Effect of *Bacillus subtilis* on growth performance, bone mineralization, and bacterial population of broilers fed with different protein sources

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ABSTRACT The objective of this study was to investigate the impacts of *Bacillus subtilis* (**BS**), ATCC 6051a strain, as a probiotic bacterium in broiler diets based of 2 protein sources (soybean meal [**SBM**] and cowpea seeds [**CWP**]), on growth performance (**GP**), carcass traits, bone mineralization, and microflora population (0 to 42 d age). The SBM and CWP starter, grower, and finisher diets were tested in the presence or absence of BS (5.0×10^{11} CFU spores g^{-1} feed) in a 2×2 factorial arrangement of treatments in a completely randomized design. Broilers were randomly assigned to 4 dietary treatments with 6 replicate pens per treatment (20 chicks per pen). The results showed that broilers fed CWP had comparable GP (body weight gain, feed intake, and feed conversion ratio) to the birds fed the SBM diet. Carcass, breast and legs' yield, organ size (i.e., gizzard, liver, pancreas, small intestine, cecum), and bone development were not affected by the protein source. The addition of BS in both types of diet improved BWG ($P < 0.001$) and feed efficiency, especially in the grower and finisher period ($P = 0.047$; $P = 0.043$, respectively). In addition, BS significantly decreased abdominal fat ($P = 0.026$) and cecum weight ($P = 0.034$) and increased tibia bone P concentration ($P = 0.015$). Furthermore, BS decrease cecal pH ($P = 0.010$) and reduced *Escherichia coli* and *Staphylococcus* spp. from cecum and excreta broilers ($P < 0.001$; $P < 0.0001$, respectively). It is concluded that the BS significantly improved the GP of broilers and can beneficially affect the gut and excreta bacterial community in both SBM and CWP diets.

Key words: cowpea, probiotic, broiler performance, gut microflora

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INTRODUCTION

The poultry industry is focused on an alternative to antibiotics for maintaining health and performance under commercial conditions. Probiotics, which are live cultures of harmless bacteria or yeast species, have been increasingly adopted as an alternative to antibiotic growth promoters in poultry diets (Mountzouris et al., 2010; Zhang and Kim, 2014; Gadde et al., 2017). Previous studies have shown that dietary supplementation with *Bacillus* spp.-based probiotics (*Bacillus coagulans*, *Bacillus subtilis* [**BS**], *Bacillus licheniformis*, and *Bacillus amyloliquefaciens*) could be successfully used in poultry diets and have been shown to have growth-promoting effects (Wang and Gu, 2010; Liu et al., 2012; Sen et al., 2012; Ahmed et al., 2014; Jeong and Kim, 2014; Park and Kim, 2014; Zhen et al., 2018).

B. subtilis a nonpathogenic bacteria are spore-forming, and the genus has been considered as one of the most successful probiotic bacteria in poultry nutrition because of its resistance to a wide range of temperatures during the feed manufacturing process and long-term storage at ambient temperature and survive the low pH, bile, and other antimicrobial molecules in the gastrointestinal tract (**GIT**) of the host (Sen et al., 2012; Alloui et al., 2013; Goodarzi Boroojeni et al., 2016; Mahmoud et al., 2017; Dumitru et al., 2019). Dietary supplementation with BS increases the abundance of beneficial bacteria, decrease of pathogens, and improve growth performance (**GP**) and carcass quality in broiler chickens (Li et al., 2016; Bai et al., 2017; Gao et al., 2017; Mahmoud et al., 2017; Reis et al., 2017; Flores et al., 2019).

Soybean meal (**SBM**) is the premier protein source used in the poultry industry. However, sometimes it is

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difficult to formulate the least-cost diets as a result of its price fluctuation. Substituting (partly) these high-quality feed ingredients with alternative feed materials, often local products could be a valuable way to enhance the sustainability of poultry production further worldwide. Such alternative protein sources (i.e., legumes) are usually cheaper but may be less digestible so that poultry performance can be reduced, and the incidence of gut health problems can increase. Among these legumes, there are also cowpea seeds (**CWP**; *Vigna unguiculata* [L.] Walp), whose research interest has grown in the past few years. Cowpea seed is a crop with reasonable high protein content (23–32%) (José et al., 2014), 50 to 60% carbohydrate (Khalid and Elharadallou, 2013; Kirse and Karklina, 2015), and about 1% fat (Kirse and Karklina, 2015) in dry basis, representing an alternative source to soy and bean crops, under drought conditions, and is locally grown in Romania (Drăghici et al., 2016a,b). It is not only rich in nutrients but also nutraceuticals such as dietary fiber, antioxidants, and polyunsaturated fatty acids and polyphenols, minerals, and vitamins (Trinidad et al., 2010; Shetty et al., 2013; Zia-Ul-Haq et al., 2013; Baptista et al., 2017; Jayathilake et al., 2018). Additionally, these bioactive components may confer an additional prebiotic effect promoting the GP or activities of beneficial microorganisms in the GIT.

To date, there were no published studies on how the novel strain of *B. subtilis* ATCC 6051a exerts its beneficial effects in broiler chickens. Thus, the present study aimed to assess the effects of BS inclusion in broiler diets with different protein sources on GP, carcass traits, and bone development and mineralization. The impact of BS supplementation on the GIT and excreta microflora population were also evaluated.

MATERIALS AND METHODS

Probiotic Strain

A bacteria strain of BS was purchased from the American Tissue Culture Collection (ATCC 6051a). The selection criteria of BS strain was based on the probiotic properties including growth rate, identification, and characterization of capacity to ferment carbohydrate substrates (API 50 CHB), amylase and protease production, survivability in low pH, bile salts, the growth rate of spores, hemolysis production, and susceptibility to antibiotics were assessed in vitro (data not shown). The bacterial strain was grown in a nutrient medium (g/L: tryptone 10; meat extract 5; sodium chloride 5; pH medium 7.2 ± 2 before autoclaving) and incubated in a shaker-incubator (200 rpm) at 37°C for 24 h in aerobic conditions as reported previously by Dumitru et al. (2018, 2019). The inoculum was analyzed by serial 10-fold dilutions using phosphate buffered saline solution (**PBS**), and then, 1 mL from 10^{-10} to 10^{-12} was placed on nutrient agar medium (g/L: tryptone 5; meat extract 3; bacteriological agar 5; distilled water). To determine the viability of spores-forming, the vegetative cell was inactivated by thermal treatment (80°C, 10 min). Serial dilutions in PBS on nutrient medium agar were followed by incubation at 37°C in an aerobic atmosphere for 24 h. The biomass surviving spores were collected by centrifugation (5,000 rpm, 10 min, 4°C), washed twice, and then resuspended in PBS solution. The strain had the capacity to sporulate 1×10^{11} CFU spores/mL. In our study, the initial spores count was adjusted at 5×10^{11} CFU/mL and kept at 4°C until utilization in broilers feeds.

Experimental Design

The birds' care and use protocol were approved by the Animal Care and Use Committee at the National Research-Development Institute for Biology and Animal Nutrition (INCDBNA-IBNA) Baloteşti, Romania, following the principles of EU Directive 2010/63/EU and Romanian Law on Animal Protection.

A total of four hundred eighty, 1-day-old healthy mixed-sex broiler chickens (Ross 308) with similar initial weights (46.5 ± 0.23 g) were used. Two sources of protein-based diet (local CWP as a potential substitute for SBM) were tested in the presence (+) or absence (−) of BS in a 2 × 2 factorial arrangement of treatments in a completely randomized design. Broiler chicks were randomly assigned to 4 dietary treatments with 6 replicate pens per treatment and 20 chicks per pen. Before starting the experiment, the strain biomass, kept at 4°C, was adjusted at 5×10^{11} CFU spores g^{-1} feed and included and blended with diets every week. After mixing, the diets supplemented with BS were analyzed for spore counts weekly.

The feeding program was divided into 3 feeding phases: starter (days 1–10), grower (days 11–24), and finisher (days 25–42). Diets for each feeding phase were formulated to be isocaloric, isonitrogenous, with similar content of total lysine, total sulphur amino acids (**TSAA**; Table 1), calcium and available phosphorous, and to meet or exceed breeder guidelines (Ross 308, Aviagen Ltd., Midlothian, UK). Diets were manufactured in mash form, without the inclusion of growth promoters or antibiotics. However, narasin as a coccidiostat (Monteban G100, Elanco GmbH) and phytase (Axtra PHY 5,000 L, Danisco Animal Nutrition, Marlborough, UK) as exogenous enzymes were included in premixes of all 4 experimental diets. Feed and water were provided *ad libitum*. Broiler chickens were raised in a temperature-controlled room with pens of identical size (1.75 × 1.55 m). Room temperature was maintained at 34°C for the first 5 d and then gradually reduced according to standard management practices until a temperature of 22°C by using thermostatically controlled heaters, fans, and adjustable sidewall inlets. Lighting was provided for 23 h/d from 1D to 7D, and from 8D, the light decreased by 1 h a day until 20 h, according to EU legislation (EU Council Directive 2007/43/EC). Broilers were vaccinated at the hatch for Marek's, Newcastle, and Infectious Bronchitis Disease.

Table 1. Ingredient and chemical composition of diets (as-fed basis).

	Starter (day 1–10)		Grower (day 11–24)		Finisher (day 25–42)	
Items	SBM	CWP	SBM	CWP	SBM	CWP
Ingredient (%)						
Corn	55.73	46.12	56.66	47.28	64.26	54.73
Soybean meal (45.7% CP)	33.10	27.20	31.56	25.50	25.10	19.10
Corn gluten	4.30	4.30	4.00	4.00	3.50	3.50
Cowpea meal (26.6% CP)	0.0	15.00	0.0	15.00	0.0	15.00
Soybean oil	1.50	2.10	2.90	3.40	2.50	3.10
Monocalcium phosphate	1.67	1.63	1.66	1.65	1.47	1.44
Calcium carbonate	1.71	1.72	1.46	1.46	1.27	1.28
Salt (NaCl)	0.28	0.28	0.28	0.28	0.28	0.28
L-Lysine HCl	0.32	0.24	0.17	0.10	0.29	0.22
DL-Methionine	0.31	0.33	0.23	0.25	0.26	0.28
Choline-chloride (50%)	0.08	0.08	0.08	0.08	0.07	0.07
Vitamin-mineral mixture[1]	1.00	1.00	1.00	1.00	1.00	1.00
Bacillus subtilis ATCC 6051a[2]	−/+	−/+	−/+	−/+	−/+	−/+
Calculated composition						
ME (MJ/kg)	12.56	12.57	12.98	12.97	13.20	13.21
CP	23.0	23.0	22.0	22.0	19.50	19.50
Lysine, total	1.40	1.40	1.32	1.24	1.16	1.16
Lysine, digestible	1.34	1.34	1.18	1.16	1.05	1.04
Methionine + cysteine, total	1.05	1.05	0.95	0.95	0.91	0.91
Methionine + cysteine, digestible	0.97	0.97	0.87	0.86	0.84	0.83
Ca	1.00	1.00	0.90	0.90	0.79	0.79
Available P	0.45	0.45	0.45	0.45	0.40	0.40
Crude fat	4.38	5.10	5.77	6.40	5.57	6.29
Crude fiber	2.85	3.27	2.79	3.20	2.61	3.02

Abbreviations: CWP, cowpea; SBM, soybean meal.

[1]Supplied per kg diet: 12,000 IU vitamin A, 5,000 IU vitamin D3, 75 mg vitamin E, 3 mg vitamin K_3, 3 mg vitamin B_1, 8 mg vitamin B_2, 5 mg vitamin B_6, 0.016 mg vitamin B_{12}, 13 mg pantothenic acid, 55 mg nicotinic acid, 2 mg folic acid, 0.2 mg biotin, 120 mg Mn, 100 mg Zn, 40 mg Fe, 16 mg Cu, 1.25 mg I and 0.3 mg Se, 70 mg Monteban G100, 0.2 g Axtra PHY 5,000 L (1,000 FTU).

[2]- = probiotic not included in the diet; + = probiotic included in the diet.

Feed Analyses and Growth Performance

Samples of ingredients and feeds were analyzed in duplicate for DM, CP, EE, and ash content, using standard procedures by the methods of the Commission Regulation (EC) no. 152 (OJEU, 2009). The contents of neutral detergent fiber and acid detergent fiber were determined using a Fibertec apparatus (automatic system Foss-Tecator, Höganäs, Sweden). Carbohydrate content was estimated as a nitrogen-free extract. The apparent metabolizable energy (**AME**) content of the diets was calculated based on the energy content of individual feed ingredients using European tables of energy values for poultry feedstuffs equation (WPSA, 1989). Amino acids (**AA**; excluding tryptophan, which not determined) were analyzed using a high-performance liquid chromatography system (HPLC Thermo Fisher Scientific Inc., San Jose, CA), according to the conditions described by Ciurescu et al. (2018). Trypsin inhibitor activity (**TIA**) in CWP was analyzed, according to Valdebouze et al. (1980), and was expressed in units of trypsin inhibited (**TIU**). All composition data and AME values are on a DM basis.

Body weights (**BW**) of the chicks and their feed intake (**FI**) were recorded at days 1, 10, 24, and 42. Body weight gain (**BWG**) and feed conversion ratio (**FCR**) during different periods and overall were calculated.

Carcass and Bone Measurements

On day 42 of the experiment, 6 broiler chickens were randomly selected from each treatment, euthanized by cervical dislocation, and weighed individually. The carcasses (without head, neck, feet, and viscera), breast, legs, abdominal fat, liver, pancreas, heart, gizzard, and small intestine were weighed individually. The length of the small intestine (duodenum, jejunum, ileum, and cecum) was also measured and recorded. The relative weight and length of internal organs were expressed as the percentages of hot carcass weight. The tibia from the left leg was removed, deboned, packed into polyethylene bags, sealed, immediately stored in the deep freezer at −20°C, until analyzed, following the procedure described previously by Ciurescu et al. (2014). Briefly, the tibias of killed broilers were autoclaved to remove the tissues and cartilage caps for the determination of relative BW and length. The ground-dried fat-free bones were analyzed using flame atomic absorption spectrometry after microwave digestion, at specific wavelengths of 422.7 nm (Ca), 213.9 nm (Zn), 372.0 nm (Fe), and 403.1 nm (Mn). Bone P concentration was measured by UV-Vis spectrometry, and the ash content was determined by a gravimetric method. The minerals content in samples was expressed in mg or μg per g of ash.

Microbiological Analyses

The same slaughtered broilers were used for microbial counts. The intestinal content (ileum from 1 cm distal to Meckel's diverticulum to the ileo-cecal junction, and cecum) was aseptically removed and placed in sterile plastic bags on ice. Then, in the lab, 10-fold serial dilutions of 1 g of digesta (ileal and cecal content) were homogenized with 7 mL Brain Heart Infusion broth (Oxoid Ltd., England) supplemented with 2 mL glycerol, and immediately frozen at −20°C until the analysis, according to the technique previously described by Sorescu et al. (2019). After defrost, decimal dilutions in PBS (Oxoid Ltd.) were performed, after which samples were assessed for lactic acid bacteria (**LAB**) as well as *Escherichia coli* (*E. coli*; biotype β-hemolytic), *Salmonella* spp., *Clostridium* spp., Coliforms, *Bacillus* spp., and *Enterococcus* spp. The LAB was cultured on de Man, Rogosa, and Sharpe agar (Oxoid CM0361) incubated in anaerobic conditions at 37°C for 48 h (Oxoid jar with Anaerogen 2.5 L). Coliforms were cultured on MacConkey agar (Oxoid CM0007) incubated aerobically at 37°C for 24 h as red, specifically colonies. *E. coli* biotype β-hemolytic was analyzed, as reported by Dumitru et al. (2018). Briefly, it was inoculated 0.01 ml from 10^{-1} dilution on sheep blood agar [Tryptic case soy agar 5% (w/v)] and incubated at 37°C for 24 h in aerobic conditions. *Clostridium* spp. were cultured on Reinforced Clostridial Agar (Oxoid CM0151) incubated anaerobically at 37°C for 48 h. *Enterococcus* spp. were enumerated on Slanetz-Bartley agar (Oxoid CM0377) incubated at 37°C for 48 h in anaerobic conditions, according to the method of Mountzouris et al. (2007), modified by Sorescu et al. (2019). *Bacillus* spp. were counted on nutritive agar medium and *Salmonella* spp. on Salmonella–Shigella agar (Oxoid CM0099), respectively incubated aerobically at 37°C for 24 h. Every sample was repeated 3 times. The microflora enumerations were expressed as $\log_{10}$ CFU per gram.

On day 42, paper drop-sheets were placed in each pen to collect excreta samples. Approximately 8 to 10 fresh droppings (deposited within 2 h) were randomly transferred into clean plastic containers. Samples were immediately frozen on dry ice before they were transferred to the lab to be stored at −18°C until the subsequent determination of microbiological assay. Lactic acid bacteria and *Salmonella* spp. followed the same method from intestinal microbial populations' analysis. Enterobacteriaceae and *E. coli* were enumerated using Levine medium agar (g/L: pancreatic digest of gelatine 10; lactose 10; potassium phosphate 2; eosin Y 0.4; methylene blue 0.065; bacteriological agar 15; pH 7.1 ± 0.2); 1 g of excreta sample was homogenized with 9 mL of Lauryl sulphate broth (enrichment medium) incubated at 37°C for 48 h, in aerobic conditions. Coagulase-positive *Staphylococci* were enumerated on Baird-Parker Agar (Oxoid Ltd.) supplemented with egg yolk tellurite emulsion incubated aerobically at 37°C for 48 h.

To measure the pH, about 1 g of the ileal and cecal digesta of each bird was collected and transferred into 9 mL distilled water (1:10 dilution); then, the pH values were measured (mean of 3 readings) using a portable pH meter (series pH 7 + DHS, XS Instruments, Italy).

Statistical Analysis

The data were subjected to two-way ANOVA using the GLM procedure of SPSS, version 20.0 (SPSS Inc., Chicago, IL). Data were analyzed as a 2 × 2 factorial arrangement of dietary treatment. The statistical model included the effects of protein sources (**Ps**), probiotic addition (**BS**), and their interactions. Replicate-pen was used as the experimental units for the analysis of GP (final BW, BWG, FI, and FCR), whereas data on the carcass, tibia bone traits, pH, and bacterial population of digesta (ileum and ceca), and excreta were based on individual broilers (n = 6). Tukey's *post-hoc* test was used to separate means when interactive effects significantly differed. The results were expressed as treatment means with their pooled SEM. A P-value ≤ 0.05 was considered statistically significant, and P-value between 0.05 to 0.10 was classified as a tendency.

RESULTS

Nutrient Composition of Cowpea

The nutrient composition and AA profile of CWP (cv. Ofelia) that were used for this study are shown in Tables 2 and 3. The analytical results show that CWP contained high levels of CP (295.4 g/kg DM) and AME values (12.8 MJ/kg DM). This cultivar was almost devoid of EE and calcium contents but was high in phosphorus (11.3, 23.1, and 62.6 g/kg DM, respectively). The TIA was low (10.7 TIU/mg; data not shown). Analysis of essential AA (Table 3) showed that all essential AA, except for the TSAA, were present in excessive amounts. As with the characteristics of legumes, CWP was high in lysine (6.8–7% of the protein) but low in TSAA (methionine + cysteine, 2%), compared with the requirements of broiler chickens in the starter phase.

Growth Performance

The main effect of Ps, probiotic addition, or their interaction (Ps × BS) on GP of broiler chickens is reported in Table 4. The results show that broilers fed diets containing CWP had comparable GP (BW, BWG, FI, and FCR) to those fed SBM throughout the study period (day 1 to day 42; $P > 0.05$). Inclusion of BS as probiotic in broiler diets increased final BW ($P < 0.001$) as well as BWG during the grower ($P < 0.01$), finisher ($P < 0.01$), and overall study period ($P < 0.001$) and tended ($P = 0.059$) to increase FI during overall study period (day 0–42). Moreover, during the grower and finisher phase, better FCR ($P = 0.047$ and $P = 0.043$, respectively) was noticed in birds fed BS spores when compared with the birds fed diets without BS. There was no significant interaction between the main factors (Ps × BS) for all GP variables

Table 2. The chemical composition of cowpea seeds (cv. Ofelia), as g/DM.

Nutrients[1]	DM	CP	EE	Fiber	Ash	NFE	NDF	ADF	AME (MJ)[2]	Ca	P
CWP	899.7	295.4	11.3	53.9	44.5	494.6	232.1	44.7	12.8	23.1	62.6

Abbreviations: ADF, acid detergent fiber; AME, apparent ME; Ca, calcium; CP, crude protein; CWP, cowpea; DM, dry matter; EE, ether extract; NFE, nitrogen-free extract; NDF, neutral detergent fiber; P, phosphorous.
[1]In duplicate samples. Calculated value.
[2]European Table of Energy Values for Poultry Feedstuffs (WPSA, 1989).

measured. However, the addition of BS in the diets led to a slight increase FI, resulting in a tendency ($P = 0.083$) for FI during the grower phase when the interaction for main factors was analyzed. Mortality was low (<2.5%) and unrelated to the treatments (data not shown). All deaths occurred within the first week of age and were attributed to stress because of transportation.

Carcass and Bone Traits

The influence of Ps and probiotic addition on carcass, breast and legs' yield, abdominal fat, and organ weights (i.e., heart, liver, gizzard, pancreas, small intestine, and cecum) as well as cecum and small intestine length of birds are presented in Table 5. No significant interaction between the main factors (Ps × BS) was noticed for all carcass characteristics measured, except for liver weight ($P = 0.048$). Feeding broilers up to 6 wk of age with CWP diets did not significantly affect the main traits of the carcass, except for small intestine as well as jejunum portion, whose weights have increased ($P = 0.044$ and $P = 0.028$, respectively) when compared with the birds fed SBM diets. Likewise, no difference was noted on carcass, breast, and legs' yield, and organs size when diets were supplemented with BS, only a tendency to increase the pancreas weight ($P = 0.057$) was observed. Nevertheless, the addition of BS in broilers' diets significantly decreased abdominal fat ($P = 0.026$) and the cecum weight ($P = 0.034$). Furthermore, cecum length ($P = 0.086$) tend to be influenced by BS addition in comparison to the unsupplemented treatments.

Results of the assessment of tibia bone development and bone mineralization in broiler fed diets with different Ps in the presence (+) or absence (-) of BS are summarized in Table 6. Tibia bone development (i.e., relative weight and length) as well as ash, Ca, P, and Fe contents were not affected ($P > 0.05$) by CWP diets, except for Zn concentration whose value tended to decrease ($P = 0.092$), compared with the birds fed SBM diets. The dietary inclusion of BS significantly increases P concentration (7.9%; $P = 0.015$), whereas tibia bone development parameters, ash content as well as Ca, Fe, and Zn values were similar between supplemented and unsupplemented treatments. Therefore, as P content was increased by the diet, a decrease of the Ca/P ratio (7.1%; $P = 0.015$) in BS treatments was observed. There was no interaction between the main factors (Ps × BS) for all tibia parameters measured (at day 42), except for P content ($P = 0.018$) and Ca/P ratio ($P = 0.026$).

Table 3. Amino acids (AA) profile (g/100 g) of cowpea seeds (cv. Ofelia).[1]

AA	CWP	AA	CWP
Lysine	1.844	Phenylalanine	1.394
TSAA	0.679	Tyrosine	0.712
Threonine	1.218	Serine	1.969
Leucine	1.834	Glycine	0.730
Isoleucine	1.203	Alanine	1.058
Arginine	1.784	Aspartic acid	2.585
Valine	1.153	Glutamic acid	5.674

Abbreviations: AA, amino acids; CWP, cowpea; TSAA, total sulfur amino acids (methionine + cysteine).
[1]In duplicate samples.

The Microbial Population of Gut Digesta, Excreta, and pH

The effect of treatments on the pH value and microbial populations of broiler gut digesta (ileum and ceca) are presented in Table 7. The interaction between the main factors (Ps × BS) had no significant effects on microbial populations of ileal and cecal digesta but tended ($P = 0.053$) to lower the pH value in the ileum.

At day 42, birds fed diets supplemented with BS showed a significant decrease in the ileum coliforms ($P < 0.001$) and *E. coli* ($P = 0.040$) counts, whereas *Enterococcus* spp. and *Bacillus* spp. numbers were increased ($P = 0.016$; $P = 0.024$, respectively) compared with treatments without BS. The cecal digesta assay results show that pH value significantly decreases ($P < 0.010$) as well as *Clostridium* and *E. coli* bacteria counts ($P < 0.0001$ and $P < 0.001$, respectively), whereas *Enterococcus* spp. and *Bacillus* spp. numbers increased ($P = 0.016$ and $P = 0.024$, respectively) by dietary supplementation of BS (ATCC 6051a strain). Nevertheless, LAB populations were not affected by dietary supplementation of probiotic in both analyzed parts of the gut. When compared SBM diets with the new local Ps tested, we observed that the LAB, *Enterococcus* spp., and *Bacillus* spp. counts remained comparable in the ileum and cecal part of the GIT. Nevertheless, there was a tendency to decrease the number of coliforms and *E. coli* ($P = 0.081$; $P = 0.078$, respectively) in the cecal digesta.

However, there was no interaction between the main factors (Ps × BS) for the excreta bacterial population of broilers at 42 d of age (Table 7). As shown in the table, the LAB population in the BS treatments was higher ($P > 0.05$), whereas *Enterococcus* spp. and *Staphylococcus* spp. bacteria was lowest ($P = 0.010$ and $P < 0.0001$, respectively) than those without BS. When we compare birds fed SBM and CWP diets, the LAB, *Enterococcus* spp., as well as *E. coli* and *Staphylococcus* counts remained comparable among.

Table 4. Effects of the diets with different protein sources without and with *Bacillus subtilis* ATCC 6051a (BS) on performance variables (mean values[1]) of broilers.

Items	Protein source	Probiotic inclusion[2]	Starter (day 0–10)			Grower (day 11–24)			Finisher (day 25–42)			Overall (day 0–42)			
			BWG (g)	FI (g)	FCR (g/g)	BWG (g)	FI (g)	FCR (g/g)	BWG (g)	FI (g)	FCR (g/g)	BW (g)	BWG (g)	FI (g)	FCR (g/g)
1	SBM	No	244	298	1.22	769	1,245	1.61	1,705	3,180	1.86	2,766	2,720	4,725	1.73
2	CWP	No	243	299	1.23	761	1,230	1.61	1,677	3,120	1.86	2,728	2,682	4,650	1.73
3	SBM	Yes	245	300	1.22	806	1,265	1.57	1,745	3,210	1.84	2,841	2,795	4,770	1.71
4	CWP	Yes	247	303	1.22	799	1,280	1.60	1,719	3,195	1.85	2,810	2,764	4,780	1.73
SEM			5.27	5.74	0.01	7.16	9.25	0.01	9.94	10.91	0.02	21.78	12.43	11.96	0.01
Main effects[3]															
Protein source (Ps)															
SBM			244	299	1.22	788	1,257	1.59	1,725	3,195	1.85	2,803	2,757	4,745	1.72
CWP			245	301	1.23	780	1,255	1.60	1,698	3,155	1.86	2,769	2,723	4,720	1.73
Probiotic inclusion (BS)															
No			244	298	1.22	765^{b}	1,235	1.61^{a}	1,691^{b}	3,145	1.86^{a}	2,747^{b}	2,707^{b}	4,690	1.73
Yes			246	301	1.22	802^{a}	1,272	1.58^{b}	1,733^{a}	3,203	1.84^{b}	2,825^{a}	2,780^{a}	4,775	1.72
P-value															
Ps effect			0.474	0.317	0.348	0.364	0.843	0.444	0.123	0.554	0.771	0.111	0.207	0.346	0.546
BS effect			0.256	0.098	0.347	0.010	0.203	0.047	0.010	0.175	0.043	0.001	0.001	0.059^{T}	0.353
Ps x BS effect			0.877	0.933	0.758	0.556	0.083^{T}	0.892	0.732	0.326	0.755	0.413	0.853	0.648	0.726

[a,b]Means with different superscripts in a row differ significantly ($P < 0.05$), T = tendency to be influenced by treatment.
Abbreviations: CWP, cowpea; Ps, protein sources; SBM, soybean meal.
[1]Data are means of 6 replicate pens with 20 birds per pen.
[2]*Bacillus subtilis*, ATCC 6051a strain: 5.0×10^{11} CFU/g^{-1} feed.
[3]Data were analyzed as a 2 × 2 factorial arrangement.

Table 5. Effects of the diets with different protein sources without and with *Bacillus subtilis* ATCC 6051a (BS) on the carcass traits (mean values[1]) of broilers (day 42).

Items	Protein source	Probiotic inclusion[2]	Carcass[4]	Breast[5]	Legs[5]	Abdominal. Fat[5]	Organs				SIW[6]				SIL[6]			
							Heart[5]	Liver[5]	Gizzard[5]	Pancreas[5]	Duodenum[5]	Jejunum[5]	Ileum[5]	Cecum[5]	Duodenum[6]	Jejunum[6]	Ileum[6]	Cecum[6]
1	SBM	No	72.47	37.96	27.71	1.38	0.63	2.85	1.81	0.22	0.84	2.92	2.15	0.77	1.75	4.29	4.25	1.97
2	CWP	No	71.88	36.95	26.40	1.29	0.54	2.89	1.83	0.26	0.96	3.42	2.87	0.93	1.70	4.52	4.39	1.70
3	SBM	Yes	72.60	37.34	27.54	1.78	0.53	3.33	1.96	0.28	0.93	2.92	2.50	0.64	1.79	4.22	4.37	1.63
4	CWP	Yes	71.68	38.37	26.14	1.56	0.59	2.77	1.81	0.33	0.89	3.64	2.43	0.72	1.74	4.53	4.44	1.52
SEM			0.31	0.44	0.24	0.07	0.01	0.08	0.07	0.02	0.03	0.14	0.13	0.05	0.04	0.09	0.08	0.07
Main effects[3]																		
Protein source (Ps)																		
SBM			72.53	37.65	27.62	1.58	0.58	3.09	1.88	0.25	0.89	2.95^{b}	2.33	0.71	1.77	4.26	4.32	1.80
CWP			71.77	37.66	26.27	1.42	0.57	2.83	1.82	0.30	0.93	3.54^{a}	2.65	0.83	1.72	4.53	4.41	1.61
Probiotic (BS)																		
No			72.17	37.46	27.05	1.67^{a}	0.59	2.87	1.82	0.24	0.90	3.18	2.52	0.85^{a}	1.73	4.41	4.32	1.84
Yes			72.14	37.85	26.84	1.34^{b}	0.56	3.05	1.89	0.31	0.91	3.28	2.47	0.68^{b}	1.77	4.38	4.41	1.58
P-value																		
Ps effect			0.249	0.993	0.103	0.268	0.650	0.086^{T}	0.632	0.152	0.537	0.028	0.209	0.176	0.588	0.153	0.619	0.193
BS effect			0.952	0.666	0.602	0.026	0.344	0.230	0.643	0.057^{T}	0.909	0.693	0.853	0.034	0.708	0.863	0.663	0.086^{T}
Ps x BS effect			0.798	0.277	0.903	0.655	0.285	0.048	0.522	0.946	0.251	0.660	0.129	0.657	0.996	0.824	0.843	0.581

[a,b]Means with different superscripts in a row differ significantly ($P < 0.05$). T = tendency to be influenced by treatment.
Abbreviations: CWP, cowpea; SBM, soybean meal; SIL, small intestine length; SIW, small intestine weight.
[1]Data are means of 6 birds per treatment.
[2]*Bacillus subtilis*, ATCC 6051a strain: 5.0×10^{11} CFU/g^{-1} feed.
[3]Data were analyzed as a 2 × 2 factorial arrangement.
[4]Represents as weight (g) of without head, neck, feet, and viscera carcass as 100 g of live body weight.
[5,6]Calculated as weight or length (g or cm) of organs as 100 g of carcass weight.

Table 6. Effects of the diets with different protein sources without and with *Bacillus subtilis* ATCC 6051a (BS) on tibia bone development and mineralization (mean values[1]) of broilers (day 42).

Items	Protein source	Probiotic inclusion[2]	Tibia, weight[4]	Tibia, length[4]	Ash, %	Minerals content				
						Ca, mg/g	P, mg/g	Fe, µg/g	Zn, µg/g	Ca: P Ratio
1	SBM	No	0.63	0.46	57.01	319.89	167.41	174.42	346.37	1.91
2	CWP	No	0.64	0.47	58.20	319.36	158.80	195.45	333.88	2.01
3	SBM	Yes	0.68	0.46	58.63	316.66	167.98	131.62	347.40	1.89
4	CWP	Yes	0.62	0.46	58.01	325.73	183.88	144.70	319.81	1.77
SEM			0.02	0.01	0.42	2.76	3.25	13.80	5.59	0.03
Main effects[3]										
Protein source (Ps)										
SBM			0.65	0.46	57.82	318.28	167.70	153.02	346.88	1.90
CWP			0.63	0.47	58.11	322.55	171.34	170.08	326.85	1.89
Probiotic (BS)										
No			0.64	0.46	57.61	319.63	163.10^{b}	184.93	340.13	1.96^{a}
Yes			0.65	0.46	58.32	321.20	175.93^{a}	138.16	333.60	1.83^{b}
P-value										
Ps effect			0.401	0.953	0.762	0.500	0.405	0.395	0.092^{T}	0.866
BS effect			0.744	0.900	0.451	0.802	0.015	0.123	0.551	0.009
Ps x BS effect			0.361	0.921	0.345	0.969	0.018	0.887	0.492	0.026

[a,b]Means with different superscripts in a row differ significantly ($P < 0.05$). T = tendency to be influenced by treatment.
Abbreviations: CWP, cowpea; SBM, soybean meal; SIL, small intestine length; SIW, small intestine weight.
[1]Data are means of 6 birds per treatment.
[2]*Bacillus subtilis*, ATCC 6051a strain: 5.0×10^{11} CFU/g^{-1} feed.
[3]Data were analyzed as a 2 × 2 factorial arrangement.
[4]Calculated as weight or length (g or cm) of bone as 100 g of carcass weight.

DISCUSSION

The results of chemical analysis of CWP seeds in the current work are comparable with those already reported by Tshovhote et al. (2003), who found that the protein content ranged from 25.35 to 26.43%, and the fiber content was of 5.15 and 5.81%, respectively, for 3 cowpea cultivars (Glenda, Agrinawa and Indigenous cowpea). Nevertheless, the CP and EE contents in local CWP (cv. Ofelia) are lower (283 g/kg DM and 73 g/kg DM, respectively) than in soybeans (410 g/kg DM and 200 g/kg DM respectively) as recorded by NRC (1994). The fiber contents of CWP seeds are equal to or lower than other legumes such as peas (*Pisum sativum*) and lentils (*Lens culinaris* medik.), as previously reported by Ciurescu et al. (2018). When evaluated, the content of total AA diverse reports on this issue have shown that the limiting AA in CWP is TSAA, followed by tryptophan and threonine, whereas CWP is an excellent source of lysine. Similar results were found by Tshovhote et al. (2003), Vasconcelos et al. (2010), and Anjos et al. (2016). Nutritionally, CWP protein has a good AA profile because it presents all the essential AA (i.e., those that cannot be synthesized by the broiler body and therefore, must be obtained by the diet) in high quantity. The amount of TIA in CWP seeds (cv. Ofelia) was lower than the values reported by Vasconcelos et al. (2010). It can be concluded that the similarities and the difference of chemical composition between CWP seeds in this experiment and other reports might be because of variety differences.

Earlier studies that investigated the effect of the use of CWP as a replacement for SBM showed inconsistent results. The results of the current study are in line with the findings of Abdelgani et al. (2013), who reported that fed graded levels of raw cowpea (0, 5, 10, and 15%) did not affect broilers' performance and carcass characteristics. Similarly, results were reported by Embaye et al. (2018) as they fed graded levels of cowpea seeds to broilers (at 0 to 20%), indicating that cowpea can replace SBM. Moreover, in an own previous study with 500 Cobb chicks fed up to 200 g/kg of raw lentil seeds (cv. Eston and cv. Anicia), no adverse effect on performance, carcass, digestive organ sizes, and cecal pH were noted, compared with broilers fed SBM (Ciurescu et al., 2017). On the contrary, Gumaa-Balaiel (2014) recommended up to 10% inclusion of untreated cowpea seeds in broiler diets. Also, Akanji et al. (2016) attributed the depression in growth to the presence of antinutritional factors (i.e., protease inhibitors) as they interfere with the digestion of protein and utilization of minerals. In this study, the good performances of broiler suggest that the CWP (cv. Ofelia) studied contain levels of antinutritional factors less harmful. In addition, the studied diets were formulated to be iso-nitrogenous, isocaloric, and with similar content of total lysine and TSAA by replacing SBM with CWP.

Feeding probiotics (e.g., BS ATCC 6051a strain) in adequate amounts might confer beneficial effects on the health status and productivity growth on the chickens. There are no studies reporting the effects of BS in broilers fed legume seeds–based diets on GP,

Table 7. Effects of the diets with different protein sources without and with *Bacillus subtilis* ATCC 6051a (BS) on the gut and excreta microflora population (log_{10} CFU/g) and digesta pH (mean values[1]) of broilers (day 42).

			Ileum							Cecum							Excreta			
Items	Protein source	Probiotic inclusion[2]	pH	LAB	Coliforms	*Clostridium* spp.	*Entero coccus* spp.	*Bacillus* spp.	*E. coli*	pH	LAB	Coliforms	*Clostridium* spp.	*Entero coccus* spp.	*Bacillus* spp.	*E. coli*	LAB	*Entero coccus* spp.	*E. coli*	*Staphylo coccus* spp.
1	SBM	No	5.67	9.76	6.98	5.88	6.12	0.67	1.37	7.00	9.26	7.76	6.93	5.64	0.01	5.25	10.00	12.44	11.22	9.00
2	CWP	No	5.00	9.14	7.12	5.51	6.01	2.07	3.67	6.96	9.10	7.24	6.82	6.10	0.67	3.37	10.05	12.45	11.27	8.94
3	SBM	Yes	4.83	8.99	6.04	5.43	7.18	4.09	1.05	6.83	9.20	7.45	5.55	6.11	4.63	1.81	10.00	12.42	10.99	9.00
4	CWP	Yes	5.33	9.07	5.09	5.49	6.39	2.72	0.02	6.80	8.93	7.20	5.75	5.70	3.65	2.06	10.06	12.44	11.22	8.88
SEM			0.14	0.15	0.20	0.20	0.14	0.42	0.46	0.09	0.12	0.11	0.13	0.19	0.26	0.39	0.11	0.03	0.20	0.12
Main effects[3]																				
Protein source (Ps)																				
SBM			5.24	9.38	6.51	5.66	6.65	4.08	1.21	6.92	9.23	7.61	6.24	5.88	2.31	3.53	10.01	12.43	11.11	9.00
CWP			5.17	9.11	6.09	5.50	6.20	2.40	1.84	6.88	9.02	7.21	6.29	5.90	2.16	2.06	10.05	12.45	11.24	8.91
Probiotic (BS)																				
No			5.33	9.45	7.05^{a}	5.70	6.07^{b}	1.37^{b}	2.53^{a}	6.98^{a}	9.18	7.50	6.88^{a}	5.87	0.33^{b}	4.31^{a}	10.02	12.45^{a}	11.24	8.97^{a}
Yes			5.08	9.04	5.56^{b}	5.46	6.79^{a}	3.40^{a}	0.53^{b}	6.82^{b}	9.07	7.33	5.64^{b}	5.91	4.14^{a}	1.28^{b}	10.03	12.43^{b}	11.11	8.94^{b}
P-value																				
Ps effect			0.770	0.331	0.178	0.700	0.121	0.982	0.500	0.329	0.391	0.081^{T}	0.868	0.941	0.771	0.078^{T}	0.177	0.363	0.182	0.234
BS effect			0.385	0.143	0.001	0.571	0.016	0.024	0.040	0.010	0.637	0.495	0.000	0.931	0.000	0.001	0.122	0.010	0.187	0.000
Ps x BS effect			0.053^{T}	0.220	0.114	0.598	0.229	0.109	0.081^{T}	0.223	0.810	0.335	0.568	0.233	0.131	0.614	0.453	0.305	0.363	0.327

[a,b]Means with different superscripts in a row differ significantly ($P < 0.05$). T = tendency to be influenced by treatment.
Abbreviations: CWP, cowpea; LAB, lactic acid bacteria; Ps, protein sources; SBM, soybean meal.
[1]Data are means of 12 birds per treatment.
[2]*Bacillus subtilis*, ATCC 6051a strain: 5.0×10^{11} CFU/g^{-1} feed.
[3]Data were analyzed as a 2 × 2 factorial arrangement.

carcass trait, and bone mineralization as well as bacterial population. In the present study, the inclusion of BS as probiotic in broilers' diets significantly increased BWG during grower and finisher period and better BWG at the end of the experiment ($P < 0.001$). Broilers fed diets containing BS also showed lower FCR during grower as well as the finisher period. These results support the previous reports that other strains of BS, or a probiotic with a predominance of BS, improved BWG and FCR of broiler chicks (Jeong and Kim, 2014; Li et al., 2016; Gao et al., 2017; Mahmoud et al., 2017; Zhen et al., 2018; Flores et al., 2019). The reasons for the improvement in BWG and FCR of broilers fed a BS probiotic supplement were probably because of the increased FI or to the ability to produce some extracellular enzymes such as amylase, protease, and lipase (Dumitru et al., 2019) that improved nutrient digestibility. Another possible reason is that *Bacillus* spp. also produces some unknown growth-promoting factors by directly fermenting in the gut (Hung et al., 2012) and then increases small intestine peristalsis, improves feed digestibility and availability, and promotes GIT health (Cartman et al., 2008; Gu et al., 2015). The present study also indicates that broilers fed diets with BS addition had a significantly lower percentage of abdominal fat ($P = 0.026$), in comparison without probiotic diets, whereas the other carcass traits evaluated, including breast and legs' yield as well as heart, liver, gizzard, and pancreas size were not affected. Beneficial effects of BS on organs size were consistent with some studies conducted in broilers (Zhang et al., 2012) who reported that the relative weights of liver and bursa of *Fabricius* were unaffected by dietary inclusion of 10^8 CFU *B subtilis*/kg. Additionally, Wang et al. (2017) found that the *Lactobacillus johnsonii* (1×10^6 CFU/g diet) could decrease fat deposition, which is considered waste in the poultry industry (Liu et al., 2016). No clear mechanisms have been reported responsible for the reduction of lipid synthesis by probiotics. It might, in part, be because of an increase of beneficial bacteria such as LAB that decrease the activity of acetyl-CoA carboxylase, which is the rate-limiting enzyme in fatty acids synthesis. *B. subtilis* played an important role in broilers' health. In our study, a difference in cecum weight and a tendency on cecum length was found as effect of BS addition. To our knowledge, little is known about the BS effect on affecting these measurements in broiler chicks. Recently, Reis et al. (2017) have demonstrated that the BS strain (DSM 17299) significantly decreases the relative weight and length of the GIT at 42D, especially for duodenum and jejunum, with a strong positive correlation between duodenal relative weight and length.

In the current study, BS addition significantly improved several bone quality parameters (e.g., tibia P concentration, as well as Ca: P ratio), and this could be related to more efficient utilization of the diets due to the production of exogenous enzymes by the BS probiotic product. There are few studies conducted in broilers indicating the positive effects of *Bacillus* spp.-based probiotic on bone health (Mutus et al., 2006; Latorre et al., 2017). They observed the positive influence of probiotic bacteria on several indices of tibia bones (i.e., percentage of ash and P content, tibiotarsal index). On the contrary, Sadeghi's (2014) showed that the *BS* supplement had no significant effect on crude ash and Ca contents of tibia bones. To the best of our knowledge, there is no research on the effects of CWP on bone health in poultry. In our previous study, we found that up to 24% of chickpea seeds (*Cicer arietinum* L., cv. Burnas as partial replacement of SBM) in starter and finisher broiler turkey diets showed similar effects on bone tibia mineralization (Ciurescu et al., 2020).

The cecum is an important site of fermentation and influences poultry health and production. There is some evidence that BS spp. favor the growth of lactic acid-producing bacteria (Gao et al., 2017; Latorre et al., 2017) and may lower the pH of chickens' GIT (Wu et al., 2011). Acidification of the GIT environment was found to determine an unfavorable medium for pathogens, and addition of a probiotic bacteria can determine a barrier effect to prevent colonization by enteropathogens (Wu et al., 2011; Jeong and Kim, 2014), which corresponds to the result in the present study. Another explanation would be that BS could suppress *E. coli* while promoting anaerobic intestinal probiotics growth in symbiosis with them (Stanley et al., 2014). Ushakova et al. (2013) found that BS can secrete pathogen-suppressive substances that have bacteriostatic action on common pathogens such as *Staphylococcus aureus* and *E. coli* which were highly sensitive to sample concentrate, and the bacteriostatic effect is equivalent to normal antibiotics. Therefore, our results suggest that the dietary inclusion of BS (ATCC 6051a strain) based on in vitro enzyme production profiles (Dumitru et al., 2019) contributed to enhance performance and bone quality as well as improve GIT microbial balance in broiler consuming SBM and CWP diets.

CONCLUSIONS

Cowpea (*V. unguiculata* [L.] Walp, cv. Ofelia) seeds can be used as an alternative protein source to replace SBM in broiler chickens diets, at inclusion levels up to 150 g/kg, to support growth, without any detrimental effects on birds. Cowpea (cv. Ofelia) is an excellent source of essential nutrients, including AA such as lysine and tryptophan. In addition, this legume is rich in nutraceuticals compounds such as dietary fiber. The results obtained in this study clearly indicate that where cowpea can be grown locally, low-input farming systems would benefit from the use of this source of protein for broiler feed.

B. subtilis ATCC 6051a supplementation positively affect the GP of broilers. The probiotic tested also decreased abdominal fat and modulated GIT microflora by enhancing the proliferation of beneficial bacteria, such as *Lactobacillus* and *Bacillus* spp., and by inhibiting potential pathogens, including *E. coli* and Coliforms bacteria. In the light of these results and the limitations of the present study, additional work will be carried out to develop suitable ways of utilization of this potential probiotic strain in the feed as an antibiotics replacement.

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