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Assessment of a probiotic Containing Bacillus Subtilis on the Performance and Gut Health of Laying Japanese Quails (Coturnix Coturnix Japonica)

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ABSTRACT

The present study was carried out to determine the effects of the inclusion of a spore-forming probiotic (*Bacillus subtilis*) in laying Japanese quail diets as an alternative to growth-promoting antibiotics to help produce healthy eggs and meat. This experiment was conducted as a completely randomized design with three treatments (control, 0.05% bacitracin methylene disalicylate (BMD), or 0.1% *Bacillus subtilis*) of five replicates of 11 quails each. Feed intake and egg production were recorded daily on cage basis. Body weight was determined at the beginning and end of the trial (36 and 42 weeks). At the end of the experiment (42 weeks), antibodies against Newcastle disease and avian influenza, egg components, Haugh units, eggshell quality and breaking strength, blood parameters, cecal microbial population, villus length, and crypt depth were measured. The dietary inclusion of *Bacillus subtilis* and BMD significantly ($p \leq 0.05$) increased egg production and egg weight; however, eggshell thickness and breaking strength, Haugh units, and eggshell percentages were not affected. The dietary addition of both products significantly ($p \leq 0.05$) decreased plasma cholesterol levels and increased LDL levels, as well as antibody levels against Newcastle disease and avian influenza ($p \leq 0.01$). In birds fed *Bacillus subtilis* and BMD, crypt depth was reduced, but villus height and villus to crypt ratio were significantly increased ($p \leq 0.001$) compared with those fed the basal diet. Cecal coliforms, *E. coli,* and Salmonella counts were reduced ($p \leq 0.01$) in quails fed the diets containing *Bacillus subtilis* and BMD compared those quails fed the non-supplemented diet. The results of this study demonstrated that in absence of AGPs, the inclusion of a spore-forming probiotic partially improves the performance of laying quails.

INTRODUCTION

Quail production is one of the fastest growing sectors in poultry industry across the globe. In addition to their excellent flavor, quail eggs have 3-4 times higher nutritional value than that of free-range chicken eggs. Quail eggs contains 13% protein and 140µg of vitamin B1 (Tavaniello *et al.,* 2014). They are much richer in vitamin B2, choline, iron, potassium, calcium and phosphorus than chicken eggs and contain high HDL cholesterol levels, the 'good' cholesterol, and therefore, can be consumed by elderly people (Dogan *et al.,* 2013). The health benefits of quail eggs include treatment of anemia, removal of toxins and heavy metals from the blood, strong anti-cancer activity, inhibiting cancer growth, nourishment of the prostate gland, restauration of sexual potency, memory and brain activity enhancement, and strengthening the immune system (Pellegrini *et al.,* 2015). Other advantages of quail production are minimum floor space required for production, low investments, early sexual maturity, and high egg production.

Probiotics have been described as alternatives to antibiotic growth promoters for poultry against oxidative stress, improving mucosal and general immunity and increase their performances (Gleeson *et al.,* 2012). Stressful situations in poultry farms may increase the incidence of gastrointestinal disease, particularly of diarrhea during production period and are likely to cause increased susceptibility to infections in the upper respiratory tract in part to suppress the immune system leading to growth rate deficiencies (Manafi, 2015). Probiotics can prevent illnesses during the laying period, which is one of the priorities for quail farmers to produce healthy eggs, and also to nutritionists, who are interested in minimizing gastrointestinal disorders (Pellegrini *et al.,* 2015).

It has been demonstrated that consumption of probiotic can enhance the immune system and health (Corthesy *et al.,* 2007). Therefore, probiotics can be used to directly maximize performance of birds by preventing the immunosuppression caused by prolonged stress and the presence of pathogenic bacteria, thus reducing the quail's susceptibility to diseases and the incidence of acute upper respiratory tract infections, diarrheas and their associated symptoms (Guarino *et al.,* 2009).

The use of spore-forming probiotics has several advantages. When the bacterium turn into spores, it forms two layers of protein around it. These layers protect the bacterium from environmental stressors, which allow spore-forming probiotics to be included in diets containing possible aggressive in-feed components such as coccidiostats (Tavaniello *et al.,* 2014). *Bacillus* spores are relatively inexpensive to produce, steam palatable and highly effective to modify the intestinal microflora to deactivate Salmonella species, *E. coli,* and *Clostridium perfringens.* It is believed that *Bacillus subtilis* C-3102 spores vegetate in the intestinal lumen and consume oxygen, providing a more anaerobic condition, which is favorable to native *Lactobacilli* spp. They then proliferate and produce lactic acid that controls pathogens and increases the utilization of essential minerals.

A number of studies in growing quails reported that egg production significantly increased in commercial flocks fed alternatives to antibiotic growth promoters, which have been increasingly banned worldwide (Tavaniello *et al.,* 2014). The objective of the present study was to compare the effects of the dietary inclusion of a commercial spore-forming probiotic on performance and egg quality parameters of laying Japanese quails with an antibiotic growth promoter.

MATERIALS AND METHODS

Bird management and experimental design

The experimental procedures were approved by the bioethical committee of Malayer University according to the procedures for the protection of animals reared for scientific experimental purposes (protocol number: 84/5-1-183). The quails were maintained in cages according to the guidelines of Iranian Council on Animal Care.

Quails were housed in thermostatically-controlled wire battery cages (152 × 46 × 27cm). A lighting program of 17 h light and 7 h dark was adopted during the experimental period.

One hundred and sixty five laying Japanese quails (*Coturnix coturnix japonica*) with 36 weeks of age were distributed into three treatment groups with five replicates of 11 quails each (8 females and 3 males). Birds were fed a basal diet containing corn-soybean meal (control); the control diet plus 0.05% bacitracin methylene disalicylate (BMD), or the control diet plus 0.1% *Bacillus subtilis* (Calsporin®, Calpis Co. Ltd, Japan) from 37 to 42 weeks of age. Calsporin is a preparation of *Bacillus subtilis* C-3102 (DSM 15544) with a minimum of 1.0×10^{10} viable spores per gram. The quail breeder mash diet was manufactured at the experimental facilities. The diet was formulated to supply the birds' nutritional requirements according to the recommendations of the NRC (NRC, 1994). The ingredients and chemical composition of the coccidio stat-free basal diet is shown in Table 1. The *Bacillus subtilis* and bacitracin methylene disalicylate were obtained from local market in powder form and added on top to the basal diet.

The tested products were fed to the quails three weeks before starting the experiment, which was considered as adaptation period. Feed and water were supplied *ad libitum.*

Performance parameters

Egg production was recorded daily per cage unit during the adaptation period, and the average egg production rate (hen-day percent) was calculated. Feed intake was calculated as feed offer minus feed residues in the feeder, and reported as gram per bird per day. Feed conversion ratio (FCR) was calculated as grams of feed per grams of egg produced. Weekly egg production was weighed to determine egg weight. Egg mass was then calculated as egg weight × number of eggs produced.

Table 1 – Composition of the basal diet of layer Japanese quail on as-fed basis (36-42 weeks of age)

Feed ingredients (%)	
Corn	65.3
Soybean meal	19
Eggshell powder	7.2
Corn gluten	5.00
Dicalcium phosphate	1.40
Soybean oil	1
DL-Methionine	0.31
L-Lysine	0.09
Mineral mixture[1]	0.25
Vitamin mixture [2]	0.25
Salt	0.20
Analyzed chemical composition	
Metabolizable energy (kcal/kg)	2950
Total protein (%)	18
Total calcium (%)	3.10
Available phosphorous (%)	0.45
Lysine (%)	0.85
Methionine (%)	0.52
Methionine + Cysteine (%)	0.82
Sodium (%)	0.18

[1]Mineral mixture provided 500mg of $FeSO_4$, 65 mg of $CuSO_4$, 100mg of $MnSO_4$, 0.5 mg of Iodine and 0.22gm of Selenium per kg of feed.

[2]Vitamin mixture provided 11000 IU of vitamin A, 1800 IU of vitamin D3,11 mg of vitamin E, 2 mg of vitamin K3, 4 mg of vitamin B1, 5.7 mg of vitamin B2, 2 mg of vitamin B6, 0.5 mg of folic acid, 2500 mg of choline chloride, 0.125 mg of antioxidants, 0.03 mg of Biotin and 0.024 mg of vitamin B12 per kg of feed.

Egg quality parameters

Egg components, Haugh unit, eggshell thickness, and eggshell breaking strength were measured at the end of the experimental period (42 weeks of age). Eggshell breaking strength was measured in two eggs per replicate chosen at random using an eggshell force gauge (model-II, Robotmation Co. Ltd., Tokyo, Japan). Eggshell thickness was measured using an ultrasonic thickness gauge (Echometer 1062, Robotmation Co. Ltd.). Haugh unit was determined in an egg multi-tester equipment (EMT-5200, Robotmation Co. Ltd.). Eggs were broken, the internal contents were removed, and eggshells were dried at room temperature for 48 h to determine eggshell weight, by a digital balance (0.001 g accuracy).

Serum biochemistry and immune response parameters

Two quails per cage (one male and one female) were randomly selected from all groups, and individual blood samples were collected in non-heparinized tubes at the end of the experimental period (42 weeks of age). The serum was separated and stored at -20˚C until further analyses. Each serum sample was analyzed for glucose, cholesterol, low-density lipoprotein (LDL), high-density lipoprotein (HDL), and triglyceride levels, and for alanine transaminase (ALT), gamma glutamyl transferase (GGT),alkaline phosphatase (ALP) enzyme activities. Antibody titers against Newcastle disease (ND) and avian Influenza (AI) were measured by commercial ELISA kits (Boehringer Mannheim Hitachi 704 automatic analyzer, Japan).

Intestinal morphology parameters

At the end of the experiment, four birds per treatment were randomly selected, stunned, and killed for the evaluation of ileal morphology. The digestive tract with its contents was aseptically removed, and the ileum was detached from the Meckel's diverticulum up to 1cm proximal to the ileocecal junction and later dried on desiccant paper. A 2-cm section of ileum was removed from the middle part of the ileum and gently flushed using PBS (pH 7.2).

Tissue sections were immediately fixed in 10% neutral buffered formalin, which was changed three times to complete fixation. A single 0.5cm sample was cut from each ileal section, dehydrated with increasing ethanol concentrations (70, 80, 95, and 100%), cleared in xylene, and placed into polyfin embedding wax. Tissue sections (2µm) were cut using a microtome (Leitz-1512 Microtome, Leitz, Wetzlar, Germany), floated onto slides, and stained with hematoxylin (Gill no. 2, Sigma, St. Louis, MO) and eosin (H&E) (Sigma).

Villus height and crypt depth images were taken using a digital camera under light microscopy. In total, 12 images from four sections of each ileal section were taken to measure 24 villus heights and crypt depths by imaging software. Villus length was measured from the tip of the villus to the valley, and crypt depth measured from the valley to the basolateral membrane. Intestinal morphology measurements were analyzed by the GLM procedure of SAS software (2007).

Enumeration of cecal bacterial population

At the end of the experiment, two quails per cage were bled by the jugular vein for the extraction of cecal contents, which were pooled for serial dilution. Microbial populations were determined by serial dilution (10^{-4} to 10^{-6}) of cecal samples in anaerobic diluents before inoculation onto Petri dishes with sterile agar, as described by Bryant & Burkey (1953).

Nine sterile lidded test tubes containing 9mL phosphate buffer solution as diluent were prepared and approximately 1g of the cecal contents taken by sterile swab and homogenized for 3min before being submitted to the microbiology lab under refrigeration

(Bryant & Burkey, 1953) and mixed, employing aseptic techniques. For serial dilution of the cecal contents, 1mL out of the 10mL buffer plus cecal content solution was removed using a 1000µL sampler, transferred to tube No. 1, and thoroughly mixed. This procedure was repeated until a 1:9 dilution (tube No. 9) was achieved. A volume of 1.0mL of each test tube was transferred to Petri dishes with the selective media. *E. coli* was grown on eosin methylene blue agar, Salmonella on *Salmonella Shigella* agar (Merck, Germany), and coliforms on McConkey agar (Darmstadt, Germany). Petri dishes were then aerobically incubated at 37°C for 24h. Colonies were manually counted between 24 and 48h after inoculation, adjusted to X 10^9, and reported as colony forming units (cfu), defined as distinct colonies measuring at least 1mm in diameter.

Statistical Analysis

Data were analyzed according to a completely randomized experimental design. The GLM procedure of SAS (SAS Institute, 2007) was applied for parameters measured only once during the experimental period, and the MIXED procedure of SAS (SAS Institute, 2007) for those repeatedly measured during the experimental period. Age was considered the main factor and initial values considered as a covariate effect in the model. Differences between treatment means were tested using Duncan's multiple comparison tests for main effects and Tukey's test for interactions. Statistical significance was declared at p≤0.05.

RESULTS

The 2-week pre-experimental addition of probiotic to laying quail diets showed had no influence (p>0.05) on egg production or egg weight. The dietary inclusion of *Bacillus subtilis* and bacitracin significantly increased egg production compared with the control group (Table 2). Feed conversion ratio (FCR; g feed/g eggs produced) was significantly (p≤0.05) reduced in the bacitracin and *Bacillus subtilis* groups compared with the control group. Feed intake was not affected by dietary treatments (Table 2). The egg weight in both feed-additive groups was significantly (p≤0.05) higher than that of the control group.

Table 2 – Effect of the dietary addition of *Bacillus subtilis* on the performance of laying Japanese quails.

Treatment	Egg production (hen-day %)	FCR (g feed/g egg produced)	Feed intake (g/quail/day)
Control	69.09[c]	3.57[a]	203.15
Bacitracin	72.74[a]	3.28[c]	205.83
Bacillus subtilis	72.22[b]	3.42[b]	204.35
SEM	0.13	0.04	1.03
P-Value	0.001	0.001	0.54

[a-c]Means with different letters within the same column are significantly different (p≤0.05); FCR: Feed conversion ratio; Bacitracin (bacitracin methylene disalicylate): antibiotic growth promoter; SEM = pooled standard error of column wise means comparison.

Eggshell thickness, eggshell breaking strength, Haugh units, and eggshell percentage, however, were not affected by addition of *Bacillus subtilis* or bacitracin (Table 3).

The effects of *Bacillus subtilis* and bacitracin on plasma parameters and antibody responses of quails are shown in Table 4. The addition of both feed additives significantly (p≤0.05) reduced plasma cholesterol and LDL levels during the experimental period, but had no effect on HDL levels. The dietary inclusion of *Bacillus subtilis* and bacitracin significantly (p≤0.01) improved the antibody response against Newcastle disease and avian influenza. Plasma total protein, calcium, phosphorus, glucose, and the activities of serum alanine transaminase, alkaline phosphatase and aspartate transaminase were not affected by the dietary addition either *Bacillus subtilis* or bacitracin.

The effects of *Bacillus subtilis* and bacitracin on ileal morphology of laying Japanese quails are shown in Table 5. In birds fed *Bacillus subtilis* and bacitracin, crypt depth was reduced, and villus height and villus to crypt ratio were significantly increased (p≤0.001) compared with the group fed basal diet. The number of goblet cells was significantly (p≤0.01) reduced by the inclusion of both these feed additives in comparison with the control group.

There was a significant (p≤0.01) reduction in cecal coliforms, *E. coli*, and Salmonella populations of Japanese quails fed diets containing *Bacillus subtilis* and bacitracin when compared with those fed the basal diet.

DISCUSSION

When administered in adequate amounts, probiotics, as live microorganisms, have beneficial effects on the performance and health status of the host. Studies have confirmed that selected heat-resistant spore-forming *Bacillus* species fed as probiotics can markedly reduce *Salmonella* and *Clostridium* populations, proving to be cost-effective feed additives in commercial poultry production (Tellez *et al.,* 2006). In the current study, the dietary addition of *Bacillus subtilis* increased the egg production and egg weight of Japanese quails. The absence of feed intake differences among the different

Table 3 – Effect of the dietary addition of *Bacillus subtilis* on egg weight and egg quality of laying Japanese quails.

Treatment	Egg weight (g)	Eggshell thickness (mm)	Eggshell breaking strength (kg)	Haugh units	Eggshell (%)
Control	11.15[c]	2.17	0.22	91.24	8.29
Bacitracin	11.23[b]	2.19	0.21	90.44	8.69
Bacillus subtilis	11.26[a]	2.21	0.21	87.47	8.69
SEM	0.01	0.03	0.01	2.39	0.32
P-Value	0.001	0.69	0.88	0.49	0.65

[a-c]Means with different letters within the same column are significantly different (p≤0.05); Bacitracin (bacitracin methylene disalicylate): antibiotic growth promoter; SEM = pooled standard error of column wise means comparison.

Table 4 – Effect of the dietary addition of *Bacillus subtilis* on the blood parameters and antibody response of laying Japanese quails.

Treatment	Cholesterol mg/dL	Triglyceride mg/dL	HDL mg/dL	LDL mg/dL	AST mg/dL	ALT %	ALP 10^6/µl	WBC 10^3/µl	Glucose mg/dL	Protein g/dL	Plasma Calcium mg/dL	Plasma phosphorus mg/dL	AI	ND
Control	330.40[a]	579	89.2	122.8[ab]	290.2	2	5192	15240	300	4.58	19.18	6.04	7.2[a]	7[a]
Bacitracin	285.80[ab]	607	93	135.8[a]	294.2	2.4	2516	1522	314	4.44	15.76	6.04	5.4[b]	6.4[a]
Bacillus subtilis	207.00[b]	412	90.2	85[b]	287.6	2	43425	14560	292	4.48	15.34	4.7	4.8[b]	4[b]
SEM	29.57	89.33	10.09	13.8	23.7	0.29	935	660	0.24	0.27	1.55	0.83	0.49	0.34
P-Value	0.03	0.28	0.96	0.05	0.98	0.55	0.16	0.71	0.81	0.93	0.19	0.41	0.01	0.001

[a-b]Means with different letters within the same column are significantly different (p≤0.05); HDL: High-density lipoprotein; LDL: Low-density lipoprotein; AST: aspartate aminotransferase; ALT: Alanine Aminotransferase, ALP: Alkaline phosphatase; WBC: white blood cell;ND: Newcastle disease antibody titers, AI: Avian influenza antibody titers. Bacitracin (bacitracin methylene disalicylate): antibiotic growth promoter; SEM = pooled standard error of column wise means comparison.

Table 5 – Influence of *Bacillus subtilis* addition of diets on cecal microbial populations and ileum morphological characteristics, including villus height, crypt depth, and the villus:crypt ratio of quails .

Treatment	Villus height (µm)	Crypt depth (µm)	Villus:crypt ratio	Number of goblet cells†	Coliforms ($\log_{10}$ cfu/g of DM)	*Salmonella* ($\log_{10}$ cfu/g of DM)	*E. coli* ($\log_{10}$ cfu/g of DM)
Control	469[b]	91[a]	5.15[c]	10.47[a]	3.33[a]	6.7[a]	3.25[a]
Bacitracin	660[a]	77[b]	8.53[a]	8.27[b]	2.38[b]	5.21[b]	2.19[b]
Bacillus subtilis	649[a]	80[b]	8.13[b]	8.15[b]	2.32[b]	4.42[c]	2.14[b]
SEM	0.06	0.01	0.12	0.09	0.06	0.16	0.04
P-Value	0.001	0.001	0.001	0.001	0.001	0.001	0.001

[a-c]Means with different letters within the same column are significantly different (p≤0.05); Bacitracin (bacitracin methylene disalicylate): antibiotic growth promoter; SEM = pooled standard error of column wise means comparison.
† number of goblet cells per mm of villus length.

treatments demonstrated that all groups consumed similar amounts of feed to produce eggs. Therefore, the differences in egg production among different treatments may only attributed to the effect of the inclusion of the evaluated feed additives. These findings are consistent, in general, with those of Abdel-Azeem (2005), Grimes *et al.* (2008), and Vilà *et al.* (2009), who reported the improvement of performance indexes of laying hens, turkey poults, and broilers fed probiotics. The enhancement of laying Japanese quail performance observed in the present study may be attributed to a reduction in the proliferation of pathogenic bacteria due to gut environmental changes, as a result of better intestinal microbial balance, nutrient utilization, and enzymatic activities in the birds' gastrointestinal tract (Corzo *et al.,* 2009). Moreover, *Bacillus subtilis* spores are effective and economical exclusion agents, enhancing the digestion and absorption of consumed feed, and consequently, improving body weight and feed conversion ratio (La Ragione and Woodward, 2003). Probiotic supplementation to laying Japanese quail diets significantly increased egg production and egg weight (Ayasan *et al.,* 2006). Other studies, however, did not report any beneficial effects of dietary probiotic supplementation on the egg production of laying Japanese quails (Onol *et al.,* 2003) or laying hens (Yoruk *et al.*, 2004). In the current study, and in agreement with the of Nahashon *et al.* (1996) and Tortuero & Fernandez (1995) in layers, the dietary inclusion of a probiotic containing *Bacillus subtilis* significantly increased the egg weight of Japanese quails. On the other hand, no effects of such probiotics were found by Haddadin *et al.* (1996) and Chen *et al.* (2009) in layers and Balevi *et al.* (2001) and Yoruk *et al.* (2004) in Japanese quails. These controversial results might be related to probiotic strain, concentration, and

the form of bacteria used during the preparation of probiotic (viability, dryness or their products). Different studies (Nahashon *et al.,* 1992; Tortuero & Fernandez, 1995; Nahashon *et al.,* 1996; Haddadin *et al.,* 1996) comparing diets containing higher bacterial counts. Thus, egg weight increase may be related to the vital form of probiotic bacteria at higher doses, of up to $\log_{10}$ cfu/g of probiotic product.

The addition of *Bacillus subtilis* had no effect on eggshell percentage, thickness, or breaking strength. However, Ayasan *et al.* (2006) obtained higher eggshell weight when supplementing a probiotic in laying Japanese quail diets. A similar result was found in layers fed aprobiotic-supplemented diet (Pedroso *et al.,* 2001; Onol *et al.,* 2003; Yoruk *et al.,* 2004). Mahdavi *et al.* (2005), on the other hand, reported that addition of a probiotic had no significant effect on eggshell hardness, eggshell thickness, or Haugh unit scores. A significant improvement of internal egg quality, as measured in Haugh units, was reported in layers fed a probiotic containing dried distillers grain and corn with solubles (Jensen & Maurice, 1978). Subsequent studies indicated that trace elements may be involved in the mineralization of eggshells (Jensen *et al.,* 1978). Tortuero & Fernandez (1995) reported that variations in plasma mineral levels of laying hens fed selected microbial cultures as probiotic supports the hypothesis that trace elements improve albumen quality with microbial supplementation (Tortuero & Fernandez, 1995).

In the present study, dietary probiotic inclusion significantly reduced plasma cholesterol levels relative to the control treatment, and LDL levels relative to the antibiotic treatment. These findings are in general agreement with previous studies (Haddadin *et al.,* 1996; Mahdavi *et al.,* 2005) that demonstrated the role of gastrointestinal tract microorganisms in the recycling of lipids. In Japanese quails, Genedy & Zewil (2003) reported a decline in total lipid and cholesterol blood levels of the birds fed a probiotic. In the presence of specific microorganisms such as *Bacillus subtilis*, the reabsorption of primary bile salts is prevented, which then can be converted into secondary bile salts. Moreover, such microorganisms are able to synthesize esterase and lipase enzymes, converting free fatty acids (except triglycerides) into their esterified forms in the intestine, reducing the chance of triglyceride absorption into the plasma (Pellegrini *et al.,* 2015). The mechanism of the cholesterol-lowering effects of probiotics is the enzymatic deconjugation of bile acids by the hydrolysis of bile salts which allows them to bind to cholesterol in the small intestine (Begley *et al.,* 2006).

Bacillus subtilis significantly reduced crypt depth and increased villus height and villus to crypt ratio in the ileum. An increase in villus length in broilers fed probiotics and prebiotics was reported, which was associated with an increased presence of lactobacilli and bifidobacterial, and with reduced intestinal colonization by Salmonella and coliforms (Baurhoo *et al.,* 2007). Feeding *Bacillus subtilis* in current study markedly reduced the populations of Salmonella, coliforms and *E. coli*.

The rationale of the direct administration of probiotic bacteria to broilers is to reduce infections caused by pathogens by the mechanism of competitive exclusion, in which bacteria compete for space and nutrients. Hence, the early administration of 'good' bacteria for competitive exclusion has been proposed as a method to prevent undesirable infections. There are several reports on the capacity of live bacterial cultures (Callaway *et al.,* 2008; Corrier *et al.,* 1998; Nisbet *et al.,*1998; Wagner *et al.,* 2003) and probiotic organisms (Higgins *et al.,* 2007; Higgins *et al.,* 2008; Higgins *et al.,* 2010; Patterson & Burkholder, 2003; Vicente *et al.,* 2008) to reduce the colonization of the gastrointestinal tract of poultry by opportunistic pathogens. The current study showed that the dietary inclusion of *Bacillus subtilis* enhanced the birds' immunity against Newcastle disease and avian influenza. The destruction of the bursa of Fabricius by viral agents impairs the humoral immune response of poultry. Most of probiotics benefit the host by immune regulation, particularly through the balanced control of pro-inflammatory and anti-inflammatory cytokines (Li *et al.,* 2009; Foligne *et al.,* 2010; Jobin, 2010). Several studies have provided unequivocal evidence that certain probiotics strains are able to stimulate several aspects of innate (Farnell *et al.,* 2006; Boirivant *et al.,* 2008; Romanin *et al.,* 2010; Weiss *et al.,* 2010) and humoral immunity (Galdeano *et al.,* 2009). Moreover, the extracellular enzymes produced by *Bacillus subtilis* may boost the general gut immune function (Chen *et al.,* 2009). Yet, further knowledge on how probiotics mediate these health benefits, specifically relative to the prevention of Salmonella infections, is required.

In conclusion, laying Japanese quails fed a probiotic containing *Bacillus subtilis* significantly increase egg production, egg weight, and villus height values, and reduced plasma cholesterol and LDL levels, as well as the intestinal populations of *Salmonella*, coliforms, and *E. coli* compared with the control group. The spore-forming probiotic partially enhanced the performance parameters, promoted significant changes in gut

physiology, and improved the general health of the laying Japanese quails in the current study. Considering the recent international legislation and domestic consumer pressures to withdraw antibiotics, spore-forming probiotics can offer an alternative to such compounds for the production of healthy in Japanese quail eggs.

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