Efficacy of *Bacillus subtilis* and bacitracin methylene disalicylate on growth performance, digestibility, blood metabolites, immunity, and intestinal microbiota after intramuscular inoculation with *Escherichia coli* in broilers

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ABSTRACT This experiment was conducted to evaluate the effect of *Bacillus subtilis* (BS) on broiler performance and health after intramuscular inoculation with *E. coli* and compare its effect with a growth promoter antibiotic. In a completely randomized design manner, 360 male Ross 308 chicks were divided into 6 treatments and 5 replicates of 12 chicks in each replicate. Experimental treatments included control diet, control + *E. coli* (0.5 mL of culture containing 10^8 CFU of *E. coli*/ml), control + 0.1% BS, control + 0.05% bacitracin methylene disalicylate (BMD), control + *E. coli* and BS, and control + *E. coli* and BMD in a factorial arrangement (3 × 2). Addition of BMD or BS to the control diet significantly ($P < 0.01$) increased body weight and decreased FCR, but *E. coli* challenge adversely reduced ($P < 0.01$) body weight and increased FCR, so that the addition of BMD or BS did not compensate growth reduction. *E. coli* challenged chicks had the lowest vaccine titers for ND, IB, AI, and IBD and the highest were observed in chicks fed BS. The *E. coli* challenge significantly ($P < 0.01$) increased albumin, globulin, cholesterol, triglyceride, LDL, ALT, and ALP indices. Addition of BMD and BS decreased albumin and globulin in challenged chick's plasma but had no effect on plasma lipid profile concentration. The *E. coli* challenge decreased villus height and increased crypt depth and goblet cell numbers significantly ($P < 0.01$). In birds subjected to BMD or BS, crypt depth decreased and villus height increased ($P < 0.01$), compared with the control diet. Challenge of *E. coli* significantly ($P < 0.01$) increased the bacterial population of *E. coli*, coliforms, and *Salmonella* in cecal parts of broilers' intestines. In challenged birds receiving BMD or BS, *E. coli*, coliform, and *Salmonella* populations of ceca showed a significant ($P < 0.01$) reduction. Both BMD and BS increased the digestibility of nutrients significantly ($P < 0.01$), but a reduction was observed in *E. coli* challenged groups. Results of the study suggest that spore-forming probiotics are partially effective in unsuitable rearing situations such as colibacillosis in which the load of harmful bacteria is high.

Key words: *Bacillus subtilis*, bacitracin methylene disalicylate, *E. coli* infection, performance, broiler

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INTRODUCTION

The poultry industry is a sector with rapid development among all Iranian agricultural fields (Manafi, 2015). *Escherichia coli* (*E. coli*) is a Gram-negative bacterium that can cause various forms of diseases in poultry called colibacillosis, infecting all classes and ages of poultry. The *E. coli* is ubiquitous wherever feces from animals are found and wherever related diseases continue to be at or near the top of the list of diseases of broilers, turkeys, and egg layers. There are many types of *E. coli*, and most of them are harmless, but some can cause bloody diarrhea, severe anemia, or kidney failure and urinary tract infections leading to severe mortality (Baurhoo et al., 2007). Broilers get *E. coli* infections by coming into contact mainly with contaminated feed and water. It can be transferred to meat and the human body through subsequent processing. The concern for spread is related only to pathogenic strains that cause diseases. All chicks actually become infected mainly at the hatchery in hatch cabinets, and later by coming into contact with contaminated feed and water. If the infected meat is not cooked to 71°C, the bacteria can survive and infect human beings through spreading from one farm to another by technicians or facilities. Treatment strategies include attempts to control predisposing infections or environmental factors and early use of antibacterials indicated by susceptibility tests (Vilà et al., 2009). Avian pathogenic *E.coli* is regularly found to be resistant to frequently used antibacterial agents (Vandemaele et al., 2002). Most of the

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available antibiotics used in Iran have lost their potency over *E. coli* due to their abuse, which could be found clearly in antibiogram tests. Over 60 percent of *E. coli* isolates from clinical cases have become resistant to the tetracycline family, especially oxytetracycline and chlortetracycline.

In addition, the nontherapeutic use of antibiotics originating resistance in humans is determined by the same pathway as in farm animals. This bacterial resistance can be propagated via the food chain into the intestinal flora of humans and can be transferred by plasmid to new generations as well. The aim for a global ban on feed antibiotics in many countries (including Iran) is to reduce the pool of resistant antibiotic and bacterial traits in the microbial flora of farm animals (Smith et al., 2007).

Probiotics are found to be suitable for chickens to prevent oxidative stress (Sohail et al., 2011), improving mucosal immunity (Cox et al., 2010) and general immunity (Gleeson et al., 2012) leading to an improvement in their performance. Stressful situations on poultry farms can increase the incidence of gastrointestinal disease episodes, particularly of diarrhea during rearing periods. They cause increased susceptibility of the upper respiratory tract to infections (Mackinnon, 2000) in addition to immune suppression leading to depressed performance (Pedersen and Hoffman-Goetz, 2000). It has been demonstrated that consumption of probiotics can enhance the immune system and health of all kinds of poultry (Corthesy et al., 2007 and Fang et al., 2000). Therefore, probiotics could be used directly to maximize the performance of birds by preventing the immune suppression caused by prolonged stress and pathogenic bacteria like *E. coli* (Guarino et al., 2009).

The use of spore-forming probiotics has several advantages. When the bacteria turn into spores, they form two layers of protein around the bacteria. These layers protect the bacteria from environmental stressors, which allow spore-forming probiotics to be included in diets containing possible aggressive in-feed components such as coccidiostats. *Bacillus* spores are relatively inexpensive to produce, steam pelletable, suitable for inclusion in feed premixes, and highly effective at modifying the intestinal microflora to inhibit *Salmonella* species, *E. coli,* and *Clostridium perfringens* (Hooge, 2008). It has been postulated that *Bacillus subtilis* C-3102 spores vegetate in the intestinal lumen and consume oxygen, making a more anaerobic condition favorable to native Lactobacilli, which then proliferate and produce lactic acid to control pathogens (Hooge et al., 2004). Besides inhibiting certain pathogens such as *E. coli* and *Salmonella*, the proliferation of Lactobacilli and increased lactic acid production, as a result of feeding *Bacillus subtilis,* also appears to increase utilization of calcium (Barabesi et al., 2007). Therefore, the current study was conducted to assess and compare the effects of *Bacillus subtilis* with growth promoting antibioticon growth performance, nutrient digestibilities, intestinal microbiota, blood characteristics and organ weight changes in broilers after intramuscular inoculation with *E. coli*.

MATERIALS AND METHODS

Bird Management and Experimental Design

Three-hundred-sixty one-day-old male Ross 308 chicks were obtained from a local commercial hatchery grown over a 42-day experimental period according to Malayer University approved animal care rules and protocols. The chicks were housed in thermostatically controlled pens in an environmentally controlled building. The experiment was done as a completely randomized design with 6 treatments and 5 replicates of 12 chicks in each replicate/pen. Experimental treatments (diets) include 1) control diet; 2) control+ *E. coli* (0.5 mL of culture containing 10^8CFU of *E. coli*/ml); 3) control + 0.1% *Bacillus subtilis* (Calsporin®, Calpis Co. Ltd Japan); 4) control + 0.05% bacitracin methylene disalicylate (**BMD**); 5) control + *E. coli* and *Bacillus subtilis*, and 6) control + *E. coli* and BMD. The pathogenic strain of *E. coli* used for experimental challenge of the birds was serotype PTCC-1399 and it was obtained kindly from the Iranian Research Organization for Science and Technology. It was removed and isolated from affected organs and feces of clinical cases of birds that showed clear signs of general colibacillosis. The challenge inoculum was prepared according to the method of Quinn et al. (1994). At d 10, each chick in the infected groups was intramuscularly (**I/M**) injected with 0.5 mL of the nutrient broth culture containing 10^8 colony forming unit (**CFU**) *E. coli*/mL in the bird's right muscle, after an overnight grown without washing, (Fernandez et al., 2002). Throughout the study, the birds were brooded following standard temperature regimens, which gradually decreased from 32 to 24°C, and under a 23L:1D lighting cycle. Basal diets were formulated to meet or exceed Ross 308 broiler nutrition specifications for macro- and micro-nutrients (Table 1). A 2-phase feeding program was used with a starter diet from d one to 21 and a grower diet from d 22 to 42. Body weight (**BW**) and cumulative feed intake (**FI**) were measured, and feed conversion ratio (**FCR**) was calculated at the end of experimental period.

Vaccination Schedule

The local office of the Iranian Veterinary Organization suggested the required local vaccination and was managed by the veterinarian of Malayer University, as below:

Essential Newcastle disease (**ND**) vaccination was carried out on the first d (by spray in the hatchery) and repeated on the twelfth d as CEVAC® BI L contains the Hitchner B1 strain of ND virus in live, freeze-dried form) in the drinking water, with a booster on the twentieth d as clone-30 (HIPRAVIAR®) in the drinking

Table 1. Composition and calculated analyses of the basal diets.

Item	Basal diets (%) 0 to 21 d	22 to 42 d
Corn	54.32	58.69
Soybean meal, 44% CP	39.43	31.87
Corn oil	2.16	5.83
Dicalcium phosphate	2.05	1.68
Oyster shell	0.9	0.79
Sodium chloride	0.37	0.37
Vitamin and mineral premix[1]	0.5	0.5
DL-Met, 99%	0.2	0.22
L-Lys-HCL, 78%	0.07	0.05
Calculated analysis		
ME, kcal/kg	2900	3200
CP %	22.16	19.2
Dig Lys %	1.15	0.96
Dig Met %	0.5	0.48
Dig Met+Cys %	0.83	0.78
Dig Thr %	0.79	0.71
Ca %	1	0.85
Available P %	0.5	0.42

[1]Vitamin and mineral mix supplied the following per kilogram of diet: vitamin A, 11,000 IU; vitamin D3, 1,800 IU; vitamin E, 11 mg; vitamin K3, 2 mg; vitamin B2, 5.7 mg; vitamin B6, 2 mg; vitamin B12, 0.024 mg; nicotinic acid, 28 mg; folic acid 0.5 mg; pantothenic acid, 12 mg; choline chloride, 250 mg; Mn, 100 mg; Zn, 65 mg; Cu, 5 mg; Se, 0.22 mg; I, 0.5 mg; and Co, 0.5 mg.

water. Vaccination against infectious bronchitis (**IB**) ensued twice as the following: first spray at commencement of the experiment and the booster in the drinking water on the tenth d, both as H-120 (CEVAC®). Vaccination against infection bursal disease (**IBD**) was carried out twice: first on d 15 and second on d 24, both as Gambo-l (CEVAC®) in the drinking water. The booster B1 neurotropic vaccine strain virus (ND 6/10) (CEVAC®) was provided in the drinking water at the age of 21 d, after measuring the hemagglutination inhibition (**HI**) titer test of sera to determine levels of antibodies to ND in the blood.

Blood Characteristics

At d 42, two chicks from each pen were selected and blood samples were collected in heparinized tubes by puncturing the brachial vein to measuring the plasma triglyceride, cholesterol, low-density lipoprotein (**LDL**), very low-density lipoprotein (**VLDL**), high-density lipoprotein (**HDL**), plasma albumin and globulin, alanine transaminase (**ALT**), and alkaline phosphatase (**ALP**). The concentration of triglyceride, total cholesterol, HDL, VLDL, and LDL in the serum samples was analyzed with an automatic biochemical analyzer (Hitach Boahringer Munnhein/ 717) using the colorimetric method. Plasma was used for determination of albumin and globulin concentrations according to procedures described by Corzo et al. (2009). ALT and ALP activities were measured with an automatic biochemical analyzer (Hitachi 717, Boehringer Mannheim, Ingelheim am Rhein, Germany) using an Elitech Diagnostic kit (catalog No. A.110537). Antibody titers against ND, AI, IB, and IBD were measured by the ELISA technique using commercial kits of Synbiotics Laboratories, Kansas City, Missouri, USA, by Boehringer Mannheim Hitachi 704 automatic analyzer, Japan.

Intestinal Morphology Parameters

At 42 d of age, upon obtaining the permission of the Ethical Committee of the University, 4 birds of each treatment were randomly selected, stunned, and killed by cutting the jugular vein for evaluation of ileal morphology. The digestive tract with contents was removed aseptically and the ileum was separated from the Meckel's diverticulum up to one cm proximal to the ileocecal junction and then dried with desiccant paper. A 2-cm section of ileum was taken from the middle of the ileum and was gently flushed with PBS (pH 7.2). Tissue sections were immediately fixed in 10% neutral buffered formalin and changed 3 times for completing the fixation. A single 0.5-cm sample was cut from each ileal section, dehydrated with increasing concentrations (70, 80, 95, and 100%) of ethanol, cleared with xylene, and placed into polyfin embedding wax. Tissue sections (2 μm) were cut by microtome (Leitz-1512 Microtome, Leitz, Wetzlar, Germany), floated onto slides, and stained with hematoxylin (Gill no. 2, Sigma, St. Louis, MO) and eosin (Sigma). To measure villus height and crypt depth, images from samples were taken using a digital camera with light microscopy. Twelve images from 4 tissue sections of each ileal section were taken, and 24 villus heights and crypt depths were measured by imaging software. Measurements for villus lengths were taken from the tip of the villus to the valley between individual villi, and measurements for crypt depth were taken from the valley between individual villi to the basolateral membrane (Xu et al., 2003). For determining the number of goblet cells in one mm of villus length, all samples were dehydrated in ethanol and embedded in paraffin wax. Sections were stained with Alcian blue and periodic acid-Schiff (**PAS**) to visualize goblet cells, 1% acidic (pH 0.3) Alcian blue for mast cells, or phloxine-tartrazine for Paneth and intermediate cells (Rohana and Thomas, 2009).

Enumeration of Cecal Bacterial Population

On d 42, 2 chicks from each pen were stunned and slaughtered by neck cut for the extraction of cecal contents. The cecal contents of each bird were pooled for serial dilution. To avoid cross contamination, the absence of *Bacillus subtilis* spores was confirmed in the feces of birds from the negative control group. Microbial populations were determined by serial dilution (10^{-4} to 10^{-6}) of cecal samples in anaerobic diluents before inoculation onto Petri dishes of sterile agar as described by Bryant and Burkey (1953). *E. coli* was grown on eosin methylene blue agar, *Salmonella* in *Salmonella Shigella* (Merck, Germany), and coliform was grown

Table 2. Effect of different dietary treatments and challenge with *E. coli* on performance of broilers from one to 42 d of age.

	Body weight, g			Feed intake, g			Feed/gain, (g/g)		
	Chal	Unchal	Means	Chal	Unchal	Means	Chal	Unchal	Means
Diet 1	2127.4	2305.2	2216.3[b]	4221	4363	4292.30	1.99	1.90	1.94[a]
Diet 2	2199.2	2490.4	2344.8[a]	4216	4337	4276.60	1.92	1.74	1.86[b]
Diet 3	2190.4	2504.0	2347.2[a]	4282	4382	4332.50	1.96	1.75	1.83[b]
Means	2172.3[B]	2433.2[A]		4239.80[B]	4361.13[A]		1.95[A]	1.78[B]	
CV, %		7.39			2.20			6.46	
Interaction		ns			ns			ns	

Chal: Challenged diets with 0.5 mL of culture containing 10^8 CFU of *E. coli*/mL; Unchal: unchallenged.
Diet 1: Control diet; Diet 2: bacitracin methylene disalicylate (BMD) (0.05%); Diet 3: BS (*Bacillus subtilis*) (0.1%).
[a,b]Means followed by different lowercase letters in the same line are different from each other in diet factor.
[A,B]Means followed by different uppercase letters in the same column are different from each other in challenged factor.

on McConkey agar (Darmstadt, Germany). *E. coli* was incubated aerobically at 37°C. Plates were counted between 24 and 48 h after inoculation. Colony-forming units were defined as distinct colonies measuring at least one mm in diameter. Then, 9 sterile test tubes with lids containing 9 mL of phosphate buffer solution as diluent were prepared. Approximately one g of the cecal contents were taken by sterile swab and homogenized for 3 min before transferring to a microbiology lab in cold condition (Bryant and Burkey, 1953) and mixed employing aseptic technique. Then one mL out of 10 mL of buffer plus cecal sample in the test tube was removed by 1,000 μl sampler and was transferred to the tubes and mixed thoroughly. Similarly, it was transferred to other new tubes, and this procedure was repeated until a dilution of 9. Later, one mL of contents of each test tube was transferred to one of 3 selective media agar in petri plates, respectively, and each petri plate was incubated at 37°C for 24 hours. Finally, the intestinal bacterial colony populations formed in each plate and were counted and adjusted to X 10^9 manually and then reported.

Measurement of Visceral Organs

Each killed bird was weighed individually, then the abdominal cavity of each bird was opened and the liver, kidney, spleen, pancreas, heart, and bursa of Fabricius were removed and weighed on a mono pan balance (0.1 mg accuracy). The weights were adjusted to g/kg of live body weight and treatment means were calculated.

Digestibility Traits

All diets were ground through a one-mm screen in a Wiley mill before analyzing for dry matter (**DM**), crude protein (**CP**), and gross energy (**GE**) (AOAC International, 2000). For apparent total tract digestibility, Cr_2O_3 (0.2%) was added into the diets as an indigestible marker during d 35 to 42. Digestibility of the nutrients and energy was calculated by the method described by Hahn et al. (2006). During d 38 to 42, fresh feces from 2 broilers in the same pen were collected, pooled, and frozen until being lyophilized and ground. Feces and feed samples were ground through a one-mm screen. Samples were then used to determine DM content by oven drying at 105°C for 24 hours. Nitrogen content of the diets was determined by the combustion method of AOAC International (2000) (Model FP2000; Leco Corp., St. Joseph, MI), and gross energy was determined by a Parr 1261 adiabatic calorimeter (Parr Instrument Co., Moline, IL) according to Meng et al. 2006). Chromium was determined by UV absorption spectrophotometry (Shimadzu UV-1201; Shimadzu, Kyoto, Japan) and digestibility of DM and nitrogen (**N**) were calculated using the indirect-ratio method.

Statistical Analysis

Data were analyzed as a completely randomized design in a factorial arrangement (3 × 2) using the GLM procedure of SAS (SAS Institute, 2011). The model equation included main effects (diets and *E. coli* challenged) and their interactions. MIXED procedure of SAS (SAS Institute, 2011) was used for analysis of traits when the interactions were significant. Differences among treatment means were tested using Tukey's test for main effects and their interactions. Statistical significance was declared at $P \leq 0.05$.

RESULTS

Growth Performance

Addition of BMD or *Bacillus subtilis* to the control diet significantly ($P < 0.01$) increased BW and decreased FCR at 42 d of age (Table 2). Challenge with *E. coli* significantly ($P < 0.01$) reduced BW (2127.3 vs. 2433.2g) and increased FCR (1.95 vs. 1.78) and the addition of BMD or *Bacillus subtilis* in the presence of *E. coli* could not compensate the growth reduction when compared with control group (Table 2). Feed intake significantly ($P < 0.01$) decreased in the *E. coli* challenged group (4239.80 vs. 4361.13 g) compared with control birds, and BMD supplementation did not prevent FI reduction. There was no significant difference between

Table 3. Effect of different dietary treatments and challenge with *E. coli* on vaccine titer of broilers at 42 d of age.

	ND (Log^2)			IB (GMT^3)			AI (Log^2)			IBD (GMT^3)		
	Chal	Unchal	Means	Chal	Unchal	Means	Chal	Unchal	Means	Chal	Unchal	Means
Diet 1	4.01	4.56	4.29^{b}	11508^{d}	12174^{c}	11841	3.05	3.45	3.25^{b}	559.3	685.3	622.3
Diet 2	4.04	4.98	$4.51^{a,b}$	11293^{d}	13154^{b}	12223	3.18	3.90	$3.54^{a,b}$	570.2	692.5	631.3
Diet 3	4.21	5.27	4.74^{a}	$11557^{c,d}$	14239^{a}	12898	3.13	4.07	3.60^{a}	566.3	719.5	642.9
Means	4.09^{B}	4.94^{A}		11452	13189		3.12^{B}	3.81^{A}		565.3^{B}	699.1^{A}	
CV, %		13.64			10			11.2			13.4	
Interaction		ns			$P < 0.002$			ns			ns	

Chal: Challenged diets with 0.5 mL of culture containing 10^8 CFU of *E. coli*/mL; Unchal: unchallenged.
Diet 1: Control diet; Diet 2: bacitracin methylene disalicylate (BMD) (0.05%); Diet 3: BS (*Bacillus subtilis*) (0.1%); ND = Newcastle disease; IB = Infection bronchitis; AI = Avian Influenza; IBD = Infection bursal disease.
[a–d]Means followed by different lowercase letters in the same line are different from each other in diet factor.
[A,B]Means followed by different uppercase letters in the same column are different from each other in challenged factor.

Table 4. Effect of different dietary treatments and challenge with *E. coli* on blood metabolites of broilers at 42 d of age.

	Albumin (g/dl)			Globulin (g/dl)			Cholesterol (mg/dl)			Triglyceride (mg/dl)		
	Chal	Unchal	Means	Chal	Unchal	Means	Chal	Unchal	Means	Chal	Unchal	Means
Diet 1	3.18^{a}	2.80^{b}	2.99	3.68	2.97	3.33^{a}	202.84	185.15	193.99^{a}	160.49	153.31	156.89^{a}
Diet 2	2.28^{c}	2.26^{c}	2.27	2.79	2.66	2.73^{b}	193.90	181.31	$187.61^{a,b}$	155.03	147.45	$151.24^{a,b}$
Diet 3	2.35^{c}	1.69^{d}	2.02	2.91	2.33	2.62^{b}	191.49	163.49	177.49^{b}	148.35	136.67	142.51^{b}
Means	2.60	2.25		3.13^{A}	2.66^{B}		196.1^{A}	176.7^{B}		154.62^{A}	145.81^{b}	
CV, %		21.09			17.86			9.28			8.21	
Interaction		$P < 0.003$			ns			ns			ns	

Chal: Challenged diets with 0.5 mL of culture containing 10^8 CFU of *E. coli*/mL; Unchal: unchallenged.
Diet 1: Control diet; Diet 2: bacitracin methylene disalicylate (BMD) (0.05%); Diet 3: BS (*Bacillus subtilis*) (0.1%).
[a–d]Means followed by different lowercase letters in the same line are different from each other in diet factor.
[A,B]Means followed by different uppercase letters in the same column are different from each other in challenged factor.

Table 5. Effect of different dietary treatments and challenge with *E. coli* on blood metabolites of broilers at 42 d of age.

	HDL (mg/dl)			LDL (mg/dl)			ALT (%)			ALP ($10^6/\mu l$)		
	Chal	Unchal	Means	Chal	Unchal	Means	Chal	Unchal	Means	Chal	Unchal	Means
Diet 1	62.53	64.51	63.52^{b}	78.01	74.09	76.05^{a}	5.11	4.81	4.96^{a}	169.36^{a}	160.25^{b}	167.51
Diet 2	70.72	69.03	69.87^{a}	75.95	72.03	$73.99^{a,b}$	4.98	4.96	4.97^{a}	166.55^{a}	168.46^{a}	164.81
Diet 3	68.18	66.09	67.14^{a}	75.82	67.90	71.86^{b}	3.97	4.15	4.06^{b}	167.56^{a}	160.06^{b}	163.81
Means	67.14	66.54		76.59^{A}	71.34^{B}		4.69	4.64		167.83	162.93	
CV, %		6.48			6.57			10.87			3.63	
Interaction		Ns			ns			ns			$P < 0.02$	

Chal: Challenged diets with 0.5 mL of culture containing 10^8 CFU of *E. coli*/mL; Unchal: unchallenged.
Diet 1: Control diet; Diet 2: bacitracin methylene disalicylate (BMD) (0.05%); Diet 3: BS (*Bacillus subtilis*) (0.1%); HDL: High-density lipoprotein; LDL: Low-density lipoprotein; ALT: Alanine transaminase; ALP: Alkaline phosphatase.
[a,b]Means followed by different lowercase letters in the same line are different from each other in diet factor.
[A,B]Means followed by different uppercase letters in the same column are different from each other in challenged factor.

the control and the *E. coli* challenged treatment by addition of *Bacillus subtilis* as a probiotic. The results of group comparisons showed that inoculation of *E. coli* in the diets significantly ($P < 0.01$) reduced BW and FI, and increased FCR.

Vaccine Titers

The effects of *E. coli*-challenge, AGP, and *Bacillus subtilis* supplementation on vaccine titers are shown in Table 3. Compared with the control group, the *E. coli* challenged chicks had the lowest vaccine titers against all diseases and the highest vaccine titers were observed in broilers fed *Bacillus subtilis*. Addition of BMD and *Bacillus subtilis* did not improve vaccine titers in *E. coli* challenged birds. The results of group comparisons showed that inoculation of *E. coli* in the diets significantly ($P < 0.01$) reduced all vaccination titers.

Blood Characteristics

The effects of *E. coli* challenge with or without BMD and *Bacillus subtilis* on blood metabolites are shown in Tables 4 and 5. Challenge with *E. coli* significantly ($P < 0.01$) increased albumin, globulin, cholesterol, triglyceride, LDL, and ALP concentrations of plasma when compared with the respective control group. *Bacillus subtilis* inclusion significantly ($P < 0.01$) decreased albumin, globulin, cholesterol, triglyceride, LDL, and ALT concentrations and increased HDL concentration

Table 6. Effect of different dietary treatments and challenge with *E. coli* on ileum morphological characteristics of broilers at 42 d of age.

	Villus height (μm)			Crypt depth (μm)			Villus height to crypt depth ratio			Number of Goblet cell[†]		
	Chal	Unchal	Means	Chal	Unchal	Means	Chal	Unchal	Means	Chal	Unchal	Means
Diet 1	4.21^{b}	4.70^{b}	4.46	0.98	0.86	0.92^{a}	4.31^{c}	5.49^{b,c}	4.89	9.68	8.77	9.22^{a}
Diet 2	4.49^{b}	5.74^{a}	5.11	0.86	0.78	0.82^{b}	5.24^{c}	7.41^{b}	6.33	9.03	8.15	8.59^{b}
Diet 3	4.29^{b}	6.47^{a}	5.38	0.88	0.75	0.81^{b}	4.92^{c}	8.74^{a}	6.83	9.14	7.66	8.41^{b}
Means	4.33	5.64		0.91^{A}	0.79^{B}		4.82	7.22		9.29^{A}	8.19^{B}	
CV, %		21.44			11.4			31.7			9.78	
Interaction		$P < 0.02$			ns			$P < 0.03$			ns	

Chal: Challenged diets with 0.5 mL of culture containing 10^8 CFU of *E. coli*/mL; Unchal: unchallenged.
Diet 1: Control diet; Diet 2: bacitracin methylene disalicylate (BMD) (0.05%); Diet 3: BS (*Bacillus subtilis*) (0.1%).
[a–c]Means followed by different lowercase letters in the same line are different from each other in diet factor.
[A,B]Means followed by different uppercase letters in the same column are different from each other in challenged factor.
[†]Number of goblet cells in each one mm of villus length.

Table 7. Effect of different dietary treatments and challenge with *E. coli* on cecal microbial populations at 42 d of age.

	Coliforms ($\log_{10}$cfu/g of DM)			Salmonella ($\log_{10}$cfu/g of DM)			*E. coli* ($\log_{10}$cfu/g of DM)		
	Chal	Unchal	Means	Chal	Unchal	Means	Chal	Unchal	Means
Diet 1	4.49^{a}	2.71^{d}	3.61	4.53	3.61	4.07^{a}	4.80	4.18	4.49^{a}
Diet 2	3.44^{b}	2.25^{e}	2.98	3.27	2.12	2.69^{b}	2.97	2.25	2.89^{b}
Diet 3	3.80^{c}	2.16^{e}	2.85	3.08	1.69	2.38^{b}	3.11	2.67	2.61^{b}
Means	3.91	2.37		3.63^{A}	2.47^{B}		3.62^{A}	3.04^{B}	
CV, %		27.83			33.43			10.5	
Interaction		$P < 0.001$			ns			ns	

Chal: Challenged diets with 0.5 mL of culture containing 10^8 CFU of *E. coli*/mL; Unchal: unchallenged.
Diet 1: Control diet; Diet 2: 0.05% bacitracin methylene disalicylate (BMD); Diet 3: 0.1% BS (*Bacillus subtilis*).
[a–e]Means followed by different lowercase letters in the same line are different from each other in diet factor.
[A,B]Means followed by different uppercase letters in the same column are different from each other in challenged factor.

of plasma compared with the respective control group. Addition of AGP and *Bacillus subtilis* decreased albumin and ALT and decreased ALP in challenged birds' plasma compared with the control group. The results of group comparisons showed that inoculation of *E. coli* in the diets significantly ($P < 0.01$) increased globulin, cholesterol, triglyceride, LDL, and ALP, and reduced albumin, but did not affect HDL and ALT values.

Intestinal Morphology Parameters

The influences of *E. coli* and BMD or *Bacillus subtilis* supplementation on villus height, crypt depth, and goblet cell numbers are shown in Table 6. Challenge with *E. coli* did increase crypt depth (0.91 vs. 0.79 μm) significantly ($P < 0.01$), when compared with the control group; also, the number of goblet cells in each one mm of villus length increased (9.29 vs. 8.19) significantly ($P < 0.01$) in the ileum of *E. coli* challenged birds, but addition of AGP or *Bacillus subtilis* did not affect it in the challenged birds. In birds subjected to BMD or *Bacillus subtilis*, crypt depth was decreased and villus height was increased ($P < 0.01$) when compared with the control diet.

Cecal Microbial Population

Challenge with *E. coli* significantly ($P < 0.01$) increased the population of *E. coli*, coliforms, and *Salmonella* of ceca (Table 7). In the *E. coli* challenged birds that received BMD or *Bacillus subtilis*, the population of cecal *E. coli*, coliforms, and *Salmonella* showed a significant ($P < 0.01$) reduction. Significantly, the lowest population of coliforms, *E. coli*, and *Salmonella* in ceca was observed in birds fed *Bacillus subtilis*. Overall, the results of group comparisons showed that inoculation of *E. coli* in the diets significantly ($P < 0.01$) increased crypt depth, number of goblet cells, and microbial populations, whereas it decreased ($P < 0.01$) villus height and its ratio to crypt depth.

Relative Organ Weights

The relative weights of spleen, heart, and pancreases were significantly ($P < 0.01$) decreased, and bursa of Fabricius weight was increased (1.67 vs. 1.41 g/kg BW), compared with the control group in *E. coli* challenged chicks (Table 8), and the relative weight of the kidney remained unchanged. *Bacillus subtilis* inclusion resulted in a significant increase ($P < 0.01$) in the spleen, pancreas, and heart in challenged and non-challenged birds. The results of group comparisons showed that inoculation of *E. coli* in the diets significantly ($P < 0.01$) reduced all of the organ weights except the weight of bursa of Fabricius, and *Bacillus subtilis* inclusion to challenged birds intensified this effect.

Table 8. Effect of different dietary treatments and challenge with *E. coli* on on relative organ weight (g/kg of BW) of broilers at 42 d of age.

	Kidney			Spleen			Pancrease			Heart			bursa of Fabricius		
	Chal	Unchal	Means	Chal	Unchal	Means	Chal	Unchal	Means	Chal	Unchal	Means	Chal	Unchal	Means
Diet 1	6.82	7.27	7.04^{b}	1.39^{b}	1.73^{a}	1.56	$3.64^{b,c}$	$3.80^{a,b}$	3.72	3.18^{d}	3.73^{c}	3.45	1.87	1.53	1.69^{a}
Diet 2	7.15	8.30	7.72^{a}	1.34^{b}	1.78^{a}	1.57	3.27^{c}	4.22^{a}	3.74	3.76^{c}	4.18^{a}	3.97	1.50	1.33	1.41^{b}
Diet 3	7.06	8.19	7.63^{a}	1.67^{a}	$1.58^{a,b}$	1.62	$3.83^{a,b}$	$3.94^{a,b}$	3.89	$3.90^{b,c}$	$4.06^{a,b}$	3.98	1.64	1.38	1.51^{b}
Means	7.01	7.92		1.47	1.69		3.58	3.99		3.61	3.99		1.67^{A}	1.41^{B}	
CV, %		10.44			15.8			15.8			9.56			16.55	
Interaction		ns			$P < 0.001$			$P < 0.01$			$P < 0.04$			ns	

Chal: Challenged diets with 0.5 mL of culture containing 10^8 CFU of *E. coli*/mL; Unchal: unchallenged.
Diet 1: Control diet; Diet 2: 0.05% bacitracin methylene disalicylate (BMD); Diet 3: 0.1% BS (*Bacillus subtilis*).
[a–d]Means followed by different lowercase letters in the same line are different from each other in diet factor.
[A,B]Means followed by different uppercase letters in the same column are different from each other in challenged factor.

Digestibility of Utrients

As shown in Table 9, both of the BMD and *Bacillus subtilis* groups increased the digestibility of CP, CF, and GE significantly ($P < 0.01$). The *E. coli* challenged group reduced the digestibility of DM, CP, CF, and GE significantly ($P < 0.01$) with respect to control. Overall, the results of group comparisons showed that inoculation of *E. coli* in the diets significantly ($P < 0.01$) decreased gross energy and nutrient apparent digestibility.

DISCUSSION

Colibacillosis continues to be a serious problem in poultry production causing mortality and condemnations (Piercy and West, 1976 and DeRosa et al., 1992). By increasing restriction of AGP usage in the poultry industry, it can be speculated that colibacillosis would become an even greater problem on commercial farms. Therefore, there is a real need to find suitable alternatives to antibiotic growth promoters. The applied experimental model used in the current study was a severe *E. coli* challenge to the birds inoculated with 10^8cfu of *E. coli* directly into the intramuscular cavity at 10 d of age. The data in this study demonstrated that the *E. coli* challenge severely retarded the growth and feed intake of birds and increased FCR. Both BMD and *Bacillus subtilis* inclusion into the basal diet improved BW and FCR, and in challenged birds, the improvements of BW and FCR by adding these feed additives were almost comparable to those of control groups. The earlier reports on the beneficiary efficacy of Lactobacillus-based probiotics on poultry performance vary from positive to negative impact. Studies involving *Bacillus spp.* specifically have shown their growth benefits in turkey poults (Grimes et al., 2008), broilers (Vilà et al., 2009), and also in swine (Alexopoulos et al., 2004; Davis et al., 2008). *Bacillus subtilis* spores may be successful competitive exclusion agents (La Ragione and Woodward, 2003) and proliferation of *Lactobacillus* spp. may improve the intestinal microflora balance, digestion, and absorption of ingested feed enhancing the BW and FCR. The other beneficial effect of inclusion of bacteria spp. in poultry diets goes back to their efficiency in reducing or preventing colonization of undesirable bacteria such as *Salmonella* levels in the intestinal tract of poultry (Knarreborg et al., 2008). Also, *Bacillus subtilis* has been approved as a safe feed additive by the European Union and is a commercially available product that improves broiler performance equal to AGPs (Lund et al., 2005).

Results of the current study showed that *E. coli* challenge declined vaccine titers of ND, IB, and IBD. The bursa of Fabricius and thymus are considered central lymphoid organs that play essential roles in the general immunity of broilers. Immunization of commercial chicken flocks is the principle method adopted to control the common viral diseases such as ND and IBD vaccine titers. Numerous factors can cause suboptimal vaccine response (Allan et al., 1972). Damage of the bursa of Fabricius due to viral (IBD) or bacterial (*E. coli)* agents has a direct adverse effect on humeral immune response. Nakamura et al. (1992) reported marked lymphocytic depletion in the bursa was due to the experimental inoculation with *E. coli.* This observation was confirmed by the report of Hassan and Hassanein (1999). In a study by Mahmoud et al. (2007), experimental inoculation of *E. coli* induced marked bursal lymphocytic depletion for about 2 weeks. As it revealed in the current study, challenge of *E. coli* increased the weight of the bursa of Fabricius significantly; this might be a result of depletion of its cell and hypertrophy of this organ for compensating the cell depletion. Infection with *E. coli* significantly compromises the immune responses of chickens; however, *E. coli* infection is a secondary localized or systemic disease occurring when host defenses have been impaired (Nakamura et al., 1986). In the current study, *Bacillus subtilis* enhanced vaccine titers more than that of AGP. *Bacillus subtilis,* in vegetative form, produces extracellular enzymes that may enhance digestibility and absorption of nutrients in addition to overall gut immune function (Samanya and Yamauchi, 2002 and Chen et al., 2009). As obtained in this study, addition of *Bacillus subtilis* to control and *E. coli* challenged diets increased digestibility of crude protein considerably.

Table 9. Effect of different dietary treatments and challenge with *E. coli* energy and nutrient apparent digestibility at 42 d of age.

	DM			CP			CF			GE		
	Chal	Unchal	Means	Chal	Unchal	Means	Chal	Unchal	Means	Chal	Unchal	Means
Diet 1	75.35	79.24	77.29	65.12^{c}	72.38^{b}	68.75	78.24	85.35	81.80^{b}	75.43^{c}	81.32^{b}	78.38
Diet 2	76.43	80.20	77.85	71.72^{b}	75.17^{a}	73.44	81.06	85.35	81.97^{b}	73.57^{d}	85.52^{a}	79.38
Diet 3	76.17	79.53	78.31	70.81^{b}	75.34^{a}	73.09	79.07	84.86	83.19^{a}	71.35^{d}	82.79^{b}	77.07
Means	75.98^{B}	79.66^{A}		69.21	74.29		79.46^{B}	85.18^{A}		73.45	83.21	
CV, %		3.21			5.2			3.94			6.89	
Interaction		ns			$P < 0.001$			ns			$P < 0.001$	

Chal: Challenged diets with 0.5 mL of culture containing 10^8 CFU of *E. coli*/mL; Unchal: unchallenged.
Diet 1: Control diet; Diet 2: 0.05% bacitracin methylene disalicylate (BMD); Diet 3: 0.1% BS (*Bacillus subtilis*); DM = Dry matter; CP = Crude protein; CF = Crude fiber; GE = Gross energy.
[a–d]Means followed by different lowercase letters in the same line are different from each other in diet factor.
[A,B]Means followed by different uppercase letters in the same column are different from each other in challenged factor.

In findings of the current trial, albumin, globulin, cholesterol, triglyceride, and LDL concentrations were increased in *E. coli* challenged bird's plasma. On the other hand, *Bacillus subtilis* inclusion decreased albumin, globulin, cholesterol, triglyceride, and LDL concentrations in blood plasma. There are reports on the positive effects of probiotics in decreeing the lipid moieties from plasma by several mechanisms (Ooi and Liong, 2010). Previous in vitro studies have evaluated a number of mechanisms proposed for the cholesterol-lowering effects of probiotics and prebiotics. One of the purported mechanisms includes enzymatic deconjugation of bile acids by bile-salt hydrolase (Begley et al., 2006). The hypocholesterolemic effect of probiotics is attributed to their ability to bind cholesterol in the small intestine. Usman (1999) reported that *Lactobacillus gasseri* could remove cholesterol from laboratory media via binding onto cellular surfaces. The present results and the findings of Yoon et al. (2004) are similar. However, Kim et al. (2003) reported that broilers fed a diet containing *Bacillus subtilis* caused a reduction in cholesterol level of broiler blood serum. Similar results were obtained by addition of probiotics containing *Aspergillus niger* and prebiotics containing *Taraxacum officinale* as feed supplement (Al-Kassie et al., 2008). The *E. coli* challenged group resulted in increased ALT and ALP values in the current study. Similar changes in the values of ALP and ALT were found by Sjoukje et al. (2006) in mice after intravenous injection of *E. coli*. The results of El Garawany et al. (2005) describes a 2-fold increase in the levels of ALP and 60% in ALT values after intraperitoneal injection of *E. coli* at a dose of one mg/kg of body weight. This influence is probably related to endotoxins released by *E. coli*, the impaired barrier, and absorptive function of the intestinal wall, which allow the passage of toxic substances from the intestinal content into the blood with consequent damage to kidney and liver functions (Petrov et al., 2011). *Bacillus subtilis* addition in this study decreased ALT activity in *E. coli* challenged and non-challenged birds, but had no effect on ALP activities. In the current study, the lowest population of *E. coli*, coliforms, and *Salmonella* in ceca was found in birds that were fed *Bacillus subtilis*. Studies have demonstrated that *Bacillus spp.* and especially *Bacillus subtilis* spores are the most successful competitive exclusion agents (La Ragione and Woodward, 2003). The inclusion of *E. coli* into the broiler's diet has led to lesser body weight and smaller body mass resulting in the lighter relative organ weights of broilers (Fritils et al., 2000). *Bacillus subtilis* is a strain of bacteria originally found in soil. When fed to poultry, it may increase the occurrence of beneficial bacteria and suppress the destructive bacteria (Blair et al., 2004). It modulates the intestinal microbiota and favors the growth of lactic acid with putative health-conferring properties (Knarreborg et al. 2008). Knap Kehlet et al. (2011) reported that *Bacillus subtilis* reduced the *Salmonella* load in the gastrointestinal tract of the chickens and the related surrounding environment, thereby reducing the risk of infection among the birds and *Salmonella* infecting slaughterhouses, leading to potentially improving overall food safety. A spore monoculture is readily produced with a long storage life, and, in the case of *Bacillus subtilis*, is avirulent (La Ragione and Woodward, 2003). Therefore, in *Bacillus subtilis* fed groups, reduction in negative microbial population along with healthy intestinal walls reduces bacterial toxin production and its absorption into the blood, whereupon decreasing ALT and ALP activities. Results of this study indicate that in birds subjected to BMD or *Bacillus subtilis*, crypt depth was decreased and villus height was increased when compared with control and *E. coli* challenged broilers. Baurhoo et al. (2007) reported that an increase in villi length could be associated with increased lactobacilli and bifidobacteria colonization and decreased *E. coli* and *Salmonella* population of broiler intestines. In the current study, the effects of *E. coli* challenge on the measured organ weights showed a decrease in weight in response to *E. coli* but bursa of Fabricius weight, as discussed earlier, was increased, which might be from lymphocytic depletion of this organ followed by hypertrophy for compensating the cell depletion. BMD and *Bacillus subtilis* inclusion increased in all measured organ weights and decreased bursa of Fabricius relative weight proving that such products can compete with harmful bacterial deleterious effects on the intestine and other related organs. The BMD increased digestibility of CP

and, in the case of *Bacillus subtilis*, of GE, but *E. coli* challenged groups significantly reduced the digestibility of DM, CP, CF, and GE. Interestingly, the lowest digestibility of GE was observed in treatments challenged by *E. coli* and supplemented with bacitaracin or *Bacillus subtilis*. The improvement in energy and nutrient digestibility might be related to better balance of intestinal microflora (Helander et al., 1998) as was gained in the current study. Addition of bacitaracin or *Bacillus subtilis* significantly lowered the harmful bacterial load of the intestine. Furthermore, previous studies reported that probiotics could stimulate the secretion of digestive enzymes from the pancreas, gut mucosa, and bile flow (Samanya and Yamauchi, 2002 and Chen et al., 2009). Therefore, the improvement of the FCR and digestibility of energy and nutrients in this study could be explained by a better balance of intestinal microflora.

In conclusion, the data from the current study indicates that inclusion of *Bacillus subtilis* as a probiotic had a positive effect on performance, gut health, blood metabolites, and intestinal health and integrity leading to a balance of intestinal microbial population. It could be concluded that the current tested spore-forming probiotic having *Bacillus subtilis* is found to be partially effective in an unsuitable rearing situations such as colibacillosis challenge, in which the risk of harmful bacteria is raised.

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