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Effects of Probiotic on Immune Response and Intestine Morphology of Broiler Chicks Exposed to Stress Induced by Corticosterone

Research Article

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ABSTRACT

This experiment investigated the effects of different doses of *Bacillus subtilis* spore as a probiotic on the immune response**,** intestinal morphology and ileal dry matter digestibility in broiler chicks exposed to stress induced by corticosterone (CORT). Two hundred and eighty-eight one-day-old Ross 308 male broiler chicks were randomly assigned to six treatments in a completely randomized factorial design with and without CORT injection and 3 levels (0, 0.8×10^6, and 1.6×10^6 cfu/g) of *B. subtilis* spore supplementation. At 7 to 9 days of age (for 3 days), the chicks received one of the subcutaneous injections of CORT or corn oil (as control) at 2 mg/kg BW twice a day. The same injections were repeated at 25 to 27 days of age. Corticosterone injection led to significant ($P<0.05$) changes in intestinal morphology including villus height, ratio of villus height to crypt depth, and dry matter digestibility (as measured by TiO_2 marker). These same parameters, however, increased in the experimental chicks relative to the control as a result of probiotic supplementation. Heterophil/lymphocyte ratio increased ($P<0.05$) as a result of corticosterone injection but exhibited no significant effect of probiotic supplementation. While corticosterone injection decreased lymphocyte density in the medulla of the bursa of Fabricius, this adverse effect was reversed by probiotic supplementation, which was more effective at a 1.6×10^6 concentration than at 0.8×10^6. As a general conclusion, it may be claimed that administration of *B. subtilis*-based probiotic alleviates certain negative effects of the stress induced by the corticosterone injection.

KEY WORDS broiler chicks, *Bacillus subtilis* spores, corticosterone, probiotic, stress.

INTRODUCTION

Although the secretion of stress hormones provides birds for fight or flight that is essential for survival in nature but it has been shown that they inhibits different functions of the immune system, leading not only to the incidence of diseases and mortality in birds and also to their reduced performance (Virden and Kidd, 2009). During their production, industrial poultry flocks are prone to such stressful conditions as overcrowding, infections, feed withdrawal, and catching as well as unfavorable temperatures, humidity, light, and unsuitable ventilation (Lin *et al*. 2007; Virden and Kidd, 2009). Despite their inherent differences, stressors induce stress through similar physiological pathways that include: 1) sympathetic adrenal medullary axis, which results in the release of catecholamines; and 2) the limbic-hypothalamo-pituitary-adrenocortical axis. Stress is defined in the literature as any non-specific response of the biological system that menaces its homeostasis (Virden and Kidd, 2009). The effects of a stressor vary with its type, severity,

duration, and cyclicity as well as the species affected and the organism's previous experience. In stress conditions, the hypothalamic-pituitary-adrenal cortical system is activated by the corticotrophin-releasing hormone (CRF) secreted by the hypothalamus, which, in turn, stimulates the release of adrenocorticotropic hormone (ACTH) from the pituitary. The ACTH causes the cells of the cortical part of the adrenal gland to proliferate, thereby secreting more corticosteroids. Corticosterone (CORT) is the major corticosteroid in avian species. Continued high levels of corticosterone induce immunosuppression as well as changes in the metabolism of glucose and minerals (Hu *et al*. 2010). High levels of corticosteroids reportedly disrupt some of the immune system functions in birds such as proliferation of lymphocyte and production of immunoglobulin and cytokine as well as cytotoxicity and anti-inflammatory agents. Administration of exogenous CORT has been shown to result in increased plasma CORT concentration (Mehaisen *et al*. 2017). Poultry treated with CORT or ACTH demonstrated immune suppression. Intestinal morphology and gut integrity in birds could be affected by some stressors (Song *et al*. 2014). Quinteiro-Filho *et al*. (2010) showed that corticosterone is an effective factor in intestinal mucosa damage (Quinteiro-Filho *et al*. 2010). Retarded proliferation of intestinal epithelial cells due to increased CORT levels leads to decreased villus height (Hu and Guo, 2008). This impresses the digestion process and the absorptive capacity in the gut. In addition, corticosterone may increase the permeability of intestinal mucosa into pathogenic antigens by inducing the production of the pro-inflammatory agent. This is because excessive release of pro-inflammatory agents might cause injuries to intestinal tissues (Deng *et al*. 2012). Some of the adverse changes in the immune system following increased corticosterone levels include: Induced atrophy in some lymphoid organs (Yang *et al*. 2015), disruptions in normal heterophil/lymphocyte (H/L) ratio, and changes in the levels of tumor necrosis factor alpha, interleukin (IL)-2, and immunoglobulin (Ig) G (Yang *et al*. 2015).

Nutritional manipulations in diets and application of various additives are considered as practical strategies aimed at moderating the effects of stress. Poultry nutritionists have shown more interest in probiotic additives due to their potential effects on the intestine structure and its microbial ecosystem, the immune system, improved physiological conditions, and the overall performance of birds. Probiotics may reverse the impaired villus-crypt structure of heat-stressed birds.

Yang *et al*. (2015) suggested that CORT exhibits immune suppressive effects in the poultry and that reduced CORT levels may, therefore, be beneficial for restoring the normal functioning and development of the immune system (Yang *et al*. 2015). Moreover, probiotics seem to be useful for ameliorating the adverse effects of stress in poultry. However, certain studies found no significant effects of probiotics in poultry. The *Bacillus subtilis* spore-based probiotic from among probiotic microorganisms is commonly used because this strain is safe and resistant to feed-processing conditions while it also has a long shelf-life (Sen *et al*. 2011). The present experiment was carried out to determine the effects of dietary supplementation of a *Bacillus subtilis*-based probiotic on the immune response, intestine morphology, and ileal dry matter (DM) digestibility in broiler chickens subjected to stress induced by CORT injection.

MATERIALS AND METHODS

Experimental chickens, diets and treatments

All procedures were reviewed and approved by the Institutional Animal Care and Use Committee of Guilan University. A total of 288 one-day-old male broiler chicks (Ross 308) were procured from a local hatchery, weighed, and reared in an environmentally controlled pen. Pen temperature was adjusted to about 32 ˚C during the first 5 days to be later decreased gradually to 21 ˚C (75% RH) up to d 28 when it was maintained constant until 42 d of age. A lighting program of 23L:1D was maintained during the first 2 days and one of 21L:3D until the last day of the experimental period. Water and feed were made available *ad libitum* throughout the experimental period.

In a completely randomized design, the one-day-old chicks were randomly assigned to experimental treatments that consisted of a 2 × 3 factorial arrangement with 2 levels of CORT or corn oil injections (Yang *et al*. 2015) and 3 levels (0%, 0.02%, and 0.04% of the diet) of a probiotic containing 4×10^9 cfu/g of *Bacillus sultilis* spores supplied by Biochem Co. (Gallipro®200). Each of the six treatments was replicated 4 times with 12 birds in each replicate. At 7 days of age, the chicks received one of the two subcutaneous injection treatments of corn oil or CORT (2 mg/kg BW) twice per day for three consecutive days. At 25 days of age (25 to 27 days of age for 3 days), the same injection protocol was repeated. The ingredients and chemical compositions of the basal experimental diets are reported in Table 1. Vaccination was conducted based on the routine regional vaccination (Table 2).

Sampling and measurements

Histomorphometry of small intestinal

Samples from approximately 2 cm of the middle section of the duodenum, jejunum, and ileum were obtained from two euthanized chicks per replicate (8 chicks per treatment) at 10 and 42 days of age for histomorphometry of the small intestinal.

Table 1 Ingredients and chemical composition of the basal experimental diets

Ingredients	Growth period		
	Starter (1-10 d)	Grower (11-24 d)	Finisher (25-43 d)
Corn	52.84	58.84	62.5
Soybean meal	39.5	33.5	29
Oil	3	3.3	4.4
Di calcium phosphate	1.95	1.75	1.65
$CaCO_3$	1.06	0.98	0.9
Salt	0.19	0.19	0.19
Sodium bicarbonate	0.25	0.25	0.25
Premix vitamin[1]	0.25	0.25	0.25
Premix mineral[2]	0.25	0.25	0.25
Choline Cl 60%	0.05	0.05	0.05
DL-Met	0.34	0.31	0.26
L-Lys	0.22	0.23	0.22
L-Thr	0.1	0.09	0.08
Total	100	100	100
Calculated composition			
Metabolizable energy (kcal/kg)	2910	3000	3110
Crude protein %	22	19.7	18
Cl %	0.2	0.2	0.2
Na %	0.18	0.18	0.18
Ca %	0.94	0.84	0.78
Avail. P %	0.465	0.42	0.4
Dig. lysine %	1.24	1.11	1
Dig. valine %	0.935	0.84	0.762
Dig. arginine	1.37	1.21	1.09
Dig. Met + Cys %	0.92	0.84	0.755
Dig. threonine %	0.84	0.75	0.68

[1] The premix provided in kg of diet: vitamin A: 11000 IU; vitamin E: 65 mg; vitamin D: 4500 IU; vitamin K_3: 2.5 mg; vitamin B_{12}: 0.017 mg, Niacin: 60.0 mg; D-patothenic acid: 17 mg; Riboflavin: 6.5 mg; Pyridoxine: 4 mg; Thiamine: 3 mg; Folic acid: 1.5 mg and Biotin: 0.18 mg;.
[2] The premix provided in kg of diet: Mn: 100 mg; Zn: 100 mg; Fe: 40 mg; Cu: 16 mg; I: 1 mg and Se: 0.3 mg.

Table 2 The vaccination program used in the experiment

Chickens age	Vaccine	Route of administration
Day 6	Newcastle B1	Drinking water
Day 11	Bronchitis	Drinking water
Day 14	Gambro D78	Drinking water
Day 18	Newcastle Clone	Drinking water
Day 24	Gambro D78	Drinking water

The intestine tissue samples were gently flushed with physiological saline and fixed in 10% buffered- formalin (Awad *et al.* 2009).

Each sample was prepared after staining with hematoxylin and eosin using standard paraffin embedding procedures.

Villus height and crypt depth were measured to calculate the ratio of villus height to crypt depth. After removal and weighing abdominal organs (liver, pancreas, spleen and bursa of Fabricius), the bursa was flushed with physiological saline and fixed in 10% buffered-formalin for histological microscopy (Muniz *et al.* 2006).

Dry matter digestibility

Titanium dioxide as a nutrient marker was added to finisher diets at 5 g/kg diet from 24 to 28 days of age. When the birds were 28-days-old, two birds from each cage were randomly selected and euthanized based on the principles of experimental animal care (Smeets *et al.* 2015). The intestinal contents of two birds were collected from the ileum (between Meckel's diverticulum and the ileo-cecal junction) by gently finger-stripping the intestinal segment. The digesta contents collected from the birds of two cages were pooled to represent one replicate (4 replicates per treatment).

The digesta samples were kept at –20 ˚C until analysis. Representative samples of diets and ileal digesta were analyzed for DM using atomic absorption (Guzman-Cedillo *et al.* 2017). The digestibility for DM were calculated as follows:

$$\text{DM digestibility (\%)} = \frac{[DM_{diet} - (\frac{TiO_{2diet}}{TiO_{2excreta}} \times DM_{excreta})]}{DM_{diet}} \times 100$$

Blood sample for H/L calculation

Forty-eight birds 28 days of age (2 chicks/replicate) were randomly selected and blood samples were taken from the wing vein. One drop of each blood sample was immediately smeared on a glass slide, allowed to dry, and fixed using alcohol. One hundred leukocytes, including both granular and non-granular, were counted per slide using a light microscope (Olympus BH-2) at 1000 times magnification to calculate the H:L ratio.

Statistical analysis

One-way ANOVA was performed using the generalized linear model (GLM) procedure of SAS software 9.2 (SAS, 2008). Pens were used as the experimental units for all the data. Significance was declared at $P < 0.05$.

RESULTS AND DISCUSSION

Table 3 shows the effects of CORT injection and *B. subtilis* spore administration on growth performance of the chicks. For injection effect (Table 3), there was statistically a tendency toward significant difference (P=0.073) between average weight gain of CORT group compared to the control. Feed intake was not affected by either CORT injection or probiotic administration. Feed conversion ratio (FCR) was significantly increased in response to CORT injection (P<0.05). Probiotic supplementation led to improved weight gain and FCR than the control group (P<0.05). The main and interaction effects of CORT and probiotic administration on villus height (VH), crypt depth (CD), and ratio of villus height to crypt depth (VH:CD) of the duodena of broiler chicks are presented in Table 4. Compared to the oil-treated group, the CORT injection group showed significantly decreased (P<0.05) duodenum VH at 42 days of age while its effect at 10 days of age was not significant. Probiotic supplementation led to significant increases in duodenum VH at both sampling ages. The crypt depth (CD) of the duodenum was not affected by the treatments (P>0.05) at each of the sampling ages. The ratio of VH:CD of the duodenum at 42 days of age decreased significantly (P<0.05) as a result of CORT injection. Probiotic supplementation at a level of 0.04% led to significant (P<0.05) increases in the VH:CD ratio of the duodenum at 10 and 42 days of age but adding it at 0.02% brought about a significant increase in this parameter only at 42 days of age (P<0.05). The interaction effects of CORT and probiotic on duodenum VH and CD were not significant (P>0.05). Corticosterone injection decreased significantly (P<0.05) jejunum VH at 42 days of age (Table 5). This is while probiotic administration led to significant (P<0.05) increases in VH:CD ratio in the jejunum at both sampling ages.

Jejunum CD was affected neither by CORT injection nor by probiotic administration (P>0.05). The mean values for ileal VH, CD, and VH:CD ratio are shown in Table 6. Corticosterone injection decreased ileal VH:CD ratio on day 42 (P<0.05) while ileal VH increased as a result of 0.04% probiotic supplementation at the same sampling age. Ileal VH:CD ratio was significantly (P<0.05) affected by 0.04% probiotic supplementation at both sampling ages.

Table 7 reports the effects of CORT and *B. subtilis* spore administration on the relative weights of some organs. Clearly, the liver's relative weight was affected neither by CORT injection nor by probiotic supplementation at 10 days of age. On day 42, however, it decreased significantly in response to CORT injection.

A reverse trend was observed for the effect of probiotic supplementation on the liver's relative weight with CORT injection.

The interaction effect of CORT injection and *B. subtilis* spore on the liver's relative weight was not significant. The relative weight of pancreas was not affected by CORT injection but it decreased as a result of both CORT injection and probiotic administration (P<0.05).

The interaction effect of CORT and probiotic administration on the relative weight of pancreas was not significant. This is while CORT injection led to significant (P<0.05) decreases in the relative weight of spleen at both sampling ages.

Probiotic administration led to a significant (P<0.05) increase in the relative weight of spleen at 10 days of age but it showed no significant effect at 42 days of age. The interaction effect of CORT injection and *B. subtilis* spore on the relative weight of spleen was only significant (P<0.05) at 42 days of age. The relative weight of bursa of Fabricius at both 10 and 42 days of age decreased significantly as a result of CORT injection. Probiotic supplementation, however, increased it significantly at both sampling ages (P<0.05). The interaction effect of CORT and *B. subtilis* spore on the relative weight of bursa of Fabricius was not significant.

Table 8 reports the values for ileal DM digestibility at day 28. Clearly, CORT injection decreased but 0.04% probiotic supplementation increased DM digestibility at this age (P<0.05). It is seen that corticosterone injection led to a significant (P<0.05) increase in H:L ratio compared to the control but this effect was not significant for probiotic supplementation.

The histomorphological results at days 10 and 42 are presented in figures 1 to 4 for bursa of Fabricius. As can be seen, the lymphocyte density in the medulla of the bursa of Fabricius decreased in the CORT injection groups when compared with the oil injection ones.

Table 3 Main and interaction effects of corticosterone (CORT) injection and probiotic (*Bacillus subtilis* spore, BSS) supplementation on broiler chickens performance parameters

Main effects	42 days of age		
	Average body weight	Average feed intake	FCR
Type of injection			
Oil (Control)	2468.5	4384	1.78[b]
CORT.	2406.32	4382	1.82[a]
SEM	23.16	38.23	0.012
P-value	0.073	0.98	0.0154
Probiotic			
0% BSS	2357[b]	4371	1.854[a]
0.02% BSS	2440[ab]	4375	1.793[b]
0.04% BSS	2514[a]	4401	1.752[b]
SEM	28.37	46.83	0.014
P-value	0.004	0.88	0.005
Injection × probiotic			
Oil × 0% BSS	2372.2	4332	1.826
Oil × 0.02% BSS	2473.96	4393	1.78
Oil × 0.04% BSS	2559.24	4423	1.728
CORT × 0% BSS	2342.86	4409	1.882
CORT × 0.02% BSS	2406.47	4357	1.811
CORT × 0.04% BSS	2469.62	4380	1.775
SEM	40.12	66.23	0.021
P-value	0.75	0.61	0.88

The means within the same column with at least one common letter, do not have significant difference (P>0.05).
FCR: feed conversion ratio.
SEM: standard error of the means.

Table 4 Main and interaction effects of corticosterone (CORT) injection and probiotic (*Bacillus subtilis* spore, BSS) supplementation on morphology of duodenum

Main effects	10 days of age			42 days of age		
	VH (µm)	CD (µm)	VH:CD	VH (µm)	CD (µm)	VH:CD
Type of injection						
Oil (control)	1125.86	127.74	8.85	1571[a]	200	7.91[a]
CORT	1082.87	129.69	8.42	1377[b]	197.5	6.97[b]
SEM	22.29	2.79	0.233	51.52	5.85	0.216
P-value	0.189	0.626	0.209	0.02	0.76	0.05
Probiotic						
0% BSS	1009.67[c]	129.65	7.83[b]	1212[b]	197.3	6.15[b]
0.02% BSS	1105.01[b]	132.04	8.39[b]	1653[a]	208.17	7.95[a]
0.04% BSS	1198.42[a]	124.45	9.68[a]	1557.46[a]	190.77	8.17[a]
SEM	27.3	3.42	0.286	63.1	7.14	0.264
P-value	0.005	0.301	0.0008	0.008	0.254	0.005
Injection × probiotic						
Oil × 0% BSS	1042	125.00	8.4	1308.3	204.33	6.45
Oil × 0.02% BSS	1113	132.88	8.38	1743.6	206.67	8.45
Oil × 0.04% BSS	1222	125.33	9.77	1662.9	189.00	8.80
CORT × 0% BSS	977	134.30	7.3	1116.6	190.33	5.93
CORT × 0.02% BSS	1096	131.20	8.41	1562.3	209.67	7.44
CORT × 0.04% BSS	1174	123.58	9.58	1452	192.53	7.54
SEM	38.6	4.84	0.405	89.23	10.1	0.522
P-value	0.82	0.439	0.344	0.98	0.626	0.77

VH: villus height; CD: crypt depth and VH:CD: ratio of villus height to crypt depth.
The means within the same column with at least one common letter, do not have significant difference (P>0.05).
SEM: standard error of the means.

Table 5 Main and interaction effects of corticosterone (CORT) injection and probiotic (*Bacillus subtilis* spore, BSS) supplementation on morphology of jejunum

Main effects	10 days of age			42 days of age		
	VH (µm)	CD (µm)	VH:CD	VH (µm)	CD (µm)	VH:CD
Type of injection						
Oil (control)	885.88[a]	106.9	8.35	1210[a]	155.96	7.84
CORT	756.69[b]	98.8	7.66	1139[b]	145.41	7.70
SEM	32.49	3.50	0.281	21.91	4.06	0.172
P-value	0.0093	0.121	0.102	0.039	0.19	0.573
Probiotic						
0% BSS	748.54	108.3	6.90[b]	1115[b]	159.7	6.79[b]
0.02% BSS	862.82	102.4	8.43[a]	1247[a]	152.17	8.23[a]
0.04% BSS	852.50	97.9	8.68[a]	1158[ab]	140.19	8.31[a]
SEM	51.31	4.29	0.345	28.98	4.98	0.211
P-value	0.246	0.255	0.0037	0.008	0.098	0.0001
Injection × probiotic						
Oil × 0% BSS	798.00	112.7	7.49	1193	167.50	7.20
Oil × 0.02% BSS	873.63	108.0	8.10	1287	155.42	8.12
Oil × 0.04% BSS	947.67	101.0	9.44	1151	141.05	8.22
CORT × 0% BSS	655.33	93.0	6.31	1011	155.93	6.39
CORT × 0.02% BSS	809.67	93.8	8.75	1208	152.00	8.34
CORT × 0.04% BSS	753.00	99.6	7.92	1166	139.33	8.39
SEM	56.28	6.67	0.488	37.95	7.04	0.298
P-value	0.517	0.904	0.084	0.071	0.67	0.178

VH: villus height; CD: crypt depth and VH:CD: ratio of villus height to crypt depth.
The means within the same column with at least one common letter, do not have significant difference (P>0.05).
SEM: standard error of the means.

Table 6 Main and interaction effects of corticosterone (CORT) injection and probiotic (*Bacillus subtilis* spore, BSS) supplementation on morphology of ileum

Main effects	10 days of age			42 days of age		
	VH (µm)	CD (µm)	VH:CD	VH (µm)	CD (µm)	VH:CD
Type of injection						
Oil (control)	635.44	107.67	5.90	825.50[a]	146.00	5.67[a]
CORT	603.64	103.89	5.86	697.33[b]	138.44	5.04[b]
SEM	26.84	0.431	0.177	12.6	4.28	0.152
P-value	0.413	0.543	0.883	0.0001	0.228	0.0109
Probiotic						
0% BSS	612.87	112.86	5.41[b]	714.08[b]	146.50	4.87[b]
0.02% BSS	576.08	102.32	5.62[b]	748.17[b]	142.33	5.28[b]
0.04% BSS	669.66	102.16	6.61[a]	822.00[a]	137.83	6.01[a]
SEM	32.88	5.28	0.217	15.5	5.24	0.186
P-value	0.157	0.285	0.0023	0.0004	0.517	0.0015
Injection × probiotic						
Oil × 0% BSS	678.5	119.47	5.68	823.50[a]	152.67	5.39
Oil × 0.02% BSS	565.5	100.65	5.62	783.67[ab]	148.00	5.30
Oil × 0.04% BSS	662.3	102.9	6.44	869.33[a]	137.33	6.38
CORT × 0% BSS	547.25	106.25	5.15	604.67[c]	140.33	4.35
CORT × 0.02% BSS	586.66	104	5.64	712.67[b]	136.67	5.25
CORT × 0.04% BSS	677	101.42	6.67	774.67[ab]	138.33	5.64
SEM	46.5	7.47	0.307	21.92	7.14	0.263
P-value	0.207	0.533	0.395	0.007	0.613	0.196

VH: villus height; CD: crypt depth and VH:CD: ratio of villus height to crypt depth.
The means within the same column with at least one common letter, do not have significant difference (P>0.05).
SEM: standard error of the means.

The suppression effect of CORT injection was more pronounced when measured at 10 days of age than it was at day 42 (Figures 1 and 2 *vs*. Figures 3 and 4), which is in agreement with the relative weights of the bursa of Fabricius at these two ages (Table 7).

In the present study, feed intake of the chicks was not affected by CORT injection which is consistent with the report by Yang *et al*. (2015). Changes in feed intake directly affect the amount of nutrients received by the bird and lead to changes in body weight gain. Corticosterone induces a change in metabolic pathways such as increased gluconeogenesis and protein degradation, resulting in reduced protein retention, which in turn leads to a decrease in body weight or a reduction in feed use efficiency (Lin *et al*. 2004) which are in line with the results of the current study. *B. subtilis* spores administration also had no significant effect on feed intake which is agreement with the observation of previous research (Deniz *et al*. 2011; Opalinski *et al*. 2007). Body weight and FCR were significantly improved in *B. subtilis* – supplemented groups. Increased body weight and improved FCR were also reported by other researchers using probiotic based on *Bacillus subtilis* spores (Deniz *et al*. 2011; Gao *et al*. 2017; Zaghari *et al*. 2015). *Bacillus subtilis* spores can improve the digestive capacity of the intestine by secretion of a wide range of digestive enzymes such as protease, amylase and lipase activity, modulation of gut microbiota and intestinal micro structures (Gao *et al*. 2017; Rajput *et al*. 2013; Zhang *et al*. 2016).

The histomorphological changes observed in the current study in response to CORT injection are consistent with those reported in the literature (Feng *et al*. 2012; Guo, 2008); however, there are published studies that reported results contrary to ours (Quinteiro-Filho *et al*. 2010). Hu and Guo (2008) reported reducing values of duodenal and jejuna VH as a result of CORT administration and attributed this to the delayed proliferation of intestinal epithelial cells. In their investigation of the effects of such stressors as high density (16 chicks/m^2) and salmonella challenge, Gomez *et al*. (2014) concluded that declining performance in exposed chicks might be related to alterations in the functions of the HPA axis (Gomes *et al*. 2014). Song *et al*. (2014) reported reductions in jejunum VH and VH:CD ratio in chicks exposed to heat stress (Song *et al*. 2014). Stress hormones mobilize the body's metabolic activities to provide glucose, and thus cell proliferation, migration and differentiation of crypt stem cells can be affected by lowering protein synthesis (Hu and Guo, 2008). The structure and integrity of the intestine play an important role in its functions. The absorption of nutrients as one of the main functions of the intestine depends on its surface area.

CORT reduces the height of the villi and consequently nutrients absorption surface area in small intestine (Hu *et al*. 2010). The observed changes in intestinal structure in the present study are consistent with the FCR results in the corticosterone groups.

Probiotic supplementation in the current study revealed beneficial effects on VH and VH:CD ratio. These findings are supported by those reported elsewhere (Awad *et al*. 2009; Sen *et al*. 2011). Low VH:CD ratio has been claimed to be associated with poor nutrient absorption and lower performance (Awad *et al*. 2009). The effects of probiotics on intestinal integrity and its morphological parameters might be improved through different modes of action such as improved microbial fermentation and short-chain fatty acid production in the small intestine, which in turn supply the energy required for the development of enterocytes.

Pathogenic bacteria in the gut produce different types of antigens and toxins; causing inflammation, reducing the length of the villus and increasing the intestinal cell turnover rate which in turn enhance the maintenance cost of the intestine for nutrients (Yegani and Korver, 2008). Therefore, probiotic administration which can improve the intestine structure by restricting the population of pathogenic bacteria indirectly reduce the maintenance cost of the intestine.

Increased production of volatile fatty acids may also result in greater proliferation of epithelial cells in the intestine of chicks fed dietary probiotics with the consequent increase in relative intestinal weight (Gao *et al*. 2017). Despite this, the results regarding the effects of probiotic supplementation in poultry production are very diverse due to several influencing factors, including quality and quantity of selected probiotic and the physiological and environmental conditions of the birds (Otutumi *et al*. 2012).

Gross and Siegel (1983) compared plasma CORT concentration and H/L ratio responses to various stressors to conclude that H/L ratio was a more suitable index of stress in poultry. The validity of H/L ratio as a physiological index of stress in birds has been comprehensively studied by Scanes (2016). The results obtained from the current study indicate that CORT injection leads to a significant ($P<0.05$) increase in H:L ratio, a finding that is in line with those of previous report (Mehaisen *et al*. 2017).

H/L ratio has been reported to increase significantly with exogenous CORT injection because of the decrease in peripheral L and the increase in H counts (Mehaisen *et al*. 2017). In addition to their direct effects on lymphocyte function, corticosteroids induce a lymphocytopenia that may result from either lympholysis or redistribution of lymphocytes due to their circulation in other body parts (Cooper, 1984).

Table 7 Main and interaction effects of corticosterone (CORT) injection and probiotic (*Bacillus subtilis* spore, BSS) supplementation on organs relative weight (% of body weight) at 10 and 42 days of age

Main effects	Day 10				Day 42			
	Liver	Pancreas	Spleen	Bursa	Liver	Pancreas	Spleen	Bursa
Type of injection								
Oil (control)	3.57	0.528	0.101^{a}	0.206^{a}	2.153^{b}	0.175	0.117^{a}	0.104^{a}
CORT	3.49	0.531	0.077^{b}	0.179^{b}	2.292^{a}	0.185	0.101^{b}	0.077^{b}
SEM	0.09	0.014	0.0027	0.004	0.022	0.0048	0.0026	0.0044
P-value	0.53	0.905	0.002	0.0001	0.0004	0.163	0.0002	0.0004
Probiotic								
0% BSS	3.38	0.540	0.086^{b}	0.183^{b}	2.271^{a}	0.182	0.109	0.090^{ab}
0.02% BSS	3.68	0.509	0.089^{ab}	0.203^{a}	2.228^{ab}	0.173	0.106	0.0799^{b}
0.04% BSS	3.53	0.541	0.097^{a}	0.192^{ab}	2.168^{b}	0.185	0.114	0.102^{a}
SEM	0.11	0.017	0.0044	0.0056	0.027	0.0059	0.0032	0.0054
P-value	0.17	0.379	0.047	0.031	0.050	0.328	0.360	0.003
Injection × probiotic								
Oil × 0% BSS	3.45	0.550	0.089	0.198	2.267^{ab}	0.180	0.113^{a}	0.098
Oil × 0.02% BSS	3.77	0.509	0.101	0.209	2.087^{c}	0.163	0.123^{a}	0.089
Oil × 0.04% BSS	3.49	0.527	0.112	0.227	2.105^{bc}	0.183	0.116^{a}	0.125
CORT × 0% BSS	3.31	0.530	0.072	0.168	2.275^{ab}	0.185	0.104^{ab}	0.083
CORT × 0.02% BSS	3.60	0.510	0.076	0.196	2.369^{a}	0.183	0.088^{b}	0.071
CORT × 0.04% BSS	3.57	0.553	0.082	0.173	2.231^{abc}	0.188	0.112^{ab}	0.078
SEM	0.15	0.024	0.006	0.007	0.0380	0.0083	0.0046	0.0070
P-value	0.68	0.651	0.589	0.233	0.0088	0.571	0.0089	0.096

The means within the same column with at least one common letter, do not have significant difference (P>0.05).
SEM: standard error of the means.

Table 8 Main and interaction effects of corticosterone (CORT) injection and probiotic (*Bacillus subtilis* spore, BSS) supplementation on dry matter (DM) digestibility and heterophil (H)/lymphocyte (L) ratio at 28 day of age

Main effects	DM digestibility (%)	H:L ratio (%)
Type of injection		
Oil (Control)	76.8^{a}	31.4^{b}
CORT	73.19^{b}	54.8^{a}
SEM	1.08	2.81
P-value	0.033	0.0001
Probiotic		
0% BSS	71.3^{b}	44.04
0.02% BSS	75.7^{ab}	40.86
0.04% BSS	77.4^{a}	44.53
SEM	1.39	3.44
P-value	0.019	0.72
Injection × probiotic		
Oil × 0% BSS	74.2	32.0
Oil × 0.02% BSS	75.6	27.3
Oil × 0.04% BSS	80.5	34.8
CORT × 0% BSS	68.3	56.1
CORT × 0.02% BSS	75.7	54.3
CORT × 0.04% BSS	74.3	54.2
SEM	1.88	4.87
P-value	0.21	0.73

The means within the same column with at least one common letter, do not have significant difference (P>0.05).
SEM: standard error of the means.

B lymphocytes (B cells) form the essential component of the humoral immune response. The environment required for the development of B cells in birds is provided by the bursa of Fabricius as a specialized organ whose genes differentiate to produce immunoglobulins.

Furthermore, it has been found that the main roles played by the bursa of Fabricius in birds are amplification and differentiation of the B-cells.

There are around 12000 follicles in a chicken's bursa of Fabricius.

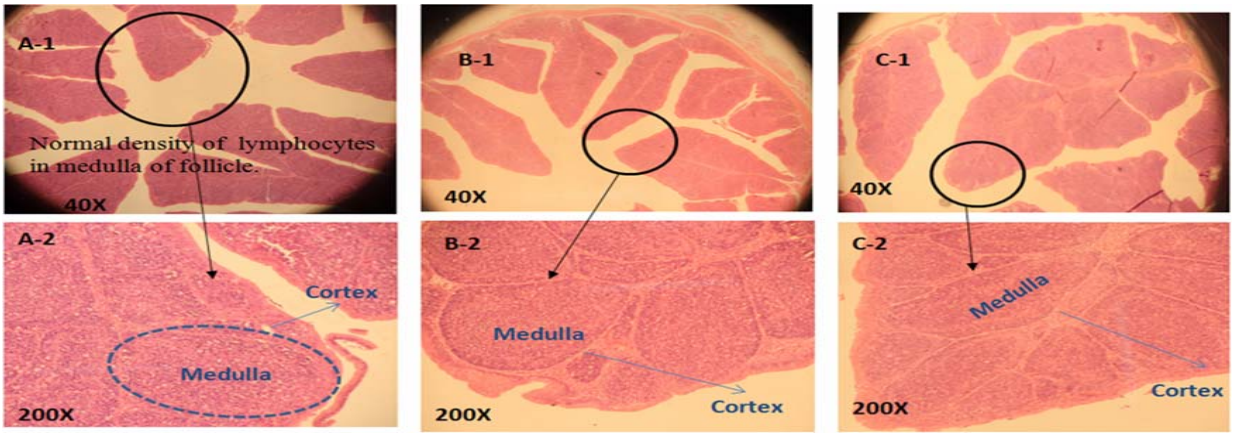

Figure 1 The bursa of Fabricius histology of chicks at 10 day of age
A-1 and **A-2**: oil injection (oil_{in}) without *Bacillus subtilis* supplementation (BSS)
B-1 and **B-2**: oil_{in} with 0.02% BSS
C-1 and **C-2**: oil_{in} with 0.04% BSS

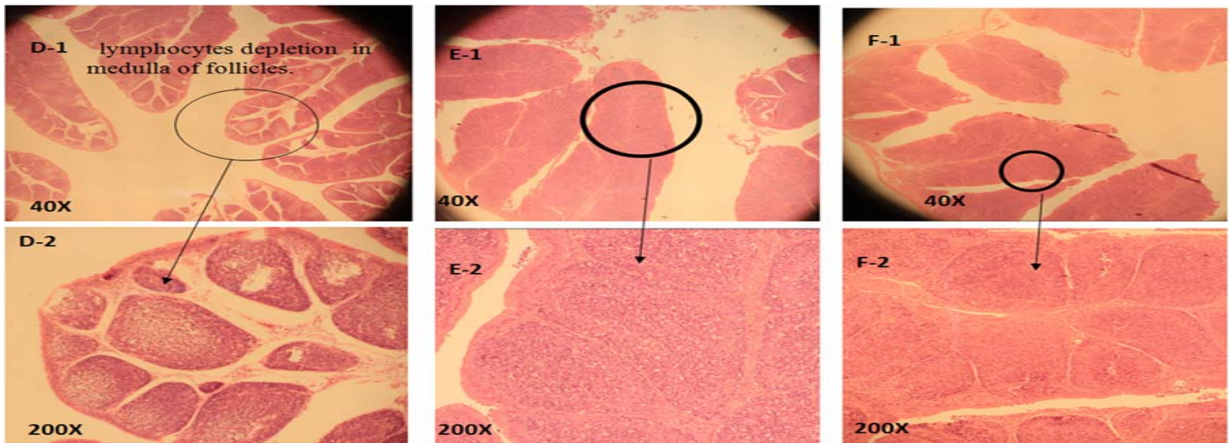

Figure 2 The bursa of Fabricius histology of chicks at 10 day of age
D-1 and **D-2**: corticosterone injection ($cort_{in}$) without *Bacillus subtilis* supplementation (BSS)
E-1 and **E-2**: $cortl_{in}$ with 0.02% BSS
F-1 and **F-2**: $cort_{in}$ with 0.04% BSS

Composed of an outer cortical and a medullary, each follicle consists of about 2 to 4 × 10^5 cells (Fellah *et al*. 2014).

CORT injection in the present study decreased the weights of immune organs including the bursa of Fabricius and spleen (Table 7). This is consistent with previously reported results (Mehaisen *et al*. 2017; Quinteiro-Filho *et al*. 2010; Yang *et al*. 2015).

Compton *et al*. (1990) demonstrated a rapid degeneration of lymphoid tissues, including bursa of Fabricius, due to apoptosis caused by elevated glucocorticoids. Probiotic supplementation was observed to undo the adverse effects of CORT injection and improve lymphocyte density in the medulla of follicles in the bursa of Fabricius (Compton *et al*. 1990). A probiotic concentration of 0.04% rather than 0.02% was found to be more effective at 42 days of age (Figures 1 to 4).

The lymphocytes in the bursa of Fabricius in avian species possess high levels of glucocorticoid receptors and are, therefore, quite susceptible to the side effects of glucocorticoids (Mehaisen *et al*. 2017).

Probiotic administration was also found to lead to significant increases in the weights of the immune organs (Table 7), which is in agreement with the results reported elsewhere (Guo *et al*. 2016; Xu *et al*. 2017). This probiotic effect has been attributed to its direct effect on the lymphatic tissue. Its indirect effects might be related to the changes that probiotic administration incurs in the microbial population of the lumen of the gastrointestinal tract.

The number of follicles has been shown to increase as a result of probiotic administration due to the high reaction of plasma cell in the medulla of chicken bursa (Alkhalf *et al*. 2010; Sikandar *et al*. 2017).

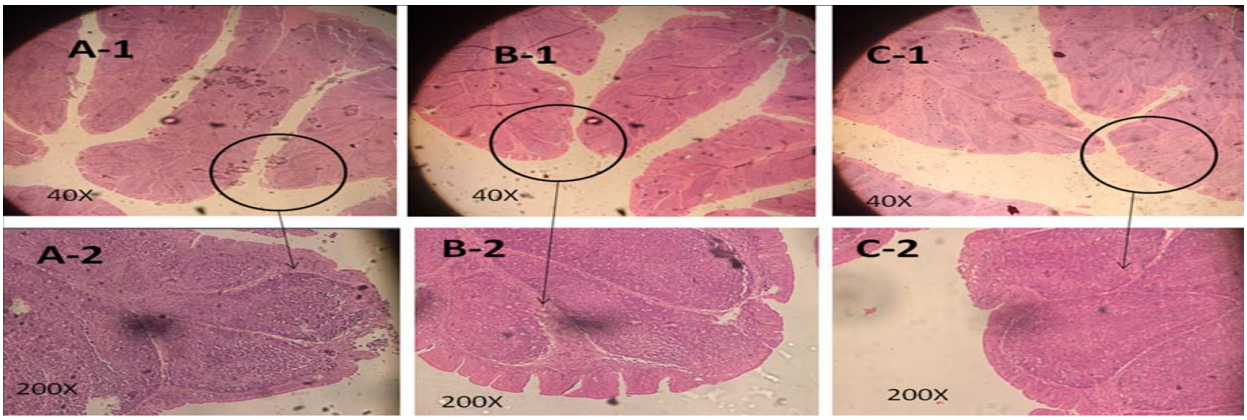

Figure 3 The bursa of Fabricius histology of chicks at 42 day of age
A-1 and **A-2**: oil injection (oil_{in}) without *Bacillus subtilis* supplementation (BSS)
B-1 and **B-2**: oil_{in} with 0.02% BSS
C-1 and **C-2**: oil_{in} with 0.04% BSS

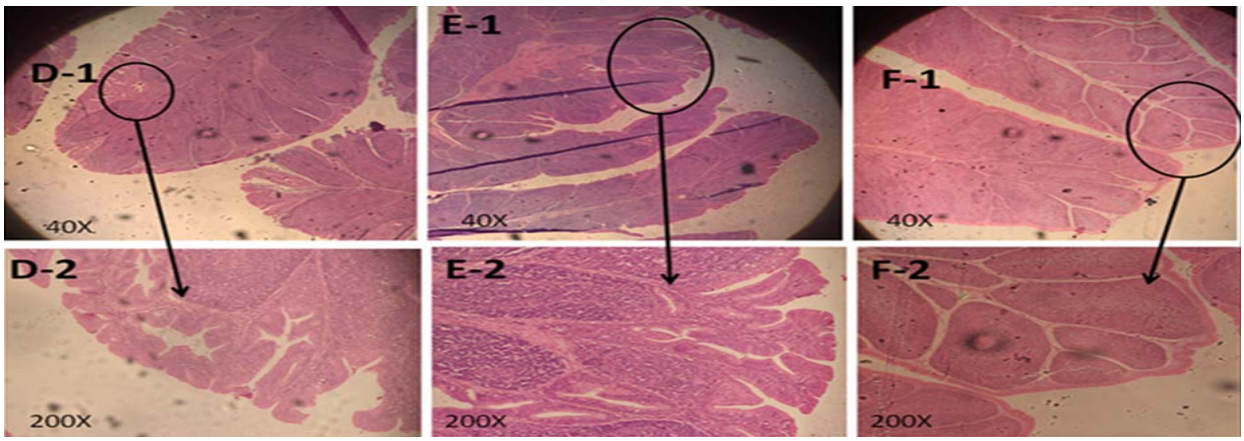

Figure 4 The bursa of Fabricius histology of chicks at 42 day of age
D-1 and **D-2**: corticosterone injection ($cort_{in}$) without *Bacillus subtilis* supplementation (BSS)
E-1 and **E-2**: $cortl_{in}$ with 0.02% BSS
F-1 and **F-2**: $cort_{in}$ with 0.04% BSS

It has been suggested that probiotic bacteria stimulated cytokine secretion by the immune system cells; hence, they suggested that some of the probiotic effects must be mediated by cytokine secretion (Alkhalf *et al*. 2010).

Corticosterone injection in this experiment led to a significant decline in DM digestibility due to the reduced VH, VH:CD ratio, a findings that is in agreement with those previously reported (Puvadolpirod and Thaxton, 2000). Increased DM digestibility as a result of *B. subtilis* supplementation through improved VH, VH:CD ratio, and enzyme (protease and amylase) activities was reported by Sen *et al*. (2011) and Gao *et al*. (2017). In some stress conditions (e.g. heat stress), the digestibility of nutrients has increased due to reduced feed intake and a decrease in the digesta passage time in the intestine (Habashy *et al*. 2017).

CONCLUSION

The results of the current study confirmed our hypothesis regarding the possible beneficial effects of *B. subtilis* administration on immune responses, intestinal morphology, and DM digestibility in broiler chicks exposed to stress.

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