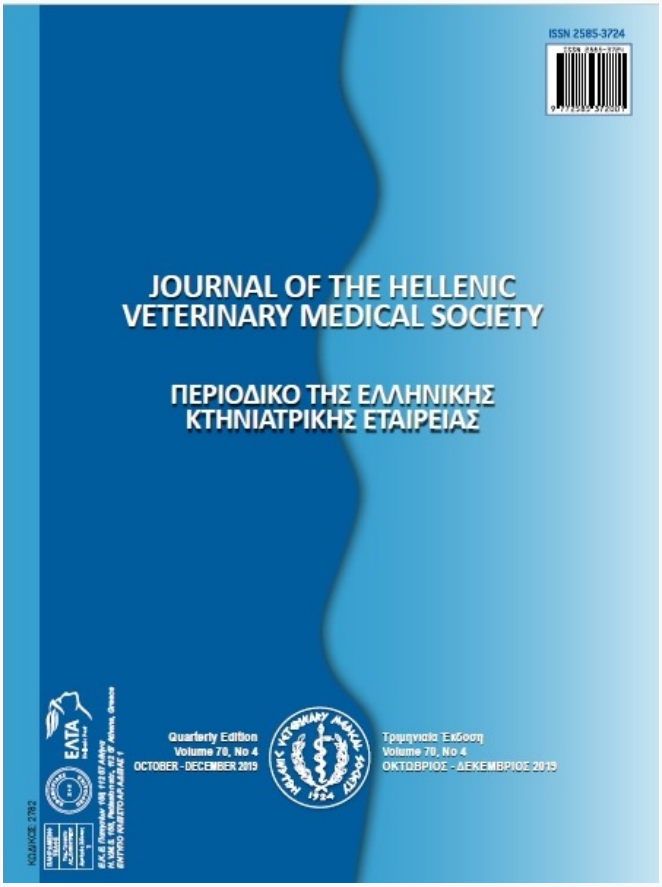

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Effects of Indigenous Spore-Forming Probiotic as Feed Supplement on Performance and Safety in Broilers

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ABSTRACT. Probiotics colonize the intestine of animals and birds and provide useful effects on their performance and immune status. This study describes a high throughput screening and characterization of spore-forming bacteria from Iranian poultry farms with the aim to identify potential probiotic native *Bacillus* spp. and determine its effects on growth performance, hemato-biochemical parameters, immunity, intestinal microflora, morphology and *MUC2* gene expression of broiler chickens. A total of 300 one-day-old female Ross 308 broilers (42.6 ± 0.6 g) were used in a 6-wk study. Broilers were randomly allotted to 1 of 3 dietary treatments consisting of 4 replicate cages with 25 broilers each: 1- Control (Corn-soy-based diet: C), 2- C + 200 g/ton of the GalliPro® (*Bacillus subtilis* DSM 17299, 4×10^9 CFU/g, as positive control group: PC), 3- C + 200 g/ton of the native probiotic (*B. tequilensis K03,* 4×10^9 CFU/g: NP) identified in this study. During the experiment parameters were measured weekly. The results revealed that birds of the NP and PC groups exhibited improved feed conversion ratio (FCR) and increased body weight (BW), carcass and breast meat yield compared with the birds of the C group ($P<0.05$). Also, lymphocytes level, antibody titers against Newcastle diseases virus (NDV) and infectious bronchitis virus (IBV) of vaccinated birds were increased, while serum triglycerides, total cholesterol levels and abdominal fat of birds fed NP and PC were decreased compared to birds of the C group ($P<0.05$). The villus height, the relative expression of *MUC2* gene and *Bacillus* spp. populations were increased, while *E. coli* was significantly decreased in the ileum content of treated groups ($P<0.05$). These results indicate that the identified native *B.tequilensis* K03 strain can improve immunity and broiler performance by modifying intestinal microflora and morphology. Studied native probiotic *Bacillus tequilensis* K03 has useful effects on health status and it can be used as poultry feed supplement.

Keywords: Broiler, Probiotic, *Bacillus*, Performance, Mucin

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INTRODUCTION

Antibiotics have been used in commercial poultry diet due to their growth-promoting and prophylactic effects for over 50 years (Coates *et al.,* 1963). Antibiotic intake of food animals, as well as the resulted antibiotic residue in food, has been noticed as one leading cause of the rapid spread of antimicrobial resistance in human populations. Reducing antibiotics in animal agriculture is one key in struggle against the spread of antibiotic resistance (Ghadban, 2002; Kabir, 2009). Increasing information on healthy food has led to increasing interests on natural food products such as probiotics. Probiotics have been demonstrated to improve intestinal microbial balance, provide protection against gut pathogens and modulate immune system. (Khaksefidi and Ghoorchi, 2006; Mingmongkolchai and Panbangred, 2018). These products have been identified as a safe feed additive in animal industry (Nawab *et al*., 2019). Lactic acid bacteria (LAB), mainly from genus *Lactobacillus*, consist the most important microbial population in the intestine of broiler chickens that have been used as probiotics in poultry industry (Huang *et al.,* 2004). Encapsulation technologies are used to keep probiotic cell viable all over storage, commercialization and use in food products, so that these cells are active during their passage through the gastrointestinal tract (Tellez *et al.,* 2012). *Bacillus* spp. is a genus of Gram-positive, rod shaped, and spore-forming bacteria. The spores present in vegetative cells allow long-term storage, and survival at the harsh environmental and processing condition and low pH of the gastrointestinal tract (Cutting, 2011).

The *Bacillus spp.* have been known as probiotics for chickens feed because it secretes antimicrobials compounds and suppress the colonization of gut pathogens (Hong *et al*., 2008; Knap *et al.,* 2011; Guyard-Nicodeme *et al.,* 2016). This probiotic with improve immunity (Melegy *et al.,* 2011) and changes in the intestine morphology of broilers (Sen *et al.,* 2012) lead to promote growth (Melegy *et al.,* 2011) and improves the quality of meat (Xu *et al.,* 2006; Yang *et al.,* 2016). Also, reported that *Bacillus spp.* decrease NH_3 emission from poultry manure (Jeong and Kim, 2014). Nonetheless, a few of them such as *B. subtilis, B. cereus,* and *B. licheniformis* are currently used in poultry industry, and the probiotic potential of other *Bacillus* spp. has been less studied (Cutting, 2011; Mingmongkolchai and Panbangred, 2018). The potential and efficacy of probiotics depend on the bacterial species and host origin, as well as on the application levels (Mountzouris *et al.,* 2007; Amerah *et al.,* 2013). Moreover, the antibiotic resistance of *Bacillus* spp. is another matter of concern. Therefore, exploring native or new probiotic strains is important to obtain very efficient probiotics for chicken feed. There is little information about the probiotic potential of *Bacillus tequilensis*, which biochemically is quite similar to *B. subtilis*, and can be differentiated by lysine decarboxylase, positive arginine hydrolases, ornithine decarboxylase and acid production from rhamnose (Gatson *et al.,* 2006). It is reported that *Bacillus tequilensis* K03 have the highest attachment ability to intestinal epithelium cells and inhibits the growth of *Salmonella* Typhimurium (Ghorban Hosseini *et al*., 2019). Therefore, in the present study, we investigated the effects a selected native strain (*B. tequilensis K03*) on performance and carcass traits, hemato-biochemical parameters, immunity, and intestinal morphology, microflora and *MUC2* gene expression of broiler chickens.

MATERIALS AND METHODS

Bacterial isolation and characterization

Bacterial isolates were obtained from fecal samples (n=86) collected from poultry farms in Golestan province in the north-east of Iran. The samples were serially diluted and spread plated on nutrient agar (QueLab-393506) followed by incubation at 37°C for 48 h. Discrete bacterial colonies (n=34) were picked and characterized according to Wu *et al.,* (2011). Then, probiotic characteristics (acid and antibiotic resistance, bile salt, the ability to attach to intestinal epithelial cells, and inhibit *Salmonella enterica* serovar Typhimurium invasion), as well as ability of producing amylase and phytase of *Bacillus* spp. isolates were analyzed (Latorre *et al.,* 2016; Thirabunyanon and Thongwittaya, 2012). The 16S ribosomal typing was also performed for identification of the selected strain (Jeevana Lakshmi *et al.,* 2013). All isolates were catalase-positive, oxidase-positive and non-hemolytic. The K03 strain was the superior bacterium, and had desirable probiotic characteristics, with production of 4.56 ± 1.1 U/ml phytase and 36.7 ± 1.3 U/ml α-amylase enzymes, and the highest adherence ability (1.9 log CFU/well) to intestinal epithelial cells. The strain had more inhibitory strength than the other isolates using exclusion assay to inhibit *Salmonella enterica* serovar Typhimurium attachment, up to 53% compared to control. The analysis of 16S rDNA gene sequences showed the highest similarity (% 99) of the K03 strain to *Bacillus tequilensis* KCTC 13622^T, in-

dicating the auto probiotic (indigenous bacteria) potential of the strain for use in chicken diet (Ghorban Hosseini *et al*., 2019).

Birds and experimental design

Three hundred one-day old healthy female broilers (Ross 308) with the initial weight of 42.6 ± 0.6 g were obtained from a local hatchery (Tehran, Iran), and randomly allocated to three dietary treatments (n=100) with four replicates (25 birds/pen) and raised for 42 days. The broiler chickens were fed a basal diet (Control; C) as well as basal diet + 200 g/ton of the GalliPro® commercial probiotic (*B. subtilis* DSM 17299, 4×10^9 CFU/g) as positive control group (PC). The birds in the native probiotic (NP) group were fed with basal diet + 200 g/ton of the native probiotic (*B. tequilensis K03,* 4×10^9 CFU/g) isolate. In broiler diets 8×10^5 viable spores/g was evaluated. Feed ingredients and nutrient composition of basal diet are shown in the Table 1. The environmental temperature was maintained at 32°C during the first week and gradually decreased (2°C per week) to 22°C, and then maintained constant until the end of the experiment. All guidelines for the ethical use and care of animal were followed, and approved by the Islamic Azad University Ethics Committee for Animal Experimentation.

Table1. Feed ingredients and nutrient composition of basal diet

Ingredients (%)	Starter (1-10 d)	Grower (11-21d)	Finisher (22-42d)
Corn	54.91	56.80	60.54
Soybean meal (44% CP)	38.00	36.22	32.03
Soy oil	2.51	3.00	3.83
Limestone	1.10	1.01	0.94
Di-calcium phosphate	1.93	1.65	1.42
Vitamin and Mineral premix*	0.50	0.50	0.50
DL-methionine	0.30	0.26	0.24
L-lysine HCl	0.25	0.13	0.10
L-threonine	0.11	0.06	0.03
Common Salt	0.20	0.23	0.24
Sodium bicarbonate	0.19	0.14	0.13
Nutrient composition			
Metabolizable energy (kcal / kg)	2950	3000	3100
Crude protein (%)	22.61	20.80	18.89
Digestible lysine (%)	1.26	1.11	0.99
Digestible methionine + cysteine (%)	0.93	0.84	0.77
Digestible threonine (%)	0.84	0.74	0.66
Calcium (%)	0.94	0.84	0.75
Available Phosphorus (%)	0.47	0.42	0.38
Sodium (%)	0.16	0.15	0.15

* The vitamin-mineral premix provided the following quantities per kg of diet: vitamin A, 9000 IU; vitamin D3, 2000 IU; vitamin E, 18 IU; vitamin K3, 2 mg; vitamin B1, 1.8 mg; vitamin B2, 6.6 mg; vitamin B3, 10 mg; vitamin B5, 30 mg; vitamin B6, 3 mg; vitamin B9, 1 mg; vitamin B12, 0.015 mg; biotin, 0.1 mg; choline chloride, 250 mg; antioxidant, 100 mg; Mn, 100 mg; Zn, 84.7 mg; Fe, 50 mg; Cu, 10 mg; I, 1 mg; Se, 0.2 mg.

Growth performance

Growth performance parameters, including body weight gain (BWG) and feed intake (FI) were measured while feed conversion ratio (FCR) was calculated for starter (1-10 d), grower (11-21 d), finisher (22-42 d), and overall period (1-42 days).

Carcass yield and relative weight of organs

On day 42, four birds from each replicates were randomly selected, weighed and slaughtered for carcass analysis, and determination of relative weight of organs. The weight of carcass, breast, thigh, gizzard, liver, heart, spleen and abdominal fat for each slaughtered bird was calculated as a relative percentage of live body weight (Zaghari *et al.,* 2016).

Hematological parameters

At the end of experiment, blood samples were collected from wing vein of four birds from each replicates, and divided into two aliquots. The first aliquot was transferred to a 2 ml heparinized tube containing EDTA to determine leukocytes, and other one in the same tube without anticoagulant and left to clot then serum was collected for humoral and biochemical analyses. Blood smears were prepared from each samples by Giemsa staining, and were examined un-

der a compound microscope for leukocyte differential count according to Beski and Al-Sardary (2015). Moreover, 100cells from the slides were evaluated to determine the heterophil to lymphocyte ratio.

Serum biochemical analysis

The collected blood samples (4 birds per replicate) in the 2 ml tube without anticoagulant left to clot, then serum was collected by centrifuging (1500 *g* for 15 min at 4°C), and stored at -20°C.

The concentration of serum total protein (TP), triglyceride (TG), glucose, total cholesterol, high-density lipoprotein (HDL) cholesterol, and low-density lipoprotein (LDL) cholesterol were measured by commercial kits (Parsazmon Co. Iran) according to the manufacturer protocols, using the Eppendorf Auto analyzer (Epos5060).

Humoral immune parameters (Antibody titers)

The broilers were vaccinated against Newcastle, Influenza, and Infectious Bronchitis as described by Rahmani *et al*. (2005). On day 28 of the experiment, four birds from each replicate were bled by wing vein for serum antibody titer analysis (Rowghani *et al*., 2007). The samples were tested for Newcastle and Influenza disease by HI test (Xu *et al*., 1997), and were analyzed by an ELISA kit (Bronchitis, IDEXX Kit) for bronchitis diseases according to manufacturer's instruction.

Determination of ileum microflora

On day 42, four birds from each replicate were randomly selected and slaughtered, and then their ileum contents (1 g) were removed to determine microflora. The samples were diluted from 10^{-1} to 10^{-7} in normal saline solution, and then to determine *Bacillus* spp., *Lactobacillus* spp., and *Escherichia coli* counts, the diluted samples were seeded on Nutrient agar (QueLab-393506), MRS agar (Merck, Germany), and MacConkey agar (Merck, Germany), respectively, and incubated at 37°C for 48 h. The numbers of colony-forming units (CFUs) were expressed as log10 CFU per gram (Wu *et al.,* 2011).

Ileum morphological examination

At the end of experiment, 4 birds per replicate were sampled, and ileum (5 cm after Meckel's diverticulum) was taken, and fixed in 10% formalin. The 5 µm sections were prepared and stained with hematoxylin and eosin (H&E) for light microscopic (E600; Nikon) examination. Morphological experiments were performed according to Iji *et al.* (2001) methods, using Image-J software (http://rsb.info.nih.gov/ij/).

***MUC2* gene expression**

Total RNA was extracted from the 20 mg of the homogenized ileum intestinal samples (3 birds from each replicates) using the RNA Isolation Kit (QIAGEN, Cat. No. 74104), and then cDNA synthesis was performed in a total volume of 25 µl from 5 µl of extracted RNA using Quantifast Reverse-Transcriptase cDNA synthesis kit (QIAGEN, Cat. No. 205311) according to the manufacturer's recommendations. Relative expression of *MUC2* gene was quantified in duplicate for each cDNA sample on the Real-Time PCR detection system (Applied Biosystems) using Quanti Fast Syber Green PCR kit (QIAGEN, Cat. No. 204052), and specific primer pairs (BX930545; F: 5′-ATGCGATGTTAACACAGGACTC-3′; R:5′-GTGGAGCACAGCAGACTTTG -3′) with cycling parameters of 95°C for 10 min for 1 cycle, 95°C for 15 s, 60°C for 20 s, and 72°C for 40 s for 40 cycles, as described previously by Forder *et al*. (2012). The melting curve of each amplicon was examined, and the expression of the *MUC2* gene was corrected based on the endogenous control expression (*GAPDH* gene: NM_204305; F: 5′-TGTGACTTCAATGGTGACAGC-3′, R: 5′-GCTATATCCAAACTCATTGTCATACC-3′) and calculated as fold change according to the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Statistical analysis

The data were analyzed using the statistical package SAS software (SAS Institute, 2007) by one-way analysis of variance (ANOVA) followed by Tukey's pairwise multiple comparison test. Values of $P<0.05$ were considered statistically significant.

RESULTS

Growth performance

The results of growth performance of broilers fed with diets containing native strain (*B. tequilensis* K03) and commercial product (*B. subtilis* DSM 17299) of *Bacillus* spp. probiotic are presented in Table 2. The results showed that dietary supplementation of *Bacillus* spp. probiotics (both native strain and commercial product) significantly improved BW, FCR of birds compared to the control group ($P<0.05$) during the overall period, while there was no significant difference *(*$P>0.05$) for starter, grower, and finisher periods. Feed intake was not affected by treatments. No significant differences were found in growth performance parameters between birds fed native strain and commercial *Bacillus* spp. probiotic supplemented diets ($P>0.05$).

Table 2. Growth performance of broiler chickens fed *Bacillus* spp. probiotics at different periods of experiment

Parameters	C	PC	NP	SEM	P-value
1 – 10 days					
BW (g)	238.3	243.7	244.8	2.0	0.4
FI (g)	266.2	235.2	233.8	11.3	0.4
FCR	1.1	0.9	0.9	0.05	0.3
11 – 21 days					
BW (g)	601.4	625.9	627.5	7.5	0.3
FI (g)	852.1	835.2	834.4	16.3	0.9
FCR	1.4	1.33	1.3	0.02	0.2
22 – 42 days					
BW (g)	1720.6	1763.7	1762.5	10.1	0.1
FI (g)	3676.9	3663.8	3662.1	19.0	0.9
FCR	2.1	2.0	2.0	0.01	0.06
1 – 42 days					
BW (g)	2602.3	2675.4	2676.9	15.3	0.05
FI (g)	4795.3	4734.3	4730.4	42.8	0.8
FCR	1.8 [a]	1.7 [b]	1.7 [b]	0.01	0.04

Different letters indicate significant differences between groups at $P<0.05$.
BW, Body weight; FI, Feed intake; FCR, Feed conversion ratio
C, Control; NP, Native Probiotic (200 g/ton, *B. tequilensis* K03, 4×10^9 CFU/g); PC, Positive Control (200g/ton, *B. subtilis* DSM 17299, 4×10^9 CFU/g); SEM, Standard error of means.

Carcass yield and relative weight of organs

The results of carcass yield and relative weight of organs of broiler chickens are shown in Table 3. The relative weight of carcass, breast, thigh, and spleen were significantly increased and abdominal fat was decreased (P≤0.05) in the birds fed with diets supplemented with *Bacillus* spp. probiotics (native strain and commercial product) as compared to the control during the overall experimental period. However, dietary *Bacillus spp*. probiotics had no significant effects on relative weight of liver, gizzard, and heart of the birds (P>0.05). No significant differences in carcass yield and relative weight of organs were observed between birds fed dietary native strain and commercial *Bacillus* spp. probiotics (P>0.05).

Table 3. Carcass yield and relative organ weight in broiler chickens fed *Bacillus spp.* probiotic diets at 42 d of age

Parameters (%)	C	PC	NP	SEM	P-value
Carcass yield	66.2 [a]	67.3 [b]	67.4 [b]	0.2	0.05
Breast	22.4 [a]	23.3 [b]	23.4 [b]	0.1	0.05
Thigh	16.1 [a]	16.8 [b]	16.8 [b]	0.1	0.04
Liver	1.6	1.6	1.8	0.03	0.11
Gizzard	1.6	1.7	1.8	0.03	0.17
Spleen	0.1 [a]	0.2 [b]	0.2[b]	0.01	0.04
Heart	0.8	0.9	0.8	0.07	0.96
Abdominal fat	1.6[a]	1.4 [b]	1.3 [b]	0.04	0.04

Different letters indicate significant differences between groups at $P<0.05$.
C, Control; NP, Native Probiotic (200 g/ton, *B. tequilensis* K03, 4×10^9 CFU/g); PC, Positive Control (200g/ton, *B. subtilis* DSM 17299, 4×10^9 CFU/g); SEM, Standard error of means.

Hematological parameters (leukocytes)

The effects of dietary supplementation of native strain and commercial *Bacillus* spp. probiotics on leukocytes differential count of broiler chickens are shown in Table 4. Diets containing native strain and commercial *Bacillus* spp. probiotics (K03 and DSM 17299, respectively) significantly increased the percentage of lymphocytes compared to the control group (P<0.05), however, no significant differences were found between K03 and DSM 17299 groups (P>0.05). There was no significant differences in the percentage of heterophile, eosinophil, basophile, monocyte, as well as heterophile/lymphocytes ratio of birds fed diets containing native strain and commercial *Bacillus* spp. probiotics compared to the control group (P>0.05).

Table 4. Hematological parameters of broiler chickens fed *Bacillus* spp. probiotics based diets at 42 d of age

Parameters (%)	C	PC	NP	SEM	P-value
Heterophile	31.6	31.5	31.3	0.3	0.9
Lymphocytes	51.8 [a]	53.4 [b]	53.9 [b]	0.3	0.02
Monocyte	7.7	7.2	7.3	0.1	0.52
Eosinophil	2.6	2.7	2.7	0.05	0.74
Basophile	2.5	2.5	2.6	0.03	0.69
Heterophile/Lymphocytes	0.6	0.5	0.5	0.01	0.56

Different letters indicate significant differences between groups at $P<0.05$.
C, Control; NP, Native Probiotic (200 g/ton, *B. tequilensis* K03, 4×10^9 CFU/g); PC, Positive Control (200g/ton, *B. subtilis* DSM 17299, 4×10^9 CFU/g); SEM, Standard error of means.

Serum biochemical parameters

The results of serum biochemical analysis of broilers fed with diets containing probiotic are shown in Table 5. The results revealed significant decrease in serum triglycerides and total cholesterol levels of birds fed dietary native strain and commercial *Bacillus* spp. probiotics (K03 and DSM 17299**)** compared to the control group (P<0.05), however, no significant differences were found between K03 and DSM 17299 dietary groups (P>0.05). No significant differences were also found in serum glucose, total protein, High density lipoprotein (HDL), and Low density lipoprotein (LDL) levels among treatments (P>0.05).

Table 5. Serum biochemical parameters in broiler chickens fed *Bacillus* spp. probiotics based diets at 42 d of age.

Parameters	C	PC	NP	SEM	P-value
Glucose (mg dl^{-1})	261.0	235.9	246.9	12.6	0.75
Total protein (g dl^{-1})	3.2	3.6	3.4	0.1	0.74
Triglycerides (mg dl^{-1})	79.5 [a]	66.9 [b]	67.9 [b]	2.2	0.02
Total Cholesterol (mg dl^{-1})	163.2 [a]	147.9 [b]	149.3 [b]	2.7	0.01
HDL (mg dl^{-1})	65.3	59.8	62.4	1.7	0.49
LDL (mg dl^{-1})	54.2	49.6	52.2	2.3	0.76

Different letters indicate significant differences between groups at $P<0.05$
C, Control; NP, Native Probiotic (200 g/ton, *B. tequilensis* K03, 4×10^9 CFU/g); PC, Positive Control (200g/ton, *B. subtilis* DSM 17299, 4×10^9 CFU/g); SEM, Standard error of means.

Humoral immune parameters (Antibody titers)

The results of humoral immune responses of birds are shown in Table 6. Results revealed a significant increase (P<0.05) in antibody titers against Newcastle diseases virus (NDV) and infectious bronchitis virus (IBV) of vaccinated birds fed with diets containing native strain and commercial *Bacillus* spp. probiotics (K03 and DSM 17299**)** in comparison with the control group (P<0.05), however, no significant differences were seen between K03 and DSM 17299 dietary groups. Moreover, diets containing *Bacillus spp*. probiotics had no significant effects on antibody titer against Influenza.

Table 6. Effect of *Bacillus* spp. Probiotics on immune response (antibody body production) of broiler chickens at 28 d of age

Parameters	C	PC	NP	SEM	P-value
Bronchitis	2676.2 [a]	2772.7 [b]	2784.5 [b]	18.88	0.01
Newcastle	3.6 [a]	4.6 [b]	4.6 [b]	0.19	0.03
Influenza	1.3	1.3	1.4	0.01	0.12

Different letters indicate significant differences between groups at $P<0.05$.
C, Control; NP, Native Probiotic (200 g/ton, *B. tequilensis* K03, 4×10^9 CFU/g); PC, Positive Control (200g/ton, *B. subtilis* DSM 17299, 4×10^9 CFU/g); SEM, Standard error of means.

Ileum microflora

The effect of treatments on ileum microflora of broilers (42 d) is shown in Table 7. The results revealed that the native strain and the commercial *Bacillus* spp. probiotics (K03 and DSM 17299**)** significantly increased the *Bacillus spp.* Populations. *E. coli* was significantly decreased in the ileum content of birds fed with diets supplemented with probiotics as compared to control (P<0.05), however, no significant differences were found between the treated groups (P>0.05). Despite the slight increase in *Lactobacillus spp.* there were no significant differences between treated and control groups.

Table 7. Ileum bacterial counts [log (cfu/g)] of broiler chickens fed *Bacillus* spp. probiotic diets at 42 d of age

Parameters	C	PC	NP	SEM	P-value
Lactobacillus spp.	6.6	7.3	7.4	0.1	0.09
Bacillus spp.	5.6 [a]	6.1 [b]	6.3 [b]	0.1	0.03
Escherichia coli	7.0[a]	6.2 [b]	6.1 [b]	0.1	0.04

Different letters indicate significant differences between groups at $P<0.05$.
C, Control; NP, Native Probiotic (200 g/ton, *B. tequilensis* K03, 4×10^9 CFU/g); PC, Positive Control (200g/ton, *B. subtilis* DSM 17299, 4×10^9 CFU/g); SEM, Standard error of means.

Ileum morphology

The result of morphological analysis of ileum is shown in Table 8. No histopathological changes were observed in the intestine tissue of any birds of all feeding groups (Fig. 1). Morphological analysis of ileum revealed significant increases (P<0.05) in the villus height in birds fed with dietary containing *Bacillus* spp. probiotics (Native strain and commercial product) as compared to the control group, however, no significant differences were found between native and commercial probiotic dietary groups (P>0.05). There were no significant differences in the villus width, crypt depth, as well as villus height/crypt of ileum between experimental and control groups (P>0.05).

Table 8. Ileum morphology of broiler chickens fed *Bacillus* spp. probiotic diets at 42 d of age

Parameters (μm)	C	PC	NP	SEM	P-value
Villus height	769.0 [a]	859.3 [b]	889.7 [b]	17.8	0.01
Villus width	153.6	154.2	165.2	4.7	0.53
Villus height/crypt	6.0	6.7	7.0	0.2	0.17
Crypt depth	133.1	134.5	132.6	3.9	0.97

Different letters indicate significant differences between groups at $P<0.05$.
C, Control; NP, Native Probiotic (200 g/ton, *B. tequilensis* K03, 4×10^9 CFU/g); PC, Positive Control (200g/ton, *B. subtilis* DSM 17299, 4×10^9 CFU/g); SEM, Standard error of means.

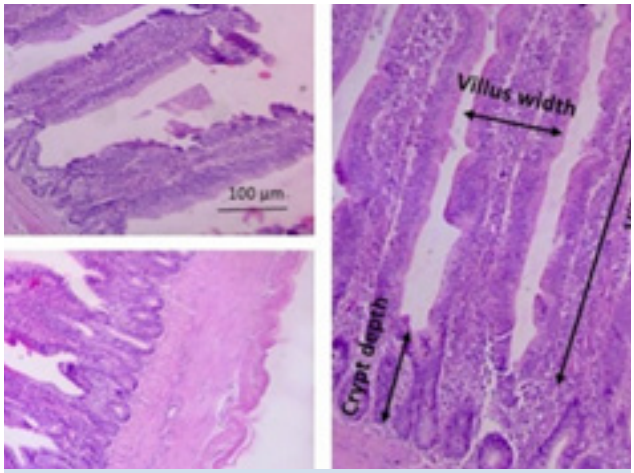

Fig. 1. Histological section (H&E) showing ileum morphology (Villus height, Crypt depth, and Villus width) of broiler chickens fed *Bacillus* spp. probiotics based diets at 42 d of age. C, Control; NP, Native Probiotic (200 g/ton, *B. tequilensis* K03, 4×10^9 CFU/g); PC, Positive Control (200g/ton, *B. subtilis* DSM 17299, 4×10^9 CFU/g); SEM, Standard error of means.

Intestinal *MUC2* gene expression

The effects of probiotic treated diets on expression of the intestinal *MUC2* gene are shown in the Fig. 2. The expression of intestinal *MUC2* gene was quantified by qPCR assay, and expressed relative to expression of the *GAPDH* gene. The relative expression of *MUC2* gene was significantly increased in the dietary native strain and commercial probiotics (K03 and DSM 17299, respectively) compared to the control group (P<0.05). No significant differences were found in *MUC2* gene expression between birds fed with K03 and DSM 17299 probiotic supplemented diets (P>0.05).

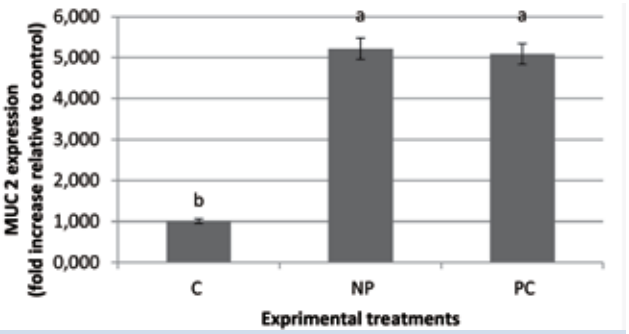

Fig. 2. The relative expression of *muc2* gene in the intestine tissue of broiler chickens fed *Bacillus* spp. probiotics diets at 42 d of age. C, Control; NP, Native Probiotic (200 g/ton, *B. tequilensis* K03, 4×10^9 CFU/g); PC, Positive Control (200g/ton, *B. subtilis* DSM 17299, 4×10^9 CFU/g); Data were normalized based on endogenous *GAPDH* gene and presented as mean fold increase relative to the control ($2^{-\Delta\Delta ct}$ method). Different letters indicate significant differences between groups at $P<0.05$.

DISCUSSION

The present study showed that consumption of diets supplemented with NP and PC significantly improved FCR and increased BW. The beneficial effects of dietary *Bacillus* spp. probiotic supplementation on FCR and increased BW of broilers are well documented in many studies (Opalinski *et al.,* 2007; Melegy *et al.,* 2011; Yang *et al*., 2016; Reis *et al.,* 2017). Spore-forming *Bacillus* spp. have been noticed as probiotic candidates due to their beneficial effects on animal health and growth, as well as their survivability under the harsh environment of the gastrointestinal tract, and stability during processing and long-term storage (Elshaghabee *et al*., 2017). Probiotics can modulate intestinal microflora, change intestinal morphology or secretion of enzymes and produce antimicrobial compounds. They can regulate immune system, increase the digestibility and the absorption of dietary nutrients and consequently improve the broiler performance (Ghadban, 2002; Elshaghabee *et al*., 2017). However, since the host origin microbes are quite familiar with the environment of gastrointestinal tract, the native and species-specific probiotic are highly preferred (Kabir, 2009). Similar studies showed that the improvement of broiler performance can be caused by beneficial changes of intestinal morphology and microflora (Ghadban, 2002; Elshaghabee *et al*., 2017). In this investigation increased BW and decreased FCR could be attributed to the growth of beneficial bacteria in the digestive tract, digestive enzymes production by these bacteria and improved digestion and absorption processes. The lack of impact in the initial period may be explained by the fact that probiotic bacteria are required to longer time for localization in the digestive tract. Our results showed that supplementation with the *B. tequilensis* K03 strain and commercial *B. subtilis* DSM 17299 have no effect on feed intake of chickens. Several studies (Opalinski *et al*., 2007; Melegy *et al*., 2011) have shown that feed intake of chickens was not affected by supplementation of *Bacillus* spp., suggesting that these strains cannot affect their appetite (Ferket and Gernat, 2006).

In our present study, increase in spleen relative weight, carcass, thigh and breast meat yield and decrease in abdominal fat of broiler chicks have been found when compared with the control group. These results are in agreement with those of Hatab *et al.* (2016), who reported that dietary supplementation with *Bacillus spp.* probiotics (*B. tequilensis* K03 strain and *B. subtilis* DSM 17299*)* rose carcass and relative organ weights due to increase of cell growth and turnover*,* while other researchers reported that (Afsharmanesh *et al*., 2014; Park *et al*., 2014; Reis *et al*., 2017; Shokryazdan *et al*., 2017), using the same or different probiotic species did not affect the relative organ weights of broilers. The reason for these contradictions may be due to differences in conditions of chickens, methods of administration, viability and concentrations of used bacteria, as well as the strain sources (Shokryazdan *et al*., 2017). Therefore, it seems probably that increase in carcass, thigh and breast meat yield of broiler in our present study can due to useful effect of probiotics in the growth of intestinal microbiota. In the report of Santoso *et al*. (2001) decrease synthesis and storage of fat in adipose tissue lead to decrease the percentage of abdominal fat.

Our results showed a significant increase in lymphocytes level, antibody titers against NDV and IBV of vaccinated birds. Lymphocytes play a crucial role in innate immune response, especially during stressful conditions, and participate in inflammation responses and phagocytosis. The increase in lymphocytes level indicates stimulation of the immune properties by *Bacillus* spp. probiotics that lead to increase in relative lymphoid organ weights (such as spleen). This assumption is supported by report of Neveling *et al*. (2017), who reported higher lymphocytes level in birds after dietary supplementation with probiotics.

It is strongly possible that probiotic microorganisms as an external organism stimulate the immune system, increase production the number of white blood cells and other immune compounds, the percentage of lymphocytes increased. Moreover, the ability of probiotics to promote humoral immunity in chickens vaccinated against Newcastle disease and infectious bronchitis reported by Rowghani *et al*. (2007), and in present study confirmed the immunostimulatory effects of the selected strain and the commercial *Bacillus* spp. probiotics. Probiotics control the balance of pro-inflammatory and anti-inflammatory cytokines. Cytokinins have an important role in immune responses. IFN-γ is a subset of the cytokine T-helper 1 that lead to killing organisms and protecting against all types of intracellular infections. Moreover interleukin-4 also can stimulate the differentiation of B cells and increase the production of antibodies to B cells (Belardelli, 1995). Therefore, the probable reason of increase in NDV and IBV of vaccinated birds is the stimulation of the immune system by probiotic native *Bacillus spp.* and probiotic Galpiro.

Our results showed a significant decrease in triglyceride and total cholesterol concentration in the serum of broilers fed with *B. tequilensis* K03 strain and *B. subtilis* DSM 17299 compared with the control group. Probiotics increase deconjucation of biliary acids excretion and since cholesterol is a substrate for the synthesis of bile acids, cholesterol molecules are used to produce bile acids (De Smet *et al*., 1998). Therefore they decrease the lipids level of blood.

In our present study, *Bacillus* spp. populations increased in the intestine of broilers fed the *B. tequilensis* K03 strain and *B. subtilis* DSM 17299. Several studies have demonstrated that dietary supplementation with *Bacillus* spp. modulate the microflora of broilers (Knap *et al*., 2011; Sen *et al*., 2012; Guyard-Nicodeme *et al*., 2016). Probiotics which increase the number of lactic acid bacteria in the gastrointestinal reduce its pH. Therefore, an unsuitable environment for the growth of harmful bacteria such as *E.coli* and *Salmonella* spp. is provided (Deniz1 *et al*., 2011). Therefore, it seems probably that probiotic native *Bacillus spp.* and probiotic Galpiro by pH reduction, increase beneficial bacteria and decrease *E. coli* population.

Our results indicated that the *B. tequilensis* K03 strain and *B. subtilis* DSM 17299 significantly increased villus height in ileum of the chickens. The effect of dietary *Bacillus* spp. probiotics on intestinal morphology of broilers has been well documented. Sen *et al.* (2012) reported the increased villus height and villus height to crypt depth ratio in chicken fed *Bacillus* spp. dietary. Deng *et al*. (2012) also found that dietary *Bacillus licheniformis* increased villus height in the ileum under heat stress conditions. It is showed that the digestive function of the intestine is related to villi structure and mucosal architecture, which influence absorptive capacity (Sen *et al.,* 2012; Neveling *et al*., 2017). Moreover, probiotics by short-chain organic acids formation stimulate the proliferation of epithelial cells and lead to increased villus height (Ichikawa *et al*., 1999).

The mucin secreted by goblet cells in the villi of the intestine is the main glycoprotein component of the mucus layer that it has role in modulation of intestinal microflora and health (Forder *et al*., 2007). In this study, intestinal *MUC2* gene expression under influence of two types of *Bacillus* spp. probiotic was significantly increased, suggesting that the probiotics may bind to specific receptor sites on the enterocyte and stimulate *MUC2* gene expression (Mattar *et al*., 2002).

CONCLUSION

From this study, it can be concluded that the identified native *B. tequilensis* K03 strain can improve immunity, hemato-biochemical parameters, as well as broiler performance, which can be explained by the modified intestinal microflora, intestinal morphology changes and increase of *MUC2* gene expression. Since the effects of selected strain (*B. tequilensis* K03) were similar with the GalliPro® commercial probiotic (*Bacillus subtilis* DSM 17299) it can be used as probiotic potential for broilers feed.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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